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RAT BRAIN TRYPTOPHAN HYDROXYLASE: PROPERTIES AND EFFECTS OF 5,7-DIHYDROXYTRYPTAMINE

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RAT BRAIN TRYPTOPHAN HYDROXYLASE: PROPERTIES AND EFFECTS OF 5,7-DIHYDROXYTRYPTAMINE

by CAROL S. CLEWANS

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ABSTRACT

RAT BRAIN TRYPTOPHAN HYDROXYLASE:

PROPERTIES AND EFFECTS OF 5,7-DIHYDROXYTRYPTAMINE

by

Carol S. Clewans

Advisor: Dr. E.C. Azmitia

It has previously been shown using anatomical methods that partial denervation of the rat hippocampus by removal of serotonergic (5-HT) fibers in the cingulum bundle induces sprouting of intact 5-HT fibers reaching the hippocampus in the fornix-fimbria (Azmitia et al., 1978; Zhou and Azmitia, 1981). The biochemical properties of sprouted fibers have remained largely uncharacterized. Thus, the rate-limiting enzyme in 5-HT synthesis, tryptophan hydroxylase (TPOH), was studied to determine whether new sprouts possess the ability to synthesize 5-HT.

Optimal assay conditions were established for measuring TPOH activity in the presence of tetrahydrobiopterin (BH_4) using the radiometric method of Ichiyama et al. (1970). The effects of reducing and phosphorylating conditions were studied. Enzyme activity was maximally stimulated by incubation in the presence of 2 mM DTT and 14 uM Fe^{++}. Phosphorylating conditions produced a slight but variable increase in TPOH activity depending on the conditions of enzyme preincubation and on the cofactor used (BH_4 or 6-methyltetrahydropterin). Neither a change in pH nor addition of an ATP-regenerating system or EDTA increased the amount of activation by phosphorylating conditions.

Unilateral stereotaxic injections of 5 ug of the specific 5-HT

neurotoxin 5,7-dihydroxytryptamine were made into the cingulum bundle of adult rats pretreated with desmethylimipramine in order to produce partial 5-HT deafferentation of the hippocampus. Three to 90 days after the injection, TPOH activity was measured in ipsilateral and contralateral hippocampi and in the midbrain, site of neuronal cell bodies of hippocampal 5-HT projections. Enzyme activity was also measured in hypothalamus and septum/preoptic area 3 to 14 days post-lesion in order to estimate the extent of retrograde degeneration of 5-HT fibers.

In both hippocampi, enzyme activity declined gradually, reaching minimal levels 7 to 14 days post-lesion. This decrease in enzyme activity was paralleled by a decrease in the Vmax and an increase in the Km of TPOH for tryptophan. TPOH activity increased from 28 to 60 days and decreased again from 60 to 90 days post-lesion. In midbrain, enzyme activity gradually increased, reaching levels significantly greater than sham at 28 days post-lesion. Enzyme activity in the hypothalamus and septum/preoptic area was unaffected 3 to 14 days after the lesion.

The results indicate that 5-HT fibers remaining in the hippocampus following partial denervation are able to compensate biochemically for those removed by cingulum bundle lesions. Changes in hippocampal TPOH can be related to simultaneous changes in midbrain TPOH and to the appearance of sprouts from intact 5-HT fibers.

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INTRODUCTION

Serotonergic (5-HT) fibers have been observed to undergo vigorous sprouting in the central nervous system following axotomy (see Bjorklund and Stenevi, 1979). Regeneration of damaged 5-HT axons has been shown to lead to normal innervation patterns in some regions of the brain and to result in the return of normal function in the spinal cord (Nygren et al., 1974) and hypothalamus (Luine et al., 1983) in particular. The question of whether reinnervation and functional restoration can also occur by collateral sprouting of chemically identical but anatomically distinct undamaged fibers has been examined in a series of studies by Azmitia and colleagues (Azmitia et al., 1978; Zhou and Azmitia, 1981; 1983) utilizing the 5-HT innervation of the hippocampus.

The hippocampus receives 5-HT input from the median raphe nucleus via two pathways (Figure 6): the supracallosal cingulum bundle and indiseum griseum and the infracallosal fornix-fimbria (Azmitia and Segal, 1978). An additional group of fibers originates in the dorsal raphe nucleus and reaches the hippocampus by a ventral route through the entorhinal cortex (Azmitia, 1978). Segregation of hippocampal 5-HT input from the median raphe nucleus in terms of pathway of projection (cingulum bundle/indiseum griseum versus fornix-fimbria) has provided the basis for studies on plasticity of the 5-HT innervation of this brain region. These studies by Azmitia and coworkers have specifically addressed the question of whether or not intact 5-HT fibers remaining in the hippocampus following removal of 5-HT fibers in the supracallosal pathways can sprout to reinnervate areas vacated by

damaged fibers, and further, whether or not these collateral sprouts have the ability to compensate functionally for the lost terminals.

In the studies by Azmitia and colleagues (Azmitia et al., 1978; Zhou and Azmitia, 1981; 1983), partial removal of 5-HT fibers in the rat hippocampus was accomplished by microinjection of the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) into the region of the supracallosal pathways. The use of 5,7-DHT, in animals pretreated with desmethylimipramine to protect catecholaminergic neurons from damage, insures specificity of denervation; thus, any reinnervation response occurring in the hippocampus can be attributed to the exclusive removal of 5-HT fibers.

Following injection of 5,7-DHT into the cingulum bundle/indiseum griseum, there is an initial decrease in the 5-HT innervation of the dorsal hippocampus as detected by anterograde transport of ^{3}H-proline, high affinity uptake of ^{3}H-5-HT (Azmitia et al., 1978), retrograde transport of horseradish peroxidase (Zhou and Azmitia, 1981), and 5-HT immunocytochemistry (Zhou and Azmitia, 1983). Twenty-eight to 90 days after injection, 5-HT fibers remaining in the hippocampus are observed to have expanded their terminal field, reestablishing a near normal pattern of innervation. Studies of turning behavior (Azmitia et al., 1978) and of acquisition of a T-maze learning task (Gage et al., 1983) indicate that 5-HT fibers sprouting in response to partial deafferentation of the hippocampus are capable of substituting functionally for those removed by neurotoxic (Azmitia et al., 1978) or mechanical (Gage et al., 1983) lesions of the supracallosal fibers.

The studies presented in this thesis were undertaken to biochemically characterize this sprouting response using a specific marker of 5-HT neurons, tryptophan hydroxylase, the rate-limiting enzyme in 5-HT synthesis. Tryptophan hydroxylase (TPOH) activity was measured by a modification of the coupled radiometric assay of Ichiyama et al. (1970) which quantitates the $^{14}CO_2$ liberated by aromatic L-amino acid decarboxylase from 5-hydroxytryptophan formed from the hydroxylation of L-[1-^{14}C-]tryptophan by TPOH. In order to measure TPOH activity under optimal assay conditions, the properties of the enzyme, including optimal pH, reducing, and phosphorylating conditions, were investigated in detail.

To follow the effect of 5,7-DHT on hippocampal TPOH activity over time, unilateral injections of 5 ug of the neurotoxin were made between the cingulum bundle and indiseum griseum of male rats, and enzyme activity was measured in hippocampi both ipsilateral and contralateral to the injection 3 to 90 days later. The kinetic parameters of TPOH from ipsilateral hippocampi of short-term (7 day) and long-term (60 day) lesioned animals were studied in an effort to detect any change in Km or Vmax resulting from the denervation. The response of the cell bodies whose axons were damaged by the lesion was examined by measuring enzyme activity in the midbrain of animals receiving cingulum bundle injections; and the extent of retrograde degeneration of 5-HT axons affected by the lesion was estimated by measuring TPOH activity in the septum/preoptic area and in the hypothalamus.

Because a unilateral injection of 5,7-DHT produced a bilateral decline in hippocampal TPOH activity, a study of the effect of the dose

and concentration of 5,7-DHT used in the injection on enzyme activity in hippocampi ipsilateral and contralateral to the injection was performed. When it was found that 2 ug of the drug caused ipsilateral damage only, the time course of TPOH activity in the hippocampus was repeated using this dose. In addition, a study of high affinity uptake of ^{3}H-5-HT into synaptosomes prepared from hippocampi of 5,7-DHT-lesioned animals was carried out to determine whether changes in this specific biochemical marker for 5-HT neurons paralleled those of TPOH activity.

LITERATURE REVIEW

Serotonin was first detected in the gastrointestinal mucosa of rabbits by Erspamer and his colleagues in the late 1930's. These investigators histochemically localized this substance to enterochromaffin cells of the digestive tract and appropriately named it enteramine. Erspamer originally proposed the use of isolated rat uterus for bioassay of enteramine, the action of which resulted in a prolonged contraction of the tissue (see Erspamer, 1966). In the late 1940's, Rapport and his group isolated and purified a vasoconstrictive substance from beef serum and called it serotonin (Rapport et al., 1948). This group was subsequently able to demonstrate the indolic nature of the molecule, its identity with 5-hydroxytryptamine, and its presence as a creatinine sulfate complex (Rapport et al., 1949; Rapport, 1949). Finally in 1952, Erspamer and Asero showed the chemical nature of enteramine to be identical to that of serotonin.

Twarog and Page first isolated serotonin from dog brain in 1953. Today serotonin (5-HT) is widely accepted as a neurotransmitter in the central nervous system. Mechanisms for its synthesis, storage, release, pre- and post-synaptic effects, and inactivation have been proposed and studied by hundreds of neuroscientists. Although much has been learned about these various aspects of the role of 5-HT in the central nervous system, much more remains to be discovered.

Figure 1 represents a serotonergic neuron and its various neurotransmitter-related activities. A brief discussion of some of these processes and how they are affected by specific drugs will aid in the understanding of information presented below.

The enzymes involved in the synthesis of 5-HT from tryptophan, tryptophan hydroxylase and aromatic L-amino acid decarboxylase, are synthesized in the neuronal cell body. Enzyme molecules are transported to terminal areas by the process of slow axonal transport (Meek and Neff, 1972), where the synthesis of 5-HT takes place. The precursor for 5-HT, tryptophan, is an essential amino acid, and therefore must be supplied by exogenous means (e.g. diet). Tryptophan is taken up into the neuron by both high and low affinity systems (Parfitt and Grahame-Smith, 1974). The data do not suggest a specific system for the transport of tryptophan: uptake of this amino acid is inhibited competitively by other large neutral amino acids (phenylalanine, tyrosine, leucine), suggesting that the tryptophan uptake mechanism possesses a wide substrate specificity (Parfitt and Grahame-Smith, 1974).

Tryptophan hyroxylase is irreversibly inhibited in vivo by p-chlorophenylalanine (PCPA) (Jequier et al., 1967). Inhibition by PCPA was thought to be due to incorporation of the amino acid analogue into the catalytic site of newly synthesized tryptophan hydroxylase (Gal et al., 1970). However, more recent evidence indicates that incorporation of a tyrosine molecule from PCPA occurs and that substitution of tyrosine for phenylalanine during protein synthesis probably occurs at the enzyme active site (Gal and Whitacre, 1982). Enzyme in terminals may also be susceptible to inhibition by PCPA (Aghajanian et al., 1973). Decarboxylase is best inhibited centrally by 3-hydroxybenzylhydrazine (NSD 1015) (Carlsson et al., 1972).

The 5-HT molecule is stored in intraneuronal granules. Storage of

5-HT is impaired by reserpine, which causes a marked depletion of brain 5-HT (Brodie et al., 1957). Intraneuronal release of 5-HT provoked by reserpine may be the result of inhibition of 5-HT binding to "serotonin binding protein," an intraneuronal protein extensively characterized by Tamir and her colleagues (Tamir and Gershon, 1981).

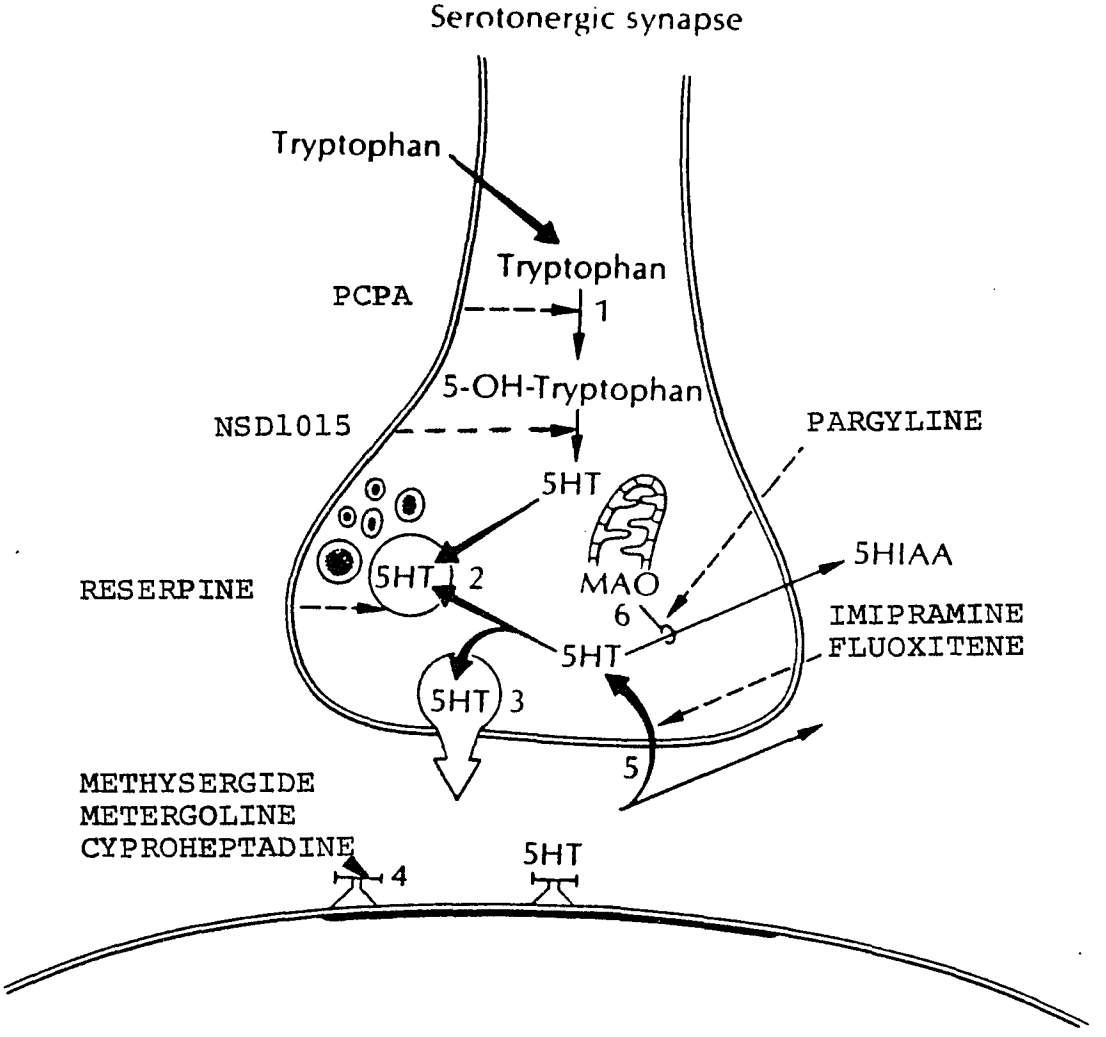
Serotonin existing outside granules, free in the cytoplasm, is subject to degradation to 5-hydroxyindole acetic acid (5-HIAA) by monoamine oxidase (MAO) probably located in the outer membrane of mitochondria (Weiner, 1960). MAO can be inhibited by a variety of drugs, the most commonly used in vivo being pargyline.

Upon depolarization of the nerve terminal, 5-HT is released by a mechanism thought to involve exocytosis. There are few drugs known to specifically inhibit the release of 5-HT, although drugs which block sodium channels or deplete extracellular calcium will inhibit depolarization-induced release. The release of newly-synthesized 5-HT induced by high K^+ concentrations in brain slices is inhibited by LSD administered to rats in vivo or included in the incubation medium in vitro (Hamon et al., 1974). LSD may inhibit 5-HT release by acting on presynaptic receptors located on 5-HT terminals (Hery and Ternaux, 1981).

At least 2 classes of post-synaptic 5-HT receptors have now been identified (Peroutka et al., 1981; Leysen, 1981). A variety of drugs are used to antagonize 5-HT at the receptor level, including methysergide, metergoline, and cyproheptadine (Peroutka et al., 1981). The action of released 5-HT is largely terminated by a high-affinity reuptake mechanism present at the presynaptic membrane (Iversen, 1971).

Inhibition of the reuptake mechanism by imipramine, chlorimipramine, or fluoxitene results in prolonged action of 5-HT at the synapse (Fuller and Wong, 1977).

Figure 1. Schematic diagram of a 5-HT terminal showing neurotransmitter-related activities and their interactions with a variety of drugs as detailed in the text. Adapted from Cooper et al. (1978).



I. Anatomy of the Serotonergic Neuronal System

With the introduction of the histochemical fluorescence technique for the localization of serotonin (5-HT) in histological sections of brain tissue by Falck in 1964, Swedish investigators embarked on a pioneering investigation of 5-HT neurons in the central nervous system. Using this technique, Dahlstrom and Fuxe (1964; 1965) demonstrated the presence of 5-HT cell bodies concentrated mainly in the raphe nuclei of the brainstem, from the level of the pyramidal decussation through the level of the interpeduncular nucleus. Fluorescent 5-HT terminals were found in widespread areas of the central nervous system, such as the spinal cord, brainstem, hypothalamus, amygdala, striatum, and cerebral cortex (Fuxe, 1965).

Further mapping studies have indicated that 5-HT axons in the spinal cord arise mainly from cell bodies in the reticular formation and raphe nuclei of the medulla oblongata (B1-B3)(Dahlstrom and Fuxe, 1965; Ungerstedt, 1971; Bowker et al., 1981), with a few projections from the midbrain reticular formation (B9) and a very few from the dorsal raphe nucleus (B7)and the nucleus raphe pontis (B5)(Bowker et al., 1981). A multitude of anatomical tracing studies mostly using autoradiographic techniques alone (Conrad et al., 1974; Bobillier et al., 1975; 1979; Parent et al., 1981) or in combination with brain lesions (Halaris et al., 1976; Azmitia and Segal, 1978; Moore et al., 1978), have contributed to our detailed knowledge of the ascending 5-HT system. Although there remain some discrepancies between various studies, the majority of ascending projections can be said to arise from the dorsal (B7) and median (B8) raphe nuclei in the midbrain and

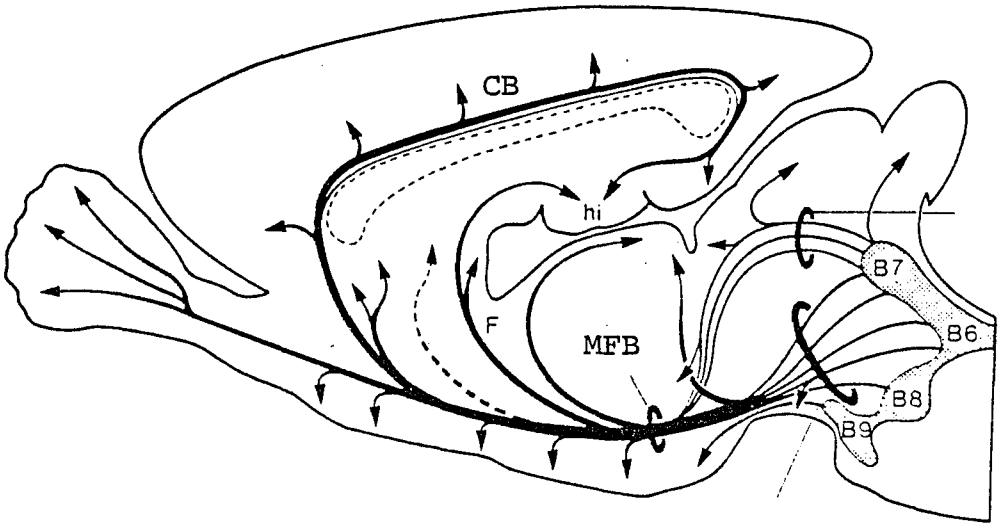
to travel in the medial forebrain bundle. As many as 6 distinct ascending tracts have been described - 4 originating from the dorsal raphe and 2 from both the dorsal and median raphe (Azmitia and Segal, 1978).

Serotonergic fibers leave the medial forebrain bundle to join other well-established neuroanatomical pathways on their way to innervate distant brain regions. For example, 5-HT fibers can be observed travelling in the stria medullaris and fasciculus retroflexus to the habenula, in the stria terminalis to the amygdala, and in the mammillothalamic tract to the thalamus (Azmitia, 1978). The hippocampus receives projections from the midbrain raphe nuclei via 3 major routes (Moore and Halaris, 1975; Azmitia and Segal, 1978). Fibers from the median raphe nucleus reach the hippocampus by way of the fornix-fimbria and the cingulum bundle. A small group of 5-HT fibers, also arising from median raphe neurons (Azmitia and Segal, 1978), is associated with the indiseum griseum along its entire length (Azmitia and Segal, 1978; Moore and Halaris, 1975; Parent et al., 1981). Fibers from the dorsal raphe nucleus enter the hippocampus by way of the entorhinal cortex via the perforant pathway (ventral pathway; Azmitia, 1978).

5-HT input travelling in the cingulum bundle is distributed mainly to the dorsal hippocampus, principally to CA1 and to the polymorphic layer of the dentate gyrus (Moore and Halaris, 1975; Azmitia et al., 1978). Projections from the median raphe carried by the fornix-fimbria are distributed to both the dorsal and ventral hippocampus in the regions CA2-CA4, with some fibers reaching the dentate gyrus (Azmitia,

1978). Dorsal raphe fibers innervate predominantly the molecular region of the dentate gyrus, most heavily in the ventral hippocampus (Azmitia and Segal, 1978).

Figure 2. Anatomy of the ascending 5-HT system in the rat. Fibers from the median raphe nucleus (B8) to the hippocampus (hi) split off the medial forebrain bundle (MFB) to ascend in the cingulum bundle (CB) and the fornix-fimbria (F). A third pathway (not shown) arises from the dorsal raphe nucelus (B7) and reaches the hippocampus by a ventral route. Adapted from Parent et al. (1981).



II. Serotonin Biosynthesis

The biosynthesis of 5-HT from L-tryptophan proceeds through a two-step sequence of enzymatic reactions: L-tryptophan is hydroxylated to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (TPOH), and then 5-HTP is decarboxylated to 5-HT by aromatic L-amino acid decarboxylase (AADC). It is now generally accepted that TPOH is the rate-limiting enzyme in this sequence of reactions.

The first direct evidence of tryptophan hydroxylating activity in the brain came from a study by Gal, Poczik, and Marshall (1963). Intracerebral injections of radioactive tryptophan resulted in the cerebral formation of radioactive 5-HT in rats and pigeons pretreated with a monoamine oxidase inhibitor. During the 4 years following this demonstration, TPOH activity was measured in vitro in a variety of mammalian tissues including neoplastic murine mast cells (Levine et al., 1964; Lovenberg et al., 1965; Hosada and Glick, 1966), human carcinoid tumor (Grahame-Smith, 1964a; Lovenberg et al., 1967), rat and bovine pineal (Lovenberg et al., 1967), and brainstem from several species (Grahame-Smith, 1964b; 1967; Gal et al., 1966; Green and Sawyer, 1966; Lovenberg et al., 1967).

III. Distribution of Tryptophan Hydroxylase

A. Anatomical

The distribution of TPOH in the central nervous system reflects the distribution of serotonergic cell bodies and their processes. Brownstein and coworkers (1975) measured TPOH activity in tissue punches of more than 70 nuclei and regions of the rat brain. Their

study revealed a 43-fold difference between the highest and lowest levels of activity measured. The raphe nuclei, which contain the soma of 5-HT neurons, had the highest levels of TPOH in the brain (with the exception of the nucleus raphe pontis). The enzyme was also highly concentrated in regions of the brain through which dense bundles of 5-HT axons travel, particularly in the medial forebrain bundle and in the area of the diagonal band of Broca. Substantial amounts of TPOH were detected in individual nuclei of the brainstem, hypothalamus and preoptic area, septum, amygdala, hippocampus, and cingulate and prepyriform cortices. The dentate gyrus, lateral habenular nucleus, entorhinal cortex, and parietal cortex contained relatively lower levels of enzyme activity, and the cerebellum possessed the lowest amount measured. No measurements were made in the spinal cord; however, TPOH activity has been detected in the spinal cord by other investigators (Meek and Neff, 1972).

B. Effects of lesions on the anatomical distribution of tryptophan hydroxylase

Following electrolytic lesion of the midbrain raphe nuclei, forebrain (everything rostral to the superior colliculi) TPOH activity is significantly reduced by 1 day post-lesion (Kuhar et al., 1971). TPOH activity declines gradually, reaching a minimum (20% of control) by 8 days post-lesion, and remaining at this level 6 days later (day 14) (Kuhar et al., 1971). From these studies, the half-life of forebrain TPOH was estimated to be about 2 days. Similarly, Meek and Neff (1972) estimated the half-life of enzyme in the spinal cord after spinal transection to be 2 days. On the other hand, Azmitia and Conrad

(1976) calculated the half-life of TPOH in the hippocampus after fornix transection to be 28 hours. In their study, a significant drop in enzyme activity was observed already 6 hours after transection and enzyme activity was maximally reduced (22% of control) in the hippocampus by 8 and 30 days post-lesion. TPOH activity in the septum remained significantly unchanged, whereas midbrain TPOH activity fell initially, only to return to normal levels by 30 days after transection.

A regional study of TPOH activity in rats receiving midbrain raphe lesions at least 2 weeks prior to sacrifice (Kuhar et al., 1972) indicated a 70-90% loss of TPOH in the hypothalamus-thalamus, an 80-90% loss in cortical and hippocampal regions, and a 45-65% reduction in the striatum.

C. Subcellular distribution

Initially, TPOH activity was almost exclusively localized in the particulate, presumably mitochondrial, fraction of brain tissue homogenates (Grahame-Smith, 1964b; Green and Sawyer, 1965; 1966; Gal et al., 1966). Subcellular fractionation studies of rabbit hindbrain (Grahame-Smith, 1967) and guinea pig brainstem (Ichiyama et al., 1968) demonstrated that particulate TPOH was contained mainly in pinched-off nerve endings, or synaptosomes. Forty to 50% of total enzyme activity was localized to the synaptosomal fraction. Moreover, enzyme from the particulate fraction was releasable into the supernatant following homogenization and centrifugation in hypotonic buffer (Grahame-Smith, 1967; Ichiyama et al., 1968; Robinson et al., 1968). Whereas particulate enzyme did not require added cofactor for activity, soluble

enzyme depended upon the addition of cofactor for optimal hydroxylating activity (Grahame-Smith, 1967; Ichiyama et al., 1968; 1970; Robinson et al., 1968), suggesting the presence of endogenous cofactor in synaptosomal particles.

Although it is now apparent that the bulk of TPOH activity is found in the soluble fraction under appropriate conditions of tissue preparation, it is still unknown whether TPOH is primarily a cytoplasmic enzyme or whether homogenization procedures used to extract the enzyme merely disrupt any association of the molecule with subcellular organelles. With respect to this latter possibility, using a specific antibody against TPOH purified from the raphe area, Joh et al. (1975) and Pickel et al. (1976) have immunocytochemically localized TPOH in the midbrain of rats to organelles having the size and distribution of microtubules and small and large synaptic vesicles. If it is the case then that TPOH is a particulate enzyme, its activity might depend on the local cellular environment, notably on membrane phospholipid composition. Tong and Kaufman (1975) failed to detect any effect of various phospholipids on TPOH partially purified from rabbit hindbrain. On the other hand, Hamon et al. (1978a) demonstrated a definite stimulatory effect of some phospholipids and gangliosides on the activity of TPOH partially purified from rat brainstem. The affinity of the enzyme for its cofactor (6-MPH_4) was enhanced in the presence of phospholipids. The data of Hamon et al. (1978a) suggest that endogenous phospholipids play a role in the regulation of TPOH activity inside the serotonergic neuron and provide support for the idea that the enzyme is actually membrane-bound. Hori et al. (1976) have provided more direct biochemical evidence for the

particulate nature of TPOH. These investigators found that after high-speed centrifugation of TPOH from bovine raphe nuclei followed by preincubation with dithiothreitol and Fe^{++}, 90% of the enzyme is recovered in the particulate fraction.

IV. Properties of Tryptophan Hydroxylation

Hydroxylation of tryptophan to 5-HTP requires both a pterin cofactor (Lovenberg et al., 1967; 1968; Jequier et al., 1969; Friedman et al., 1972) and molecular oxygen (Green and Sawyer, 1966; Lovenberg et al., 1967; 1968). It is thought by most investigators that the natural cofactor involved in tryptophan hydroxylation is tetrahydrobiopterin (BH_4) (Friedman et al., 1972; Gal, 1981). The synthesis of BH_4 from guanosine-5'-triphosphate by rat brain preparations has been recently demonstrated, although the nature and sequence of the intermediate products is still controversial (Gal, 1981; Kapatos et al., 1982). Nevertheless, many studies of the biochemical properties of TPOH have used the model cofactors 6-methyltetrahydropterin ($6\text{-}MPH_4$) and 6,7-dimethyltetrahydropterin ($DMPH_4$). As will be discussed below in detail, the kinetic properties of the enzyme are highly dependent on the nature of the cofactor used.

For each mole of cofactor oxidized, one mole of 5-HTP is formed (Friedman et al., 1972). The same holds true when $DMPH_4$ or $6\text{-}MPH_4$ is used as cofactor in the reaction (Tong and Kaufman, 1975). The stoichiometry of the reaction is as follows:

L-Tryptophan + BH_4 + O_2 $\longrightarrow$ 5-HTP + quinonoid dihydro-

biopterin + H_2O

The observation that tryptophan hydroxylation was stimulated by purified sheep liver dihydropteridine reductase plus reduced pyridine nucleotides indicated that the product of BH_4 oxidation was quinonoid dihydrobiopterin (Friedman et al., 1972). Quinonoid dihydrobiopterin had been previously shown to be the substrate for the dihydropterin reductase by Kaufman (1964). The product of this reaction, in turn, was BH_4.

$$BH_2 + NADH + H^+ \longrightarrow BH_4 + NAD^+$$

Quinonoid dihydrobiopterin reductase (QDPR) can utilize either NADH or NADPH as substrate but is far more active with NADH (Craine et al., 1972). Thus, cofactor regeneration is thought to be accomplished by QDPR using NADH in 5-HT terminals. More recently, Snady and Musacchio (1978) and Bullard et al. (1978) studied the regional and subcellular distribution of this enzyme in rat brain. Whereas Bullard et al. found no correlation between the distribution of QDPR and either TPOH or tyrosine hydroxylase, Snady and Musacchio found a high correlation between the distribution of QDPR and that of AADC, suggesting that QDPR is primarily localized in catecholamine- and indoleamine-containing neurons. Both groups of investigators found the enzyme to be distributed in the same subcellular fractions containing TPOH and tyrosine hydroxylase, i.e. in both the soluble and synaptosomal fractions.

The importance of QDPR in the maintenance of reduced levels of cofactor and consequently in the synthesis of 5-HT and catecholamines

was clinically revealed by Kaufman et al. (1975). These investigators found a variant of phenylketonuria in which the patient had <1% of normal levels of QDPR activity in liver, brain, and skin fibroblasts (Kaufman et al., 1975) and low or undetectable amounts of dopamine, 5-HT, and their metabolites in urine, serum, CSF, and brain (Butler et al., 1978).

Jequier et al. (1969) demonstrated that, as liver phenylalanine hydroxylase, rat brainstem TPOH has a lower Km for BH_4 than for $DMPH_4$. The reported Km value with BH_4 was 5 uM, whereas that for $DMPH_4$ was 30 uM. Ichiyama et al. (1970) reported a Km of 60 uM for TPOH from guinea pig brainstem with $DMPH_4$ as cofactor. In a more detailed study of the pterin specificity of partially purified rabbit hindbrain TPOH, Friedman et al. (1972) confirmed the observations of Jequier et al. that the Km for BH_4 is much lower than that for $DMPH_4$. Their Km values, however, were 4 to 6 times higher than those reported by Jequier et al. and twice as high as that reported for $DMPH_4$ by Ichiyama et al. (see Table 1). The reported Km of crude TPOH from rat midbrain with $6\text{-}MPH_4$ as cofactor is 200-220 uM (Table 1).

The activity of TPOH from rat brainstem was reported by Jequier et al. (1969) to be 1.7 times greater with BH_4 than with $DMPH_4$, although it is not clear whether the rates presented in this study were Vmax values. In contrast, Friedman et al. (1972) demonstrated a Vmax for TPOH only slightly higher with BH_4 than with $DMPH_4$. Kaufman (1974) suggests that the major difference between BH_4 and $DMPH_4$ lies in the much lower Km of the hydroxylase enzymes

(tryptophan, tyrosine, and phenylalanine) for the former, whereas the Vmax is about the same or even lower (in the case of phenylalanine hydroxylase) with BH_4 than it is with $DMPH_4$.

The Km of TPOH from rabbit brain for tryptophan has also been shown to vary with the nature of the pterin used (Friedman et al., 1972), although this does not appear to be the case for enzyme from bovine pineal (Hori and Ohotani, 1981). The use of $DMPH_4$ results in a Km of more than 5 times that obtained with BH_4 (Friedman et al., 1972). A comparison of values in the literature (Table 1) suggests that the Km of TPOH for tryptophan with $6\text{-}MPH_4$ is more similar to the Km for tryptophan in the presence of BH_4 than in the presence of $DMPH_4$, although the reported values span a wide range (17-234 uM).

The studies of Friedman et al. (1972) have also demonstrated that the affinity of brain TPOH for oxygen is sensitive to the cofactor used. The Km of TPOH for oxygen in the presence of BH_4 (2.5%) is one-eighth of that in the presence of $DMPH_4$ (20%). An oxygen concentration of 5% in the brains of animals breathing air has been reported (Jamieson and Van de Brenk, 1965), suggesting that TPOH in vivo is not normally limited by oxygen supply. However, in vivo studies suggest that 5-HT synthesis is affected by oxygen concentration. Exposure of rats to 5.6% O_2 for one hour results in a decrease of >50% in 5-HT synthesis (Davis and Carlsson, 1973), whereas exposure to 100% O_2 results in greater brain levels of 5-HT after pargyline pretreatment than those in animals breathing air (Diaz et al., 1968).

In 1971, Grahame-Smith showed that the amount of 5-HT accumulating in brains of rats pretreated with an MAO inhibitor increased with increasing brain tryptophan concentrations. 5-HT synthesis plateaued when the brain tryptophan concentration reached 70 ug/g tissue (4 x 10^{-4} M) (Grahame-Smith, 1971). From studies demonstrating that the rate of 5-HTP accumulation in the brain after treatment with an inhibitor of AADC is also dependent on brain tryptophan levels, Carlsson and Lindqvist (1972) were able to calculate a Km in vivo for TPOH. This Km value, 60 uM, approximates that observed in vitro in the presence of BH_4, lending support to the notion that BH_4 is the natural cofactor in 5-HT synthesis. Work by Fernstrom and Wurtman (1971) estimated the the concentration of brain tryptophan at 30 uM, leading to the conclusion that although TPOH functions with tissue concentrations of its substrate within the region of its Km, it is normally not saturated with its substrate in vivo (Friedman et al., 1972). Thus, changes in brain tryptophan levels by dietary manipulation have been shown to result in changes in 5-HT and 5-HIAA both in cell body regions and in terminal areas (Colmenares et al., 1975; Wurtman and Fernstrom, 1976). Similarly, many psychotropic drugs producing increases in cerebral tryptophan levels also elevate 5-HT synthesis. Conversely, drugs known to accelerate the synthesis of brain 5-HT have been shown to induce parallel changes in brain tryptophan concentrations (Gessa and Tagliamonte, 1974). On the other hand, Neckers et al. (1977) demonstrated that dietary manipulations and drug treatments producing changes in brain tryptophan actually result in reciprocal changes in TPOH assayed in vitro.

The concentration of BH_4 in rat brain has been estimated to be

from 1 to 3 uM (Levine et al., 1979; Bullard et al., 1978; Mandell et al., 1980); a concentration far below the Km of TPOH for this cofactor. Until recently, this suggested that the activity of the aromatic amino acid hydroxylases were severely limited by cofactor concentration in vivo. However, Levine et al. (1981) recalculated the concentration of BH_4 in the striatum based largely on the observation that the cofactor was highly localized to dopamine terminals in this region and on the assumption that these terminals represent 1% of striatal volume. If their intraneuronal estimate of 100 uM BH_4 can be assumed to hold true for 5-HT neurons, then 5-HT synthesis is certainly not limited by the availability of cofactor. Further work is needed to be able to directly estimate BH_4 levels in 5-HT neurons and thereby clarify the role of this cofactor in the regulation of 5-HT synthesis.

Brain TPOH is sensitive to inhibition by high concentrations (>0.2 mM) of its substrate in the presence of BH_4, but not in the presence of $DMPH_4$ (Friedman et al., 1972; Tong and Kaufman, 1975; Youdim et al., 1975). Fifty percent inhibition occurs when the concentration of tryptophan is raised to 1 mM (Friedman et al., 1972). Similarly, TPOH from bovine pineal shows substrate inhibition with BH_4 but not with $DMPH_4$ (Hori and Ohotani, 1981).

Brain TPOH does not appear to be inhibited in vitro by high levels of oxygen in the presence of either BH_4 or $DMPH_4$ (Friedman et al., 1972). On the other hand, Hori and Ohotani (1981) recently showed that pineal TPOH activated by dithiothreitol was slightly inhibited by 20% oxygen as compared to 5% oxygen, when the enzyme was assayed with BH_4 but not with $DMPH_4$. The effect of oxygen on TPOH activity

is complex, and the inhibition observed by Hori and Ohotani may be due to the oxidation of disulfide bonds and iron sites at the enzyme catalytic site (see below, Kuhn et al., 1980).

In the presence of BH_4, TPOH is significantly inhibited by 0.1 mM 5-HTP, but not by the same concentration of 5-HT (Kaufman, 1974). Jequier et al. (1969) observed significant inhibition of the enzyme in the presence of $DMPH_4$ only when the concentration of 5-HTP reached 1 mM. Since such high levels of 5-HTP were required for TPOH inhibition, these authors discounted the physiological significance of product inhibition of this enzyme.

The evidence in vivo for end-product inhibition of 5-HT synthesis is contradictory. Millard et al. (1972) reached the conclusion that 5-HT synthesis is not regulated by end-product inhibition based on the finding that the calculated turnover rate of 5-HT in animals injected with pargyline followed by radioactive tryptophan was not different from that in control (no pargyline) animals. Similarly, large increases in brain 5-HT produced by MAO inhibition failed to suppress a diet-induced increase in brain 5-HT synthesis (Jacoby et al., 1972). On the other hand, Macon et al. (1971) and Hamon et al. (1973) have provided support for the idea of end-product inhibition of 5-HT synthesis. These investigators observed a decrease in 5-HT synthesis in animals receiving MAO inhibitors 3 hours or less prior to intracisternal injection of tryptophan and in slices of striatum treated to increase intraneuronal 5-HT stores.

Figure 3. Comparison of cofactors used in assays of TPOH. 6-MPH_4 has the same structure as $DMPH_4$ without the $-CH_3$*. Adapted from Azmitia (1973).

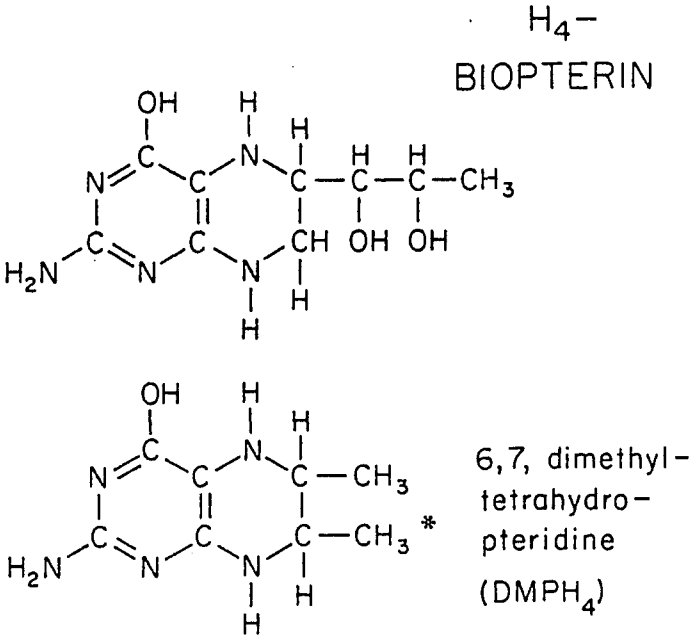


TABLE 1

KINETIC PARAMETERS OF TPOH AS REPORTED IN THE LITERATURE

Enzyme Source	Km for TRP* (uM)	Km for BH4 (uM)	Km for 6-MPH4 (uM)	Km for DMPH4 (uM)	Km for O_2*	Reference
rat brainstem partially purified	300 (DMPH4)	5		30		Jequier et al., 1969
guinea pig brainstem partially purified	300 (DMPH4)			60		Ichiyama et al., 1970
whole homogenate	20					
rabbit hindbrain partially purified	50 (BH4) 290 (DMPH4) 78 (6-MPH4)	31	67	130	2.5% (BH4) 20% (DMPH4)	Friedman et al., 1972
rabbit hindbrain partially purified		32				Tong and Kaufman, 1975
pig brainstem partially purified	500 (DMPH4)					Youdim et al., 1975
40,000xg sup.	500 (DMPH4)					
whole homogenate	20					
rat brainstem 35,000xg sup.	228-34 (6-MPH4)		202-8			Hamon et al., 1978a,b
mouse midbrain 100,000xg sup.	17 (6-MPH4)		200			Lysz and Sze, 1978
mouse midbrain 35,000xg sup.		60				Knapp et al., 1981
rat midbrain 40,000xg sup.	80-132 (6-MPH4)		210-17			Kuhn et al., 1978 1979

*Km determined in the presence of cofactor noted in parentheses.

V. In Vitro Activation of Tryptophan Hydroxylase

A. Effects of the reducing environment

Early investigators of TPOH found the use of mercaptoethanol essential for measuring TPOH activity in mouse mast cells (Hosada and Glick, 1966; Sato et al., 1967). Relatively high concentrations of mercaptoethanol (0.05 M) were needed in the incubation mixture to obtain optimal hydroxylating activity in partially purified preparations from rat brainstem, bovine pineal and mouse mast cells (Lovenberg et al., 1968). Subsequently, Ichiyama and coworkers (1970) demonstrated that hydroxylation by partially purified hindbrain enzyme almost absolutely required a sulfhydryl compound such as dithiothreitol (DTT). Reduced pyridine nucleotides (NADPH, NADH) could partially substitute for the sulfhydryl compound, but in its presence, NADH or NADPH had no further stimulatory effect. When Friedman et al. (1972) showed that optimal enzyme activity could be achieved by inclusion of the NADH-dihydropteridine reductase regenerating system in the absence of DTT (mercaptans were inhibitory under these conditions), the question arose as to whether the stimulation by DTT observed previously was due to its reducing effects on the pterin cofactor or on the hydroxylase itself. In this study, an almost complete dependence on catalase was observed in the absence of a sulfhydryl compound (Friedman et al., 1972).

Enzyme purified 15-fold from pineal was shown to be activated 50-100 times by preincubation with DTT or other thiols under anaerobic conditions (Ichiyama et al., 1974; Hori, 1975). Inclusion of Fe^{++} in the preincubation mixture markedly accelerated activation in the

presence of DTT but had little effect on enzyme activity in its absence. Hori (1975) demonstrated that inclusion of the BH_4-regenerating system in the assay could not substitute for the effect of preincubation of enzyme with sulfhydryl agent. Kinetic analysis of activation by DTT in the presence and absence of Fe^{++} suggested that pineal TPOH possesses 2 or more sulfhydryl-interacting sites: one specifically requires a sulfhydryl agent, whereas the other can be reduced nonspecifically by sulfhydryl agents, Fe^{++}, or other reducing agents.

In contrast to this, Hamon et al. (1978b) found that preincubation with DTT alone under anaerobic conditions failed to activate rat brain TPOH. Only the combination of DTT and Fe^{++} was able to stimulate enzyme activity by 73%. Removal of these compounds after preincubation by filtration of the enzyme preparation through Sephadex G25 did not reverse this activation, suggesting that the enzyme itself had been altered. Enzyme activated by DTT/Fe^{++} had a lower pH optimum and a different ionic charge than untreated enzyme. Moreover, preincubation induced a doubling of the Vmax of the enzyme with no change in the Km for tryptophan and for 6-MPH_4.

Recently, experiments by Kuhn et al. (1980) have indicated that in the previous studies, TPOH was being reactivated by preincubation with sulfhydryl agents and/or Fe^{++}. In this study, midbrain TPOH lost activity upon exposure to O_2. This loss could be prevented by the inclusion of sulfhydryl agents or other reductants, such as ascorbic acid and sodium borohydride, in the incubation medium. The activity of enzyme exposed to O_2 was reconstituted by anaerobic

incubation in the presence of DTT and Fe^{++}, the extent of reactivation being dependent on the extent of initial inactivation. These investigators propose that the catalytic activity of TPOH is dependent on the oxidation/reduction status of -SH groups and iron (see below) probably located at the catalytic site of the enzyme. The oxidized from of TPOH is inactive, but upon reduction of disulfide bonds ($S_2 \longrightarrow (SH)_2$) and iron sites ($Fe^{+++} \longrightarrow Fe^{++}$), the enzyme is able to express hydroxylating activity once again.

B. Effects of Fe^{++}

Mouse mast cell TPOH activity was shown early to be completely dependent upon exogenous ferrous ion (Lovenberg et al., 1965), whereas enzyme from pineal and brainstem did not have an absolute requirement for Fe^{++} (Lovenberg et al., 1968). However, inhibition of pineal (Jequier et al., 1969) and brainstem enzyme (Jequier et al., 1969; Ichiyama et al., 1970) by the iron chelators o-phenanthroline and α, α -dipyridyl could be reversed by the addition of ferrous ion. This suggested that Fe^{++} is a necessary component of the enzyme system. Moreover, partially purified pineal (Jequier et al., 1969) and brainstem enzyme (Ichiyama et al., 1970) was activated by Fe^{++}. Although Ichiyama and his colleagues demonstrated a stimulatory effect of Fe^{++} on TPOH activity in the presence of catalase, Friedman and his coworkers (1972) observed such stimulation only in the absence of catalase. This latter observation suggested that Fe^{++} might substitute for catalase in protecting a sensitive component of the hydroxylating system from oxidation by hydrogen peroxide.

Contrary to the above observations, Youdim et al. (1974; 1975)

could find no effect of Fe^{++} on crude soluble or partially purified brainstem enzyme, despite a 35-48% inhibition of activity by iron chelators. These investigators attributed the inhibition by iron chelators to the chemical nature of these compounds: their heterocyclic nitrogen structure resembles that of the pteridines, and thus they may inhibit TPOH activity by competing with cofactor. Hosoda (1975) subsequently demonstrated actual competitive inhibition of TPOH from mast cells by o-phenanthroline with respect to both tryptophan and $DMPH_4$.

Kuhn et al. (1980) have provided evidence that the inhibitory action of metal chelators is not the result of competitive inhibition, but is probably due to the binding of chelators directly to Fe^{++} within the enzyme. TPOH preincubated with iron chelator and then chromatographed on a column of Sephadex G25 in order to remove unbound chelator, remained inhibited to the same extent as when the chelator was present in the assay. Moreover, addition of Fe^{++} to the assay after preincubation with chelator partially reversed the inhibition.

In view of the many similarities between all the aromatic amino acid hydroxylating enzymes (tryptophan, tyrosine, phenylalanine), it is relevant to note that purified rat liver phenylalanine hydroxylase (Fisher et al., 1972) and bovine adrenal tyrosine hydroxylase (Hoeldtke and Kaufman, 1977) have been shown to contain iron. Thus, although an iron requirement for TPOH has not been demonstrated conclusively, the possibility that this is an iron enzyme cannot be ruled out.

C. Effect of Ca^{++}

1. Millimolar concentrations

Changes in impulse flow along 5-HT neurons have been shown to alter 5-HT turnover. Stimulation of the midbrain raphe nuclei increases the metabolism (Sheard and Aghajanian, 1968) and synthesis (Shields and Eccleston, 1972) of 5-HT in the forebrain. Activation of tryptophan hydroxylase in vivo, as measured by 5-HTP accumulation after AADC inhibition, occurs independently of changes in brain tryptophan levels (Herr et al., 1975). Similarly, depolarization of brain slices stimulates 5-HT synthesis in the absence of changes in intracellular tryptophan, tryptophan in the incubation medium, and tryptophan uptake (Elks et al., 1980). Thus, it is apparent that the activity of TPOH is regulated by an intrinsic mechanism called into play when the nerve terminal is depolarized.

In 1975, Knapp et al. and Boadle-Biber independently observed that crude extracts of TPOH were activated by 0.5-10 mM Ca^{++}. This lead to the suggestion that the increase in 5-HT synthesis in the brain induced by neural activation might be due to the influx of Ca^{++} associated with membrane depolarization and resulting activation of TPOH in the nerve ending.

However, it was soon suggested and then demonstrated by Hamon and his colleagues (Hamon et al., 1977; Hamon and Bourgoin, 1979) that this hypothesis could not account for the activation of TPOH by millimolar concentrations of Ca^{++} as observed by Knapp et al. and by Boadle-Biber. Hamon et al. (1977) emphasized that the millimolar concentrations of Ca^{++} used in the earlier studies were 10^3 to 10^4 times greater than those found even under extreme physiological

conditions. They further demonstrated that under these conditions, activation of TPOH was irreversible and the consequence of partial proteolysis of the enzyme by a Ca^{++}-dependent neutral proteinase (Hamon et al., 1977; Hamon and Bourgoin, 1979). Native enzyme could be activated by phosphorylating conditions, trypsin, sodium dodecyl sulfate, and some phospholipids before, but not after exposure to millimolar Ca^{++}. Based on these observations, the authors proposed that a 60,000 MW fragment removed by the proteinase may be a regulatory subunit of TPOH upon which all of these factors act. Stimulation of TPOH by DTT/Fe^{++} still occurred after exposure to millimolar Ca^{++}, indicating that activation by these agents involves another site on the enzyme, possibly the catalytic site, as suggested by Kuhn et al. (1980).

2. Micromolar concentrations and phosphorylating conditions

Boadle-Biber (1978; 1979a) demonstrated that when enzyme was prepared from brainstem slices depolarized in a K^{+}-enriched incubation medium or from slices incubated with agents promoting Ca^{++} uptake, its activity was greater than enzyme prepared from normal tissue. This increase in activity was reflected mainly in a decrease in Km for both substrate and cofactor (6-MPH_4). The activation effect of these treatments was likely related to the Ca^{++}-dependent phosphorylation mechanism to be discussed in this section.

Within the last 5 years, several groups of investigators reported a c-AMP independent activation of brainstem TPOH by ATP and Mg^{++}, implicating a phosphorylation process in TPOH regulation (Hamon et al.,

1978c; Kuhn et al., 1978; Lysz and Sze, 1978; Yamauchi and Fujisawa, 1979a). Hamon et al. (1978c) observed that inclusion of micromolar Ca^{++} in the assay further enhanced enzyme activity, but only when ATP and Mg^{++} were present. However, Vitto and Mandell (1981) could not find any further increase in activation by ATP and Mg^{++} when low levels of Ca^{++} were included in the assay.

Phosphorylating conditions have been demonstrated variously to decrease Km and increase Vmax for cofactor (6-MPH_4) only (Yamauchi and Fujisawa, 1979a), to decrease Km for both substrate and cofactor (Hamon et al., 1978c) and to decrease Km for cofactor only (Kuhn et al., 1978; Lysz and Sze, 1978).

Yamauchi and Fujisawa (1979a) have provided evidence for a Ca^{++}-dependent phosphorylation/dephosphorylation process at work in the regulation of TPOH. Following activation by ATP, Mg^{++}, and Ca^{++} and subsequent removal of the Ca^{++} by EGTA, TPOH was reversibly deactivated. Further addition of Ca^{++} and ATP resulted in restoration of initial activity. These observations, taken together with the fact that deactivation was inhibited by addition of sodium fluoride or omission of Mg^{++}, suggested that brain TPOH is regulated by a Ca^{++}-dependent protein kinase and a Mg^{++}-dependent phosphatase. In support of this, Lysz and Sze (1978) showed TPOH to be sensitive to inactivation by acid phosphatase.

Finally, activation of TPOH by phosphorylating conditions has now been shown to be completely dependent on the calcium-binding protein, calmodulin (Yamauchi and Fujisawa, 1979b; Kuhn et al., 1980) and a calmodulin-dependent protein kinase isolated from cerebral cortex

(Yamauchi and Fujisawa, 1980). Yamauchi and coworkers (Yamauchi et al., 1981) have recently demonstrated further that activation of TPOH by Ca^{++}-, calmodulin-dependent protein kinase requires an activator protein. Whether the activator protein is required for phosphorylation of TPOH by the Ca^{++}-,calmodulin-dependent kinase or for activation of the phosphorylated enzyme remains to be clarified.

Despite the evidence that depolarization of 5-HT neurons leading to an influx of Ca^{++} results in activation of TPOH, Bourgoin et al. (1980) have demonstrated that although stimulation of the nucleus raphe magnus produces a significant increase in 5-HT synthesis in the spinal cord, this increase in not associated with a persisting activation of TPOH activity in soluble extracts of the cord. That is, TPOH isolated from stimulated tissue was still activated by ATP, Mg^{++}, and Ca^{++}. Although this suggests that electrical stimulation produces an increase in 5-HT synthesis not associated with TPOH activation, more work is required before this conclusion is warranted. The neuronal environment is very different from the conditions of in vitro enzyme assays, so that phenomena occurring in vivo may be lost in vitro.

VI. Assays of Tryptophan Hydroxylase Activity

The first demonstration of TPOH activity in a cell-free preparation of mammalian brain tissue was achieved by Grahame-Smith (1964; 1967) using D-L-[3-^{14}C]tryptophan as substrate. Radioactive 5-HTP formed by hydroxylation was isolated, converted to 5-HT by purified AADC, and then quantitated. The series of column and paper chromatographic procedures used to isolate and purify 5-HTP and then 5-HT were laborious and time-consuming, making use of this assay

impractical for further studies of TPOH. Other more rapid and sensitive methods were needed before further progress was to be made.

A. Assays using ring-labelled ^{14}C-tryptophan

The major drawback of these methods was the requirement that the substrate be isolated from the product. Gal et al. (1966) attempted to separate the radioactive 5-HTP formed in the presence of a decarboxylase inhibitor from the radioactive substrate tryptophan by column and paper chromatography. An overall recovery of only 20-25% was achieved. Assays used by other groups (Grahame-Smith, 1964; 1967; Green and Sawyer, 1966) depended on the isolation and quantitation of radioactive 5-HT produced in the presence of excess AADC. The more useful method developed by Lovenberg et al. (1967) did not require recovery and quantitation of product but relied on the determination of its specific activity. The two-step assay took advantage of the greater affinity of AADC for 5-HTP as compared to tryptophan. An initial incubation of enzyme source with L-tryptophan-3-^{14}C, a known amount of 5-HTP (in excess), a monoamine oxidase inhibitor, and other appropriate reactants was followed by a second incubation with AADC. 5-HT formed in the second reaction was isolated by column chromatography, and its specific activity was determined. The amount of tryptophan converted to 5-HTP could be calculated since the specific activity of newly formed 5-HT was the same as the specific activity of 5-HTP.

B. Assay using DL-[5-^{3}H-]tryptophan

Renson and coworkers (1966) introduced an assay for TPOH which

measured the release of tritium into the incubation medium from tryptophan labelled with tritium in the 5-position. In the process of developing this assay, these investigators discovered what soon became known as the NIH shift, a general phenomenon common to all the aromatic amino acid hydroxylation reactions. The product of the hydroxylation reaction, [4-^{3}H-]5-HTP, was the result of an intramolecular migration of [^{3}H]. Under acidic conditions, tritium in the 4-position exchanged readily with water, thereby allowing quantitation of the amount of substrate hydroxylated. Lovenberg et al. (1971) were able to improve the quality of the substrate (5-^{3}H-tryptophan) by using lower specific activity tritium in its preparation and by employing a rigorous isolation and purification procedure. However, despite the better quality of the substrate, the preparation could be used only for 3 to 4 weeks without substantial increase in blank values.

C. Assay using carboxyl-labelled ^{14}C-tryptophan

Ichiyama et al. (1968; 1970) developed a rapid, sensitive, and convenient method for measuring TPOH using L-[1-^{14}C-]tryptophan as substrate. Like that of Lovenberg et al. (1967), the assay takes advantage of the differing Km's of AADC for tryptophan and for 5-HTP (2-4 orders of magnitude greater for tryptophan than for 5-HTP). However, the method of Ichiyama et al. obviates the need for product separation by measuring the release of $^{14}CO_2$ liberated from 5-HTP in the presence of excess AADC. The major disadvantage of this assay has been that at high concentrations of tryptophan, direct decarboxylation of substrate seems to occur, making it difficult to

measure TPOH activity at saturating levels of substrate.

D. Assay using ^{3}H-methyl-S-adenosylmethionine

More recently, Kizer et al. (1975) introduced an assay for TPOH sensitive enough for measuring enzyme activity in as little as 5 ug of crude brainstem homogenate (at least 100 times more sensitive than any of the other methods available). This 3-step assay is based on the enzymatic conversion of the product of hydroxylation (5-HTP) to ^{3}H-melatonin as follows:

$$\text{L-tryptophan} \xrightarrow{\text{TPOH}} \text{5-HTP} \xrightarrow{\text{AADC}} \text{5-HT} \xrightarrow[\text{transferase}]{\text{N-acetyl}} \text{N-acetyl 5-HT} \xrightarrow{\text{HIOMT}} {}^{3}\text{H-melatonin}$$

The tritiated melatonin formed by the transfer of a [^{3}H-]methyl group from [^{3}H-methyl]-S-adenosylmethionine to N-acetyl 5-HT by hydroxyindole-O-methyltransferase (HIOMT) is isolated by organic extraction into toluene and quantitated. The amount of tryptophan hydroxylated is equal to the amount of tritiated melatonin formed. Although suitable for measuring TPOH activity in small amounts of tissue, the complexity of preparation for and performance of this method makes its impracticable.

E. Fluorescence methods

In 1972, Friedman and his colleagues proposed a sensitive and direct assay of TPOH in brain tissue based on the differential fluorescent properties of 5-HTP and tryptophan. The formation of 5-HTP in the presence of a decarboxylase inhibitor (NSD 1034) is measured

fluorometrically with quenching of tryptophan being easily corrected for. This method is not restricted to the use of low tryptophan concentrations, allowing enzyme activity to be measured at its Vmax. Moreover, the use of radioactive compounds is not required. However, the number of samples in a single assay is limited by the necessity of centrifuging the samples after stopping the reaction.

Gal and Patterson (1973) introduced an assay for TPOH similarly based on the fluorescent properties of 5-HTP. These fluorescence assays have been particularly suitable for measuring TPOH activity at various stages of purification and for determining the kinetic and other properties of this enzyme.

VII. The Role of the Hippocampus in the Limbic System

The concept of the limbic lobe was introduced in the late nineteenth century by Broca to characterize the ring of cortical tissue encircling the upper brainstem. The limbic lobe consists of the cingulate, subcallosal, and parahippocampal gyri and the underlying hippocampal formation. The hippocampal formation includes the hippocampus proper (Ammon's horn), the dentate gyrus, and the transitional cortical areas situated between the neocortex and the hippocampus (entorhinal cortex, parasubiculum, presubiculum, and subiculum).

In 1937, Papez hypothesized that the limbic lobe formed an essential link between subjective (conscious) emotional experience originating in the cortex and the autonomic expression of emotions, which was dependent on the integrative action of the hypothalamus. He

suggested that cortical influences on emotive processes reached the hypothalamus only after passing through the hippocampal formation. Cortical information was "built up" in the hippocampus and then projected via the fornix to the mammillary bodies. In turn, hypothalamic information reached the cortex by way of the mammillothalamic tract to the anterior thalamic nuclei and then via a pathway from the anterior thalamic nuclei to the cingulate cortex. "Radiation of the emotive process from the gyrus cinguli to other regions in the cerebral cortex would add emotional coloring to the psychic processes occurring elsewhere," (Papez, 1937).

In the decades since the introduction of Papez' theory, neuroanatomists have demonstrated extensive, direct connections between the hippocampal formation and both cortical and subcortical regions of the brain. In the monkey, the entorhinal cortex receives input from the temporal, orbitofrontal, and prepyriform cortices and in turn projects to the dentate gyrus and pyramidal cells of the hippocampus (Van Hoesen et al., 1972). Subcortical connections to the hippocampal formation include direct afferents from the thalamus, hypothalamus, septum, preoptic area, and brainstem (Wyss et al., 1979). The major efferent pathways from the hippocampal formation to other cortical areas (perirhinal, retrosplenial, and cingulate) arise from the subicular complex (Rosene and Van Hoesen, 1977), although a few direct connections from the CA regions to these areas were demonstrated by Swanson and Cowan (1977). From these cortical areas, information is relayed to nearby neocortical association areas (Rosene and Van Hoesen, 1977). The subicular region also projects subcortically to the septal and anterior thalamic nuclei, as well as to the mammillary nuclei of

the hypothalamus, whereas the CA fields appear to project exclusively to the septum (Swanson and Cowan, 1977; Miebach and Siegal, 1977).

From the previous brief discussion, it is apparent that the hippocampal formation is in a neuroanatomical position to be able to modulate neuronal activity in widespread areas of the brain and thereby play a major role in the regulation or modulation of behavior. Rather than exerting direct control over the animal's internal environment, the hippocampus seems to function in the evaluation of and response to events in the external environment. It must be noted, however, that a close but unclear reciprocal association between the hippocampus and neuroendocrine systems, particularly the adrenal-pituitary axis, has been found (McEwen et al., 1975; deKloet et al., 1980; Feldman and Conforti, 1980; Wilson et al., 1980). Thus, there definitely exists some sort of influence of the animal's internal environment on the behaviors mediated by the hippocampus and of the hippocampus on the neuroendocrine changes occurring under various behavioral states.

Changes in locomotor activity, exploratory behavior, distractability, spontaneous alternation, acquisition and performance of certain behavioral tasks, and short term memory have all been observed in animals with hippocampal lesions (see Isaacson, 1982, for a review of the behavioral literature).

VIII. Serotonin and the Hippocampus

The increase in locomotor activity observed in rats following electrolytic lesion of the median raphe nucleus has been suggested by Jacobs et al. (1975) to be the result of a reduction of hippocampal

5-HT. In support of this, these investigators showed that neither PCPA nor median raphe lesions altered locomotor activity in hippocampectomized animals. Lorens et al. (1976) also observed an increase in activity of rats lesioned electrolytically in the raphe nuclei, but they could not detect such a change in animals lesioned with the serotonin neurotoxin 5,7-dihydroxytryptamine (5,7-DHT). However, more recently, Williams and Azmitia (1981) reported that the increase in nocturnal locomotor activity observed in rats after 5,7-DHT lesions of the fornix-fimbria was significantly correlated with a reduction in synaptosomal uptake of ^{3}H-5-HT in the dorsal hippocampus.

Further evidence of a role for 5-HT in the modulation of hippocampal function comes from electrophysiological studies. Spontaneous activity of hippocampal pyramidal cells is inhibited by iontophoresis of 5-HT (Segal, 1975). Similarly, raphe stimulation results in a long latency, long duration inhibition of hippocampal pyramidal cells which can be antagonized by the 5-HT receptor antagonists methysergide and cyproheptadine and blocked by PCPA treatment (Segal, 1975; 1976). Granule cells of the dentate gyrus have also been shown to be inhibited by iontophoretic application of 5-HT (Assaf et al., 1981) as well as by stimulation of the median raphe (Assaf and Miller, 1978). This latter group of investigators also showed that median raphe stimulation results in a potentiation of responses (population spike) in the dentate gyrus produced by perforant path stimulation (Assaf and Miller, 1978).

Studies of hippocampal theta rhythm also reveal inhibitory

influences of the serotonergic system on hippocampal electrical activity. Theta rhythm in the hippocampus consists of high amplitude slow waves (4-12 Hz) which are controlled by pacemaker cells in the medial septal area (Rawlins et al., 1979). Interference with 5-HT transmission in the hippocampus by systemic administration of PCPA and methysergide or by lesion of the fornix-fimbria and/or cingulum bundle with 5,7-DHT, results in a decrease in the threshold for septal elicitation of hippocampal theta (McNaughton et al., 1977; 1980). Using the same technique of intracerebral injection of 5,7-DHT to deplete hippocampal 5-HT (Azmitia et al., 1978), Srebro et al. (1982) have shown that behavioral modulation of neuronal transmission from the perforant pathway through the dentate gyrus is dependent on the connectivity of median raphe 5-HT neurons with the hippocampus. Thus, following 5,7-DHT lesions of the cingulum bundle and fornix-fimbria, the difference in the granule cell population spike evoked by perforant path stimulation normally observed between animals in slow-wave sleep and in animals in the still-alert state is abolished.

Gray (1982) has suggested that the function of the septo-hippocampal system is to compare actual with expected stimuli. When the animal's "plan" is interrupted by adverse conditions (e.g. novelty, error), the job of this system is to "work out what has gone wrong so that existing plans can be applied again or new ones substituted." He proposes that the ascending 5-HT projection, both to the hippocampus and septum, is mainly involved in the output phase of this behavioral modification system and subserves an overall inhibitory function.

IX. Central Nervous System Plasticity

The growth potential of the adult mammalian central nervous system (CNS) has been the subject of much research and controversy since the late nineteenth century. Initially, interpretation of observations on the CNS response to injury relied heavily on studies of normal CNS development. Ramon y Cajal stressed the applicablitiy of the laws of neurogenesis to regenerative phenomena because, "...the regenerative process of the nerves and central tracts interrupted by traumatic or toxic violence represents merely a repetition, under somewhat special environmental conditions, of the creative act of the embryonic neural appendices and nerve trunks" (1959). However, in contrast to the immature, growing CNS, Ramon y Cajal and his contemporaries could find no evidence for the (re)establishment of functional connections after damage to adult central axons. Spinal cord transection in mammals resulted in abortive regeneration. Unable to cross the glial membrane and scar tissue formed in response to injury, the numerous fine sprouts observed a week after the lesion, largely disappeared by one month (Ramon y Cajal, 1959). Attempts at regeneration by damaged axons in the cerebrum and cerebellum similar in extent to those observed in the cord were never detected, fostering the view that the brain is far less plastic than the spinal cord.

In the 1950's, Windle and his colleagues were able to observe regenerating fibers bridging the transection site of the severed spinal cord of animals receiving bacterial pyrogens to inhibit scar tissue formation (Windle, 1956). Regeneration was demonstrated only in cases where the usual dense fibrous scar at the lesion site was replaced by

loose, highly vascularized connective tissue. Other studies indicated that damaged fibers in the brain and cord could regrow along guiding structures, such as transplants of tumor cells and epidermis, placed in their proximity (see Windle, 1956). By the late 1950's, it was apparent that adult central neurons retained the ability to grow and could do so under favorable conditions.

A. Collateral sprouting

Research in the area of central neuroi plasticity acquired a new dimension when Liu and Chambers (1958) reported sprouting of intact intraspinal dorsal root axons in the cat spinal cord in response to destruction of either adjacent dorsal roots or the corticospinal tract. The suggestion that intact nerve fibers could grow new branches in response to damage of neighboring fibers was not novel. The phenomenon of collateral sprouting had previously been described in the peripheral sensory and motor nervous systems. Sprouting of residual motor axons after partial muscle denervation had been demonstrated in the rat in the late 1940's (see Edds, 1953). Reinnervation of empty neuromuscular junctions by new fibers resulted in functional recovery. Similarly, intact sensory axons were shown to sprout into zones of sensory loss in the skin (Weddell et al., 1941). Collateral sprouting in the autonomic nervous system was demonstrated by Murray and Thompson in 1957. These investigators found that structural and functional recovery of the superior cervical ganglion following removal of up to 90% of preganglionic fibers was the result of sprouting of remaining intact fibers.

The experiments of Liu and Chambers (1958) demonstrated that

collateral sprouting was not restricted to the peripheral nervous system. Subsequent work by Liu and his coworkers (McCouch et al., 1958) showed that the increase in the electrophysiological response to dorsal root stimulation and the appearance of spasticity in hemisected monkeys and cats was accompanied by sprouting of dorsal root afferents on the side of hemisection. This indicated that, as in the periphery, sprouting fibers in the CNS were able to form functional, although not necessarily appropriate, synapses. Since these classical studies, collateral sprouting has been demonstrated using a variety of techniques in many regions of the CNS. However, in only a few cases has it been shown that collateral sprouting results in the restoration of normal function.

1. Collateral sprouting following mechanical and electrolytic lesions

One of the first examples of collateral sprouting in the adult brain came from the studies of Goodman and Horel (1966) on the rat visual system. Using the Nauta stain for degenerating fibers, these investigators demonstrated that in response to visual cortex destruction, optic tract fibers sprouted to innervate areas vacated by degenerated corticofugal fibers. Interestingly, sprouting did not occur in all areas previously innervated by the damaged corticofugal fibers even though these regions were closely associated with optic tract projections. New growth was detected only in regions of major convergence of the 2 fiber systems.

Several explanations were offered to account for the observation of a highly specific sprouting response. All fibers in the denervated

areas may have been equally capable of filling vacant synaptic sites, and successful sprouting depended on factors such as relative percentage of total afferents, proximity to deafferented sites, and inherent axonal growth rate. Thus, in this study, it was possible that other fibers had sprouted in areas where optic tract fibers did not sprout and this response could not be detected by the methods used to investigate optic tract fibers only. Alternatively, fibers may have had differing affinities for synaptic sites so that if compatible sites were unavailable, fibers did not sprout.

Goodman and his colleagues hypothesized that if potential limiting factors such as competition and specificity were reduced, generalized sprouting by a neuronal system would occur. By unilateral eye removal in the rat, partial denervation (removal of crossed retinofugal fibers) of brain regions receiving visual input was accomplished and the opportunity for sprouting of compatible fibers (uncrossed retinofugal fibers from the other eye) was maximized in each area receiving retinal input (Goodman et al., 1973). Under this experimental paradigm, Goodman et al. observed generalized sprouting in all partially deafferented areas. However, other investigators have found that this is not the case in adult species other than the albino rat, such as the cat (Guillery, 1972; Hickey, 1975; Kalil, 1973) where there is no overlap of retinal terminals from both eyes. Stelzner et al. (1976) as well as others, have suggested that terminal overlap is one of the major factors in determining the success of sprouting into denervated areas.

The first electron microscopic description of collateral sprouting

in the brain came from Raisman (1969). The medial septal nucleus is the recipient of medial forebrain bundle (MFB) fibers from the brainstem which terminate on dendrites and neuronal cell bodies in the nucleus and of fimbrial fibers from the hippocampus which terminate on dendrites only. Removal of either of these inputs resulted in a change in the distribution of the remaining input. Thus, after fimbrial lesions, MFB fibers expanded their contacts with dendrites, whereas after MFB lesion, fimbrial fibers contacted not only dendrites but cell bodies as well. In a quantitative reexamination of the sprouting response to fimbrial lesions, Raisman and Field (1973) found that by one month after fimbrial lesion, normal numbers of dendritic synaptic contacts were restored by sprouting MFB fibers and the number of axon terminals making more than one synaptic contact increased significantly.

In the red nucleus, collateral sprouting has been shown to result in electrophysiologically functional synapses (Tsukahara, 1978). The red nucleus receives 2 major inputs: afferents from the cerebral cortex terminate on distal dendrites of red nucleus neurons, whereas afferents from cerebellar nuclei terminate mainly on neuronal cell bodies. Following lesions of the cerebellar nucleus interpositus in cats, electrical responses recorded from rubral neurons by stimulating the cerebral peduncles showed a component having a faster rise time and larger amplitude than the normally slow-rising corticorubral EPSP (Tsukahara et al., 1974; 1975). This suggested that corticorubral fibers had established new synaptic contacts at more proximal portions of the red nucleus neuronal membrane. Electron microscopic studies confirmed this idea by demonstrating new corticorubral synapses on

neuronal somata in response to removal of cerebellar fibers (Nakamura et al., 1974).

Cotman and his group have extensively investigated collateral sprouting in the hippocampus following a variety of extra-, inter-, and intrahippocampal lesions (see Cotman and Lynch, 1976; Cotman and Nadler, 1978; Cotman, 1978). Unilateral entorhinal cortex lesion is followed by a highly specific pattern of synaptic reorganization in the dentate gyrus. This pattern is consistent from animal to animal in adults, but differs markedly from the pattern observed in young animals (Lynch et al., 1973). In both instances, the lesion produces observable changes in the lamination pattern and/or density of all granule cell afferents. Significantly, locus coeruleus fibers, which appear to innervate interneurons in the area, do not seem to react to the lesion (see Cotman and Nadler, 1978). Sprouting of intact contralateral entorhinal afferents in the dentate gyrus results in electrophysiologically functional synapses (Steward et al., 1973) and recovery of behavioral deficits (Loesche et al., 1977).

In contrast to the remarkable plastic response of afferents to the dentate granule cells in response to removal of ipsilateral entorhinal cortex afferents, damage to input derived from contralateral hippocampal pyramidal cells (commissural fibers) has a lesser effect on the existing fiber organization of the dentate gyrus. Removal of commissural fibers has no effect on entorhinal or septal afferents (Lynch et al., 1974). Reinnervation of the deafferented zone does occur (McWilliams and Lynch, 1978), suggesting that projections from the ipsilateral pyramidal cells (associational fibers) sprout to fill

vacated synaptic sites (Cotman and Nadler, 1978).

The studies of sprouting in the hippocampus have clearly demonstrated the highly plastic nature of extrinsic hippocampal afferents and of intra- and interhippocampal connections. Reorganization of afferents in response to lesion depends on the type of afferent removed and on the extent of denervation. Based on observations of the response of the hippocampus to deafferentation, Cotman and Nieto-Sampedro (1982) have drawn the following conclusions:

1. Sprouting completely restores the synaptic input lost following partial denervation.

2. Fibers will sprout only if their terminal field overlaps that of the damaged afferents.

3. Sprouting results in a change in the density or distribution of previously existing connections; new pathways are not established.

4. When a neuron receives input from different sources, there is a definite hierarchy in the ability of the various afferents to sprout in response to deafferentation of that neuron.

Recently, Cotman and colleagues (Hoff et al., 1981a; 1981b) demonstrated synaptic turnover in areas not denervated by unilateral entorhinal lesions. Thus, in the ipsilateral inner molecular layer of the dentate gyrus, where the lesion causes no loss of synapses, there is a rapid 20% decrease in the number of synapses followed by recovery within 10 days of the lesion. A similar loss and recovery of synpases occurs in the contralateral inner molecular layer, although this loss is maximal at 60 days and recovery is not complete until 180 days post-lesion. No degenerating terminals are found at any time in these

regions.

The neurons undergoing synaptic turnover in these studies are likely those simultaneously undergoing sprouting into the denervated outer molecular layer in response to the lesion, suggesting that the two phenomena are intimately related. Sprouting and synaptic turnover may both result from the same signal, that is, the entorhinal lesion. Removal of entorhinal afferents from granule cell dendrites in the outer molecular layer might produce a change in the granule cell dendritic tree as a whole, which is then transmitted to neurons presynaptic to granule cell dendrites. Such a signal could result in a general mobilization of the presynaptic neuron's growth capablities, including both sprouting of new terminals into denervated areas and remodelling of existing ones in intact areas.

Synaptic turnover may be a normal process in the adult CNS which is greatly magnified in neurons stimulated to undergo sprouting by damage to neighboring neurons. Observations in the normal adult CNS suggest that synaptic turnover is not merely a response to a pathological condition. Sotelo and Palay (1971) have observed atypical axonal profiles with occasional sprouts in the lateral vestibular nucleus of the normal adult rat and have suggested that they represent degenerating axon collaterals and terminals with the formation of new ones. Based on these observations, these investigators have proposed that continuous axonal remodelling occurs in the normal adult brain.

2. Sprouting of monoaminergic neurons following mechanical and electrolytic lesions

Detailed study of central monoaminergic neurons has demonstrated their highly plastic nature. Monoaminergic axons have been shown to undergo extensive regeneration following axotomy (see Bjorkund and Stenevi, 1979). In addition, sprouting of intact monoaminergic neurons has been demonstrated to occur in response to lesions of other monoaminergic and non-monoaminergic fibers.

Following removal of the visual cortex, Stenevi et al. (1972) noted an increase in the number of fluorescent terminal and paraterminal catecholamine fibers in the dorsal lateral geniculate (DLG). These investigators attributed the change in adrenergic innervation of the DLG to collateral growth from intact fibers. No change in the adrenergic innervation of other areas denervated by the cortical lesion was observed, suggesting that the sprouting response was selective. Furthermore, unilateral enucleation, which also partially denervated the DLG, produced no change in the adrenergic innervation of this region, indicating that collateral growth was dependent on the type of denervation. It is possible that sprouting occurred exclusively in response to cortical ablation because only this lesion damaged adrenergic axons and resulted in the mobilization of the intact part of the neuronal system to sprout (see pruning response, below). The occurrence of sprouting in only some areas and not others may have depended on the extent of denervation, the distribution of damaged axons and their relationship to intact adrenergic axons, and the compatibility of adrenergic axons to vacated synaptic sites. A major issue not addressed in this study was whether or not in regions where sprouting was observed, adrenergic terminals and damaged terminals converged on the same dendritic tree. Recent

autoradiographic and histofluorescence evidence indicates that DLG neurons receiving both retinal and visual cortical input may also receive noradrenergic input from locus coeruleus neurons (Kromer and Moore, 1980). Electron microscopic studies are required to clarify this question.

Partial lesion of the superior cerebellar peduncle (SCP) which carries noradrenergic (NA) afferents to the cerebellum has been shown to induce a proliferation of NA terminals in the cerebellar cortex (Pickel et al., 1973; 1974) and in the hippocampus (Pickel et al., 1974). Although in these studies is was not possible to determine whether sprouting of NA fibers in the cerebellum was the result of partial removal of NA afferents to the area or to the removal of non-NA affferents, sprouting of NA fibers in the hippocampus, where no deafferentation supposedly occurred, suggests that the SCP lesion stimulated intact collaterals of damaged NA fibers to sprout. That a similar increase in NA terminals in the cerebellum occurred following stab lesions of the locus coeruleus, the cells of origin of cerebellar afferents (Pickel et al., 1973), suggests further the possibility that sprouting occurred from neurons untouched by the lesion (of either the locus coeruleus or the SCP).

When a restricted area of the cortex is destroyed by a vascular lesion, generalized sprouting of NA axons occurs in the ipsilateral and contralateral cortex and in the cerebellum (Robinson et al., 1977). Thus, removal of one part of the locus coeruleus axonal tree produces compensatory sprouting of intact branches of the same neurons. As in the studies of Pickel et al. (1973; 1974) sprouting occurs not only in

deafferented areas, but in regions far from denervated areas. This phenomenon has been termed the "pruning effect" by Schneider (1973) and is hypothesized to be the result of a neuron's attempt to reestablish a normal number of arborizations following loss of some of its branches.

If sprouting occurs from undamaged neurons, as may be the case following partial locus coeruleus lesions (see above, Pickel et al., 1973), pruning may then be the response of a whole neuronal system to damage of its parts. This possiblity postulates the existence of communication between neuronal elements. Damage to fibers in the periphery may cause retrograde changes in the neuronal cell body which could then be transmitted to neighboring cell bodies by a variety of intraneuronal connections.

Evidence exists for a transneuronal mechanism for the induction of sprouting of motor neurons in the frog (Rotshenker, 1979; 1982; Rotshenker and Reichert, 1980). Transection of or application of colchicine (in doses causing partial inhibition of retrograde axonal transport) to the nerve of one cutaneous pectoris muscle of the frog induces the formation of new synapses by the intact nerve of the normal contralateral muscle. These observations suggest that the signal for sprouting and synapse formation in the normal muscle arises in the injured neuron and is transferred transneuronally across the spinal cord to intact motor neurons.

Moore et al. (1971) demonstrated sprouting of NA fibers in the septum in response to lesions of the fornix-fimbria. These investigators attributed the collateral growth of NA fibers in the septum to the removal of non-adrenergic hippocampal afferents to the

area. As Raisman and Field (1973) have suggested, this study may constitute the light microscopic correlate to their electron microscopic observation of sprouting of MFB terminals in the septum after fornix-fimbria lesions. The electron microscopic appearance of sprouting MFB terminals suggested that they belonged to the ascending noradrenergic fiber system (Raisman and Field, 1973). However, it should be noted that the fornix-fimbria also carries NA fibers to the hippocampus. Therefore, the sprouting observed by Moore et al. may have been a pruning response of NA fibers damaged by the fornix-fimbria lesion.

Sprouting of ventral tegmental (A-10) dopaminergic (DA) axons in the olfactory tubercle occurs in response to removal of non-dopaminergic input from cells in the olfactory bulb (Gilad and Reis, 1979). It is unclear whether dopaminergic neurons in the midbrain project only to the olfactory tubercle or whether some of these axons continue anteriorly to innervate the olfactory bulb. Neither substantia nigra nor ventral tegmental lesions result in a significant change in olfactory bulb dopamine levels (Fallon and Moore, 1978), suggesting that the intrinsic dopaminergic juxtaglomerular neurons contain the vast majority of dopamine present in the bulb (Halasz et al., 1977; 1978). Dopamine has also been observed in some external tufted cells, whose axons project out of the bulb via the lateral olfactory tract (Halasz et al., 1977). However, Fallon and Moore (1978) reported the existence of a very sparse extrinsic dopaminergic innervation of the bulb in addition to that of the intrinsic systems. Assuming the existence of connections between the midbrain dopaminergic cells and the olfactory bulb, removal of the bulb

would then result in axotomy of these neurons. If this is the case, the sprouting observed by Gilad and Reis (1978) might be the attempt of damaged axons to regenerate behind the lesioned area. Alternatively, proliferation of dopaminergic terminals in the tubercle might be a pruning response of A-10 axons not damaged by the lesion.

The dopaminergic input to the olfactory tubercle appears to be directly to pyramidal cell bodies as well as to the apical dendrites of these cells (Kreiger et al., 1977). Olfactory bulb afferents also terminate on the distal segments of the apical dendrites of pyramidal cells in the tubercle (Price, 1975). Thus, sprouting of DA terminals following olfactory bulb ablation may be similar to sprouting in the dentate gyrus (Cotman and Lynch, 1976; Cotman and Nadler, 1978), in the red nucleus (Nakamura et al., 1974) , and in the septum (Raisman and Field, 1973). In all these examples, removal of some of the afferents to a neuron (cell body or dendrites) results in expansion of the remaining afferents to fill sites vacated by degenerated fibers. It must be emphasized that this type of sprouting involves inputs to the same or different regions of a single neuron.

3. Sprouting of monoaminergic neurons following neurotoxic lesions

In the vast majority of studies on collateral sprouting, the use of mechanical or electrolytic lesions resulted in damage to all fiber types present in the lesioned area. This has complicated the interpretation of these experiments. In many studies, it cannot be stated with certainty that removal of a specific fiber type is the cause of sprouting of intact fibers. Furthermore, in electron microscopic studies, where degenerating and sprouting fibers can be

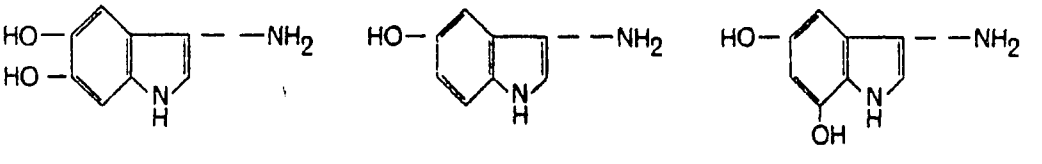
identified and these processes followed (Raisman 1969; Raisman and Field, 1973; Nakamura et al., 1974; Matthews et al., 1976a, b), the chemical identity of the involved fibers has not been conclusively demonstrated. The monoaminergic neurotoxins, 6-hydroxydopamine (6-OHDA), 5,6-dihydroxytryptamine (5,6-DHT) and 5,7-dihydroxytryptamine (5,7-DHT) offer a useful way of producing selective denervation of specific brain regions in the absence of significant scar tissue formation. By monitoring the response to selective denervation, additional insight into the nature of the sprouting signal and response will be gained.

As structural analogues of 5-HT, 5,6- and 5,7-DHT (Figure 4) are selectively accumulated by 5-HT neurons via the high-affinity uptake mechanism for 5-HT located at axon terminals. Once the toxin has reached a critical concentration inside the neuron, it exerts its cytotoxic action, the mechanism of which is controversial. Baumgarten et al. (1982) suggest that the 5-HT neurotoxins become cytotoxic by 2 mechanisms: 1) oxidation of these compounds by mitochondrial cytochrome-C oxidase produces reactive quinoidal intermediates which readily attack nucleophiles in proteins and result in the inactivation of indispensable functional groups in enzymes and structural proteins, and 2) oxidation results in the production of free radicals which also damage proteins essential for the maintenance of cellular function and structure. The role of this latter mechanism in 5,6- and 5,7-DHT toxicity is as yet largely undefined.

Intraventricular administration of 5,6- and 5,7-DHT has been used to investigate the regenerative properties of NA (Bjorklund et al.,

1975; Bjorklund and Lindvall, 1979) and 5-HT neurons (Bjorklund et al., 1973a; Bjorklund and Wiklund, 1980; Baumgarten et al., 1974; Nobin et al., 1973; Wiklund and Bjorklund, 1980). Intracerebral injection of these neurotoxins is useful for the lesioning of specific axonal bundles and for the denervation of specific terminal areas (Bjorklund et al., 1973b; Daly et al., 1973). Because both drugs have a significant damaging effect on NA and DA neurons (Bjorklund et al., 1973b; 1975; Bjorklund and Lindvall, 1979; Liston et al., 1982), the selectivity of action by and the response to these neurotoxins depend on protection of NA and DA neurons by appropriate neuronal uptake blockers. Pretreatment of animals with desmethylimipramine (desipramine, DMI) effectively prevents the neurotoxic effects of 5,6- and 5,7-DHT on NA neurons (Bjorklund et al., 1975; Gerson and Baldessarini, 1975). Amfonelic acid can be used to protect DA neurons from the effects of 5,7-DHT (Liston et al., 1982), whereas nomifensene, a potent inhibitor of both NA and DA uptake, decreases the effects of the neurotoxins on both types of catecholaminergic neurons (Baumgarten et al., 1982). None of these drugs has been shown to reduce the effects of 5,6- and 5,7-DHT on 5-HT neurons.

Figure 4. Chemical structures of 5,6-DHT, 5-HT, and 5,7-DHT. Adapted from Baumgarten et al. (1977).



5,6-Dihydroxytryptamine 5-Hydroxytryptamine 5,7-Dihydroxytryptamine

In a recent series of experiments, Mollgard and Wiklund (1979; Wiklund and Mollgard, 1979) combined intraventricular injections of 5,6- and 5,7-DHT with histofluorescent and electron microscopic analysis of the 5-HT innervation of the rat subcommissural organ (SCO) to demonstrate collateral sprouting of non-monoaminergic fibers in this region. Following removal of the 5-HT innervation of the SCO, fibers having the same ultrastructural appearance as the original 5-HT synapses reinnervate SCO cells. These investigators suggest that the reinnervating sprouts might arise from afferents to nearby SCO-associated neurons, since the ultrastructural appearance of this input is very similar to that of the new synaptic input to the SCO cells.

Azmitia et al. (1978) have used intracerebral injections of 5,7-DHT to investigate plasticity of median raphe 5-HT input to the hippocampus. The cingulum bundle and fornix-fimbria are anatomically separated by the corpus callosum along much of their length. This allows for microsurgical manipulation of either of these pathways while leaving the other intact. Following 5,7-DHT lesions of the cingulum bundle, a loss of 5-HT terminals in the dorsal hippocampus was indicated by a decrease in the uptake of ^{3}H-5-HT by hippocampal slices and a decrease in the amount of ^{3}H-proline transported from the median raphe detected by radioautography. By 28 days after the lesion, 5-HT fibers in the fornix-fimbria were observed to undergo anatomical reorganization resulting in restoration of the normal terminal density in the dentate gyrus. Moreover, reorganization of 5-HT afferents to the hippocampus also corrected the abnormal pharmacologically-induced turning behavior observed in rats 14-28 days

after the lesion. More recent study of this system using the horseradish peroxidase tracing technique, confirms that collateral sprouting of undamaged 5-HT fibers in the fornix-fimbria occurred in response to cingulum bundle lesions (Zhou and Azmitia, 1981). The studies of tryptophan hydroxylase presented in this thesis were undertaken to further characterize this sprouting response using a specific biochemical marker of 5-HT neurons.

MATERIALS AND METHODS

I. Chemicals and Drugs

The following compounds were purchased from Sigma Chemical Co. (St.Louis, Missouri): adenosine 5'-triphosphate (disodium salt), ammonium sulfate (Grade I), catalase (from bovine liver), creatine kinase (Type I), 5,7-dihydroxytryptamine creatinine sulfate, DL-dithiothreitol, ferrous ammonium sulfate, 5-hydroxytryptamine creatinine sulfate, 2-mercaptoethanol, B-nicotinamide adenine dinucleotide (reduced form, disodium salt), pargyline hydrochloride, phosphocreatine (disodium salt), pyridoxal 5'-phosphate, sucrose (Grade I), sodium phosphate (monobasic and dibasic), Trizma (Tris) acetate, Trizma (Tris) base, Trizma (Tris) hydrochloride, L-tryptophan.

The following compounds were obtained from Fisher Scientific Co. (Fairlawn, New Jersey): L-ascorbic acid, calcium chloride, dextrose, ethylenediamine-tetraacetic acid (EDTA), magnesium chloride, sodium metabisulfite.

Biopterin was purchased from Regis Chemical Co. (Chicago, Illinois). DL-6-Methyl-5,6,7,8-tetrahydropterin (6-MPH_4) came from Calbiochem (La Jolla, California). Platinum oxide (Adam's catalyst) came from Matheson Coleman and Bell (Norwood, Ohio). Perchloric acid (70-72%) was purchased from Baker Chemical Co. (Phillipsburg, New Jersey).

Desipramine was obtained from Merrill Labs (Cincinnati, Ohio). Ketalar (ketamine-HCl) was purchased from Parke Davis (Detroit, Michigan), and Rompun (xylazine) came from Haver-Lockhart (Shawnee,

Kansas). Neosporin aerosol (Polymixin B, Bacitracin, Neomycin powder) was obtained from Burroughs Wellcome Co.(Research Triangle Park, North Carolina).

II. Scintillation Compounds

Econofluor and Protosol were obtained from New England Nuclear (Boston, Massachusetts). Toluene (scintillation grade) came from Fisher Scientific Co. PPO (2,5-diphenyloxazole) and POPOP (p-bis[2-(5-phenyloxazolyl)]benzene) were purchased from Amersham (Arlington Heights, Illinois). Absolute ethanol was obtained from US Industrial Chemicals (Newark, New Jersey).

III. Isotopes

L-Tryptophan-1-^{14}C (specific activity 52.3 mCi/mmol) and ^{3}H-5-hydroxytryptamine creatinine sulfate (specific activity 33.8 Ci/mmol) were purchased from New England Nuclear.

IV. Special Equipment

Item	Source
Autoclips	Clay Adams, Parsippany, N.J.
Center wells	Kontes, Vineland, New Jersey
Centrifuge (RC2-B)	Sorvall, Norwalk, Conn.
Cornwall automatic pipettor	Becton-Dickinson, Rutherford, N.J.
Dialysis tubing	Arthur H.Thomas Co., Philadelphia, Pa.
GF/B Filters	Whatman, England
Homogenizer (glass-teflon	Arthur H.Thomas Co.

clearance .10-.15 mm)

Omnimixer	Sorvall
SM-24 rotor	Sorvall
SS-34 rotor	Sorvall
Rubber stoppers	Kontes
Stereotaxic apparatus	Prior, England
Swinnex filter (.45 um)	Millipore, Bedford, Mass.
Titertek cell harvester	Flow Laboratories, Rockville, M.D.
Tissue culture plates	Linbro Scientific, Hamden, Conn.

V. Experimental Animals

Male Sprague-Dawley rats were used in all studies reported here. Animals obtained from Perfection Breeders (Douglassville, Pa.) weighed 210-280 g at the time of use. Animals were housed in groups (3-6 per cage) until the time of experimental manipulation. Following stereotaxic surgery, animals were housed individually in a temperature-regulated room ($22\pm2^{o}C$) and were maintained on a 12-hour light/dark cycle (lights on at 8:00 h) with food and tap water available ad libitum.

VI. Brain Dissection

Rats were decapitated between 10:00 and 12:00 h using a small animal guillotine. Brains were rapidly removed and placed in ice-cold 0.01 M Tris-HCl, pH 7.4, for enzyme studies, or in ice-cold 0.32 M sucrose for studies of ^{3}H-5-HT uptake. Appropriate brain regions were dissected on ice:

The midbrain was removed from between the rostral border of the

superior colliculi and caudal border of the inferior colliculi, dorsally, and from between the caudal border of the mammillary bodies and rostral edge of the pons, ventrally. The hypothalamus extended ventrally from the caudal border of the mammillary bodies to the optic chiasm. This region was separated from the overlying thalamus by cutting around the hypothalamus with a scalpel, and then lowering a pair of curved forceps along the lateral hypothalamic border to the level of the thalamus. The preoptic area appeared on the ventral surface of the brain as a distinct oval region bilaterally adjacent to the hypothalamus. The rostral boundaries of this area were the genu of the corpus callosum and the anterior communicating artery. The septum, lying immediately dorsal to the preoptic area, was dissected away from the most ventral fibers of the genu of the corpus callosum and pooled with its ipsilateral preoptic area. (Both areas will now be referred to as the combined SPOA.) To remove the hippocampus, the brain was placed on its ventral aspect. A midline cut was made to expose the fornix-fimbria and hippocampus. The hippocampus, without the fornix-fimbria, was peeled away from the overlying cortex using blunt curved forceps. The caudate area, having a distinctly darker appearance, was then separated from the surrounding cortex.

Individual brain regions were immediately frozen on dry ice in aluminum foil for storage at -70°C or were weighed and then placed in ice-cold buffer for immediate assay.

VII. Measurement of Tryptophan Hydroxylase Activity

A. Enzyme preparation

Brain regions were either defrosted in 0.01 M Tris-HCl, pH 7.4, within one month of freezing, or used immediately after dissection. In both cases, tissues were cleared of excess buffer by gentle blotting on filter paper and weighed. Tissues were homogenized (10 passes at 3000 rpm) in 10 volumes (v/w) ice-cold 0.01 M Tris-HCl, pH 7.4, containing 2 mM dithiothreitol. Homogenates were centrifuged in an SM-24 rotor at 35,000 x g for 20 minutes at 4°C, and the resulting supernatants were assayed for enzyme activity.

B. Substrate preparation

L-[1-^{14}C-]Tryptophan (50 uC) was received in a volume of 2.5 ml (98% H_2O:2% ethanol) and brought to 0.1 M Tris-HCl, pH 7.4, in a final volume of 3 ml. Aliquots of 100 ul were prepared and stored frozen in the dark at -20°C.

Unlabelled L-tryptophan was dissolved in distilled water (8.24 mg/ml) and 100 ul aliquots were prepared and stored frozen at -20°C in the dark. At the time of assay, an aliquot of unlabelled tryptophan was diluted 1:100 in 0.08 M Tris-HCl, pH 7.4. One aliquot of radiolabelled tryptophan was reconstituted to 2.2 ml with diluted cold tryptophan and 0.08 M Tris-HCl. (In experiments where the pH of the assay mixture was a variable, all dilutions were performed with Tris-HCl buffer at the desired pH.) By varying the proportion of diluted cold tryptophan to buffer per aliquot of labelled tryptophan, the concentration of substrate in the assay was changed. In a standard assay (80 uM tryptophan), 100 ul of the final substrate solution contained 29.4 nmol of tryptophan and 140,000 to 150,000 CPM. In this instance, the specific activity of the substrate was 2.65 mCi/mmol.

C. Cofactor preparation

Tetrahydrobiopterin (BH_4) was prepared according to the method of Knapp (1982). Biopterin was pulverized using a porcelain mortar and pestle and suspended in 5 mM HCl to achieve a final concentration of 14 mM. Aliquots were prepared and stored frozen in the dark at -20°C. For each assay, the stock solution of biopterin was freshly diluted with 5 mM HCl and reduced to BH_4 by catalytic hydrogenation over platinum oxide (PtO_2). The solution was placed in an amber glass bottle containing 3-5 mg PtO_2, and the bottle and its contents were weighed. The bottle was then tightly sealed with a vented rubber stopper and securely placed in a vortex shaker. Hydrogen gas was gently bubbled through the shaking solution for 20 minutes at room temperature. Reduction of biopterin to BH_4, followed spectrophotometrically, was completely accomplished during this time period. The bottle and its contents were weighed a second time, and any volume lost by evaporation during the hydrogenation procedure was reconstituted with 5 mM HCl. The solution was then passed through a Swinnex filter into a light-proof vial on ice. All subsequent dilutions were made into 5 mM HCl and were kept on ice in the dark until delivery into the reaction tubes.

6-MPH_4 was freshly weighed and diluted into 5 mM HCl for each assay. Solutions of 6-MPH_4 were kept in the dark on ice until delivery into the reaction tubes.

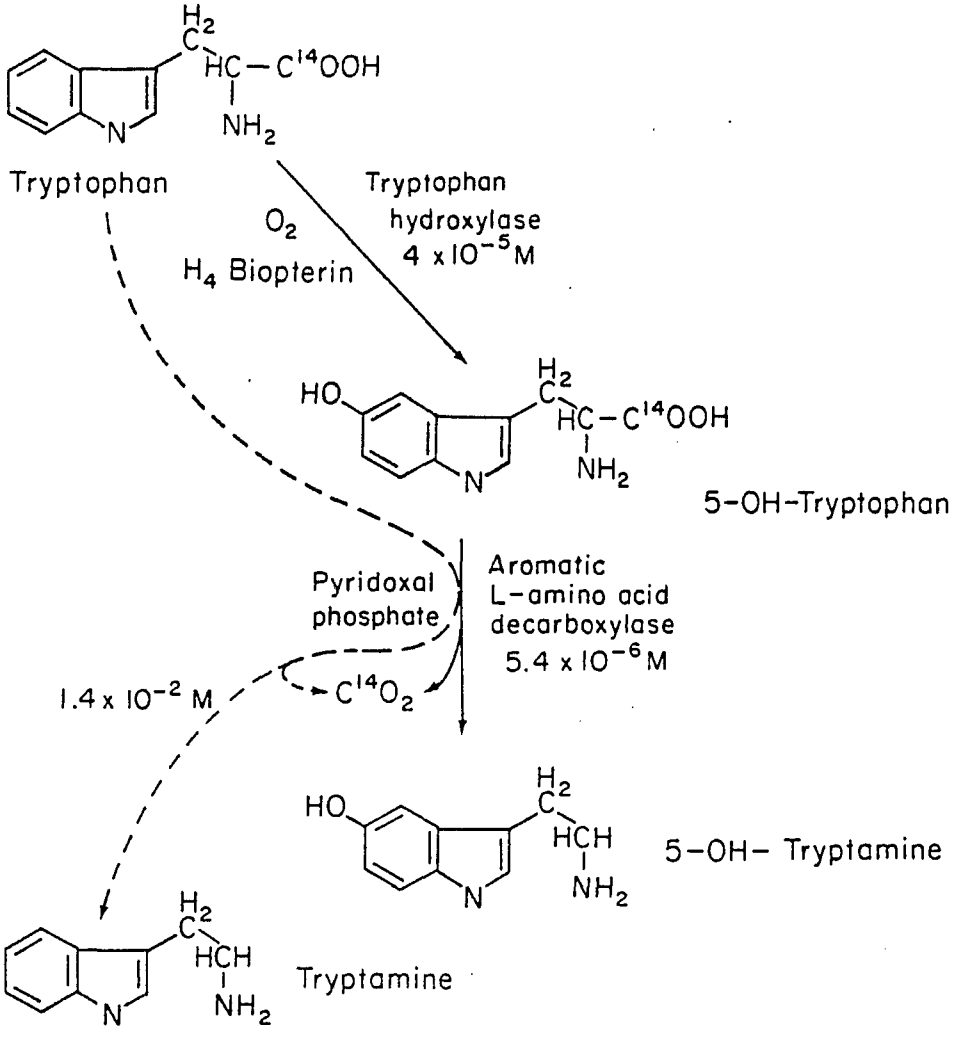
D. Aromatic L-amino acid decarboxylase (AADC) preparation

AADC was partially purified from fresh or frozen rat kidneys

according to the method of Christenson et al. (1970). Kidneys were weighed and then homogenized in a Sorvall Omnimixer in 4 volumes of ice-cold 0.02 M Tris-HCl buffer, pH 8.1. The homogenate was centrifuged at 12,000 x g for 20 minutes. The resulting supernatant was brought to 35% saturation with ammonium sulfate, stirring gently on ice for 15 minutes. The solution was centrifuged again at 12,000 x g for 20 minutes and the resulting supernatant was brought to 50% saturation with ammonium sulfate, stirring gently on ice for 15 minutes. Following a third centrifugation at 12,000 x g for 20 minutes, the supernatant was discarded and the pellet was resuspended in a volume of ice-cold 0.002 M sodium phosphate buffer, pH 7.0, equal to 0.8 times the original wet weight of the kidneys. The crude enzyme was dialyzed against 100 volumes of 0.002 M phosphate buffer overnight in the cold. After a final centrifugation for 20 min at 12,000 x g, the enzyme preparation was aliquoted and stored frozen at -70°C. All centrifugations were performed in a Sorvall SS-34 rotor at 4°C.

For each AADC preparation, enzyme activity was titrated using the TPOH assay: increasing volumes of AADC were included in the assay for TPOH until there was no further change in the number of CPM accumulated during 30 minutes of incubation (excluding the number of CPM in the blank tubes). In the same assay, the concentration of pyridoxal phosphate (PLP) required for maximal activity was determined. AADC prepared and stored in this manner was stable for up to 4 months with no apparent decrease in activity observed under the assay conditions used here.

Figure 5. Principle of the assay for TPOH developed by Ichiyama et al. (1968; 1970). Radioactive CO_2 is released from 5-HTP by the action of AADC. Direct decarboxylation of tryptophan can occur at high substrate levels (dashed line). Adapted from Azmitia (1973).



E. Standard assay for tryptophan hydroxylase

Tryptophan hydroxylase (TPOH) was assayed by a modification of the coupled radiometric method of Ichiyama et al. (1968; 1970). The assay measures $^{14}CO_2$ liberated by AADC from [1-^{14}C-]5-hydroxytryptophan (5-HTP) formed from the hydroxylation of L-[1-^{14}C-]tryptophan by TPOH (Figure 5). The difference in Km of AADC for 5-HTP (10^{-5} M) and for tryptophan (10^{-2}-10^{-3} M) makes the assay possible. High concentrations of substrate (^{14}C-tryptophan) are avoided in order to keep the direct decarboxylation of tryptophan to tryptamine to a minimum and thereby increase the specificity of the assay. Since TPOH is the rate-limiting enzyme in 5-HT synthesis, the amount of $^{14}CO_2$ released is stoichiometrically related to the amount of tryptophan hydroxylated and the amount of 5-HT produced. Unless otherwise noted, the following standard assay procedure was used for measuring TPOH activity.

Reactants were kept always on ice and were delivered to 15 ml tubes on ice in the following order and amount. (Reactants added simultaneously are listed together.):

1) 50 ul AADC preparation plus 10 ul PLP in distilled water (37 nmol)
2) 30 ul DTT/Fe^{++} in distilled water, 50 ul NADH in 0.2 M Tris-HCl, pH 7.4, 77.5 ul BH_4 in 5 mM HCl (0.37 umol DTT, 5.2 nmol Fe^{++}, 0.17 umol NADH, 42.7 nmol BH_4)
3) Enzyme preparation (25 ul hypothalamus, 50 ul midbrain or SPOA, 100 ul hippocampus)
4) 100 ul L-[1-^{14}C-]tryptophan (29.4 nmol, specific activity = 2.65

mCi/mmol)

The final assay volume for hippocampus was 417.5 ul; that for midbrain, hypothalamus, and SPOA was 367.5 ul.

Following addition of substrate, the assay mixture was sealed in test tubes with rubber stoppers from which were suspended plastic center wells containing 100 ul Protosol for collection of $^{14}CO_2$. Samples, in triplicate, were incubated at 37°C for 30 minutes in a shaking water bath. At the end of the incubation, the samples were again placed on ice. The reaction was terminated by injection of 500 ul 14% perchloric acid through the stopper using a Cornwall automatic syringe. Samples were incubated further for one hour at 37°C to insure complete collection of $^{14}CO_2$ in the Protosol. Center wells were removed directly into glass scintillation vials containing 10 ml of scintillation fluid composed of a toluene phosphor (3.99 g PPO and 0.48 g POPOP) and absolute ethanol in a 3.33:1 ratio. After at least 2 hours of equilibration, radioactivity was counted for 5 minutes in a Beckman LS7500 liquid scintillation counter with an external quench correction. The counting efficiency was approximately 83%.

Based on the assumption that the specific activity of the product ($^{14}CO_2$) is equal to that of the substrate (L-[1-^{14}C-]tryptophan), the number of pmol $^{14}CO_2$ released was determined using the specific activity of the substrate. Following conversion of CPM to DPM using the efficiency noted above, enzyme activity was calculated and expressed as follows:

pmol $^{14}CO_2$/mg wet tissue wet/30 minutes =

DPM x Ci/2.22×10^{12} DPM x mol/2.65 Ci x 1/10 mg wet weight (hippocampus) x 1/30 minutes

For midbrain and SPOA, the above calculation was made using 1/5 mg wet weight, and for hypothalamus, this number was 1/2.5.

F. Blanks

For each sample, blank tubes, in triplicate, were incubated with 77.5 ul 5 mM HCl in place of BH_4. Unless otherwise specified, all results reported are derived from CPM which have been corrected for the number of CPM in blank tubes. The number of CPM in samples were at least twice that in blanks. In some cases (see Part I, Results VII), other types of blanks were used to attempt to assess the amount of $^{14}CO_2$ released as a result of the action of AADC on tryptophan in the absence of TPOH, the action of TPOH supernatant on tryptophan in the absence of AADC, and the direct decomposition of L-[1-^{14}C-]tryptophan in the absence of both AADC and TPOH.

VIII. Properties of the Tryptophan Hydroxylase Assay

A. Buffer and molarity

In order to assess the effect of the type of buffer and its molarity on TPOH activity, enzyme activity was assayed using Tris-acetate, Tris-HCl, and sodium phosphate buffers, all at pH 7.4. The molarity of the final reaction mixture ranged from 0.5 to 0.001 M. The assay was performed on TPOH prepared from midbrains of normal animals in the presence of 10 uM tryptophan, 116 uM BH_4, 1mM DTT, 0.4 mM NADH, 0.1 mM PLP, and 50 ul AADC.

B. pH and temperature

The effect of pH and temperature on TPOH activity was investigated. Enzyme activity was assayed using 0.05 M Tris-HCl buffer at pH 6.8, 7.0, 7.2, 7.4, 7.6, and 8.1. pH was measured at room temperature. At each pH, the assay mixture was incubated on ice (4°C), at room temperature (22±2°C), and at 37°C for 30 minutes. The quenched samples were secondarily incubated for one hour at 37°C in all cases. The assay conditions were as above.

C. Linearity with tissue concentration

Linearity of enzyme activity was investigated for supernatants from midbrain, hippocampus, SPOA, caudate, and hypothalamus. The following substrate/cofactor conditions were assayed: 10 uM tryptophan/400 uM 6-MPH_4; 400 uM tryptophan/400 uM 6-MPH_4; 10 uM tryptophan/116 uM BH_4; 200 uM tryptophan/400 uM BH_4.

The assay was carried out on 6.25 ul to 100 ul of supernatant. In all cases, the total volume of the assay mixture was kept constant within a single experiment. All other conditions were standard unless otherwise noted.

D. Linearity with time

The time course of enzyme activity was studied for hippocampal and midbrain enzyme preparations only. Samples were incubated at 37°C for 0 to 60 minutes. Linearity was established for TPOH assayed under standard conditions.

E. Effect of freezing on TPOH activity

Four animals were sacrificed and their midbrains were removed. One midbrain was frozen on dry ice and stored at -70°C for future assay, while the others were pooled and immediately homogenized in 10 volumes 0.01 M Tris-HCl, pH 7.4, containing 2 mM DTT. A fraction of the homogenate was frozen at -70°C and the remainder was centrifuged to obtain the 35,000 x g supernatant. TPOH activity was measured in the supernatant in the presence of 10 uM tryptophan, 116 uM BH_4, 0.4 mM NADH, 1 mM DTT, 7 uM Fe^{++}, 50 ul AADC, and 0.1 mM PLP. The supernatant remaining after assay was frozen at -70°C for assay 18 days later using the same conditions as above.

F. Assay of TPOH at high substrate and cofactor concentrations

TPOH was prepared from midbrain and assayed using 100, 200, and 400 uM tryptophan and 200 and 400 uM BH_4 and 6-MPH_4. All other assay conditions were standard with the exception of 7 uM Fe^{++}. Two sets of blank tubes were prepared at each concentration of substrate: one lacked cofactor only and the other lacked cofactor and TPOH.

IX. Homogenization, Preincubation, and Incubation of TPOH Under Reducing Conditions

A. Homogenization

To investigate the effects of various reducing agents in the brain homogenate on TPOH activity, midbrains from several animals were pooled and homogenized in 5 volumes 0.01 M Tris-HCl, pH 7.4. Immediately after homogenization, measured aliquots of the homogenate were brought to 2 mM dithiothreitol (DTT), ascorbic acid, or metabisulfite in an

equal volume of 0.01 M Tris-HCl. The pH of the buffer plus reducing agent was rechecked and if necessary adjusted to pH 7.4 before addition to the homogenate. Assay conditions were as above with the exception of 1 mM DTT. Control samples were homogenized and incubated in the absence of reducing agent.

B. Preincubation

In studies to investigate the effects of various reducing agents on TPOH preincubated under specific atmospheric conditions, midbrains from several rats were pooled and homogenized in 5 volumes 0.01 M Tris-HCl, pH 7.4. Measured aliquots of the 35,000 x g supernatant were brought to the desired concentration of DTT, DTT plus ferrous ammonium sulfate (Fe^{++}), or Fe^{++} alone with equal volumes of the appropriate compound(s) dissolved in 0.01 M Tris-HCl, pH 7.4. In one experiment, DTT plus Fe^{++} were added to the supernatant in Tris-HCl buffer, pH 8.5, and enzyme activity was assayed at pH 7.4. Also, following preincubation at pH 7.4, supernatant was assayed at pH 7.0 and pH 7.2. In another experiment, aliquots of the homogenate were constituted to the appropriate concentration of DTT and/or Fe^{++} before centrifugation (as above). Preincubation was then carried out on the supernatant directly or on supernatant again constituted to 5 mM DTT, 50 uM Fe^{++}.

Enzyme solutions were placed either on ice or preincubated in amber glass bottles sealed with vented rubber stoppers. 100% hydrogen gas (H_2), 100% nitrogen gas (N_2), or 95% oxygen/5% carbon dioxide (O_2) was bubbled over the enzyme solution through a syringe tip placed 1/2-1 inch from the surface of the mixture. Solutions were

gassed 45 minutes at room temperature or left at room temperature without being gassed for the same time period. Following preincubation, enzyme solution was placed immediately on ice and assayed within one hour. Control samples were prepared in the absence of reducing agent and maintained on ice until time of assay. No reducing agent was included in the incubation mixture of control samples. Enzyme activity was assayed in the presence of 10 uM tryptophan, 116 uM BH_4, 0.4 mM NADH, 0.1 mM PLP, and 50 ul AADC. Specific reducing conditions are indicated in the results.

Any decrease in the volume of the enzyme preparation due to evaporation of buffer during the process of preincubation was detected by weighing the bottle and its contents before and after preincubation. When a decrease was noted, the volume was reconstituted with 0.01 M Tris-HCl.

C. Incubation

In several experiments, the reducing conditions of the enzyme assay were modified from those of the homogenate or from those of the preincubated supernatant. Desired assay conditions were achieved by adding the appropriate compound in distilled water (catalase, DTT, Fe^{++}, metabisulfite, ascorbic acid) or in 0.2 M Tris-HCl, pH 7.4 (NADH) to the assay mixture. Solutions of metabisulfite and ascorbic acid were neutralized before addition.

In some cases, the pH of the assay mixture was modified by the use of Tris-HCl buffer, pH 7.0 or 7.2, at all steps of the assay but homogenization. Control samples were prepared and assayed in the

absence of reducing agent at pH 7.4. Specific assay conditions are noted in the results.

X. Preincubation and Incubation of TPOH Under Phosphorylating Conditions

A. Effect of phosphorylating conditions on midbrain and hippocampal TPOH activity

In one series of experiments, the effects of phosphorylating conditions on TPOH prepared from midbrain and hippocampus and assayed at 10 uM tryptophan and 116 uM BH_4 were investigated. The appropriate assay conditions were achieved by the addition of ATP and/or $MgCl_2$ and/or $CaCl_2$ in 0.2 M Tris-HCl, pH 7.4. Control samples were incubated in the absence of ATP, $MgCl_2$, and $CaCl_2$. Included in the assay mixture were 1 mM DTT, 7 uM Fe^{++}, 0.4 mM NADH, 0.1 mM PLP, and 50 ul AADC.

B. Effect of DTT/Fe^{++} on activation of TPOH by phosphorylating conditions

Another experiment investigated the effects of assay reducing conditions on the activation of TPOH by 1 mM ATP and 10 mM $MgCl_2$. Midbrains were homogenized in 0.1 mM DTT and the desired concentration of DTT and/or Fe^{++} in the reaction mixture was achieved by the addition of the reducing agent(s) in distilled water. Controls were incubated in the absence of ATP and $MgCl_2$. All other assay conditions were as in A.

C. Effect of pH on activation of TPOH by phosphorylating

conditions (1)

The effect of the assay medium pH on TPOH activity measured under phosphorylating conditions was studied for midbrain enzyme assayed with 30 uM BH_4, 116 uM BH_4, and 116 6-MPH_4. In these experiments, enzyme activity was assayed with 1 mM ATP and 10 mM $MgCl_2$ at pH 7.2, 7.4, 7.6, 7.8, 8.0, and 8.2. Controls at each pH were incubated without ATP/$MgCl_2$. All other assay conditions were as in A.

D. Effect of pH on activation of TPOH by phosphorylating conditions (2)

An attempt was made to replicate the pH profile of activation of TPOH found by Hamon et al. (1978c). In this experiment, midbrains were homogenized in 0.05 M Tris-HCl, pH 7.6, containing 2 mM 2-mercaptoethanol (MCE) and assayed in 0.05 M Tris-HCl, pH 7.6, 2 mM MCE, 60 U catalase, 160 uM 6-MPH_4, and 10 uM tryptophan. Enzyme activity was assayed at pH 7.2, 7.4, 7.6, 7.8, 8.0, and 8.2. Phosphorylating conditions were 1 mM ATP and 10 mM $MgCl_2$. Controls were incubated at each pH in the absence of ATP/$MgCl_2$.

E. Effect of preincubation on activation of TPOH by phosphorylating conditions

In one series of experiments, the 35,000 x g midbrain supernatant was preincubated with 50 ul AADC, 0.1 mM PLP, 1 mM DTT, 7 uM Fe^{++}, and 0.4 mM NADH at 37°C for 10 minutes. Enzyme was preincubated in the presence and absence of cofactor (BH_4=116 uM, 6-MPH_4=200 uM) and substrate (tryptophan=10 uM) both with and without 1 mM ATP/10

mM $MgCl_2$. Following the preincubation, the samples were placed on ice for any further additions. Again, controls were samples assayed in the absence of ATP/$MgCl_2$.

F. Effect of order of addition of assay components on activation of TPOH by phosphorylating conditions

A possible effect of the order of addition of components to the assay on TPOH activation by phosphorylating conditions was investigated. In previous experiments, all preincubations had been carried out in the presence of AADC, PLP, NADH, DTT, and Fe^{++}. In this assay, these components were added after preincubation, immediately before cofactor (except when cofactor was included in the preincubation). The reaction was initiated by the addition of substrate (except when substrate was included in the preincubation). Midbrain enzyme was not preincubated (I) and preincubated in the presence and absence of 1 mM ATP, 10 mM $MgCl_2$ or 1 mM ATP, 10 mM $MgCl_2$, 10 uM $CaCl_2$ without cofactor and substrate (II), with cofactor only (III) and with substrate only (IV). For each preincubation condition, control enzyme activity was measured in the absence of ATP/$MgCl_2$/$CaCl_2$. Enzyme was assyed at 116 uM BH_4 and 10 uM tryptophan. All other conditions were as in A.

G. Effect of an ATP-regenerating system on activation of TPOH by phosphorylating conditions

The effect of an ATP-regenerating system on the activation of midbrain TPOH by ATP/$MgCl_2$/$CaCl_2$ was studied. Creatine kinase (CK, 1 mg/ml), creatine phosphate, (CP, 20 mM), ATP (1 mM), $MgCl_2$

(10 mM) and $CaCl_2$ (10 uM) were added to the assay mixture in 0.2 M Tris-HCl, pH 7.4. Enzyme activity was assayed in the presence and absence of Fe^{++}. Controls were assayed in the absence of ATP/$MgCl_2$/$CaCl_2$ and CK-CP. The concentration of BH_4 was 116 uM and that of tryptophan was 10 uM in this experiment. All other assay conditions were as in A.

H. Effect of EDTA on activation of TPOH by phosphorylating conditions

The effect of EDTA on midbrain enzyme activity assayed in the presence and absence of phosphorylating conditions was investigated. TPOH was assayed with 0, 10^{-3} M, 10^{-4} M, or 10^{-5} M EDTA under the following conditions:

1 mM ATP, 10 mM $MgCl_2$

1 mM ATP, 10 mM $MgCl_2$, 10 uM $CaCl_2$

1 mM ATP, 10 mM $MgCl_2$, 50 uM $CaCl_2$

1 mM ATP, 10 mM $MgCl_2$, 100 uM $CaCl_2$

Control samples were assayed in the absence of EDTA and phosphorylating conditions. The concentration of BH_4 was 116 uM and that of tryptophan was 10 uM. All other assay conditions were as in A.

XII. Neurotoxin Injections

Animals were injected intraperitoneally with desipramine dissolved in 0.9% saline, 10 mg/kg body weight, 30 to 45 minutes prior to injection of the neurotoxin. Injections were made into animals anaesthetized with Ketalar, 30 mg/kg intraperitoneally, followed 5

minutes later by Rompun, 12 mg/kg intramuscularly.

The skin overlying the cranium was shaved free of fur and the animal was then placed in a stereotaxic apparatus with earbars positioned to just penetrate the animal's eardrums and the incisor bar set to 3.2 mm below the interaural line. After cleansing the animal's skin with alcohol, a 3 cm midline incision was made beginning immediately posterior to the eyes. A surgical scalpel was used to remove the underlying fascia. With the aid of an operating microscope, an opening in the skull at the level of the injection site was made using a dental drill. The dura was pierced with a fine syringe tip.

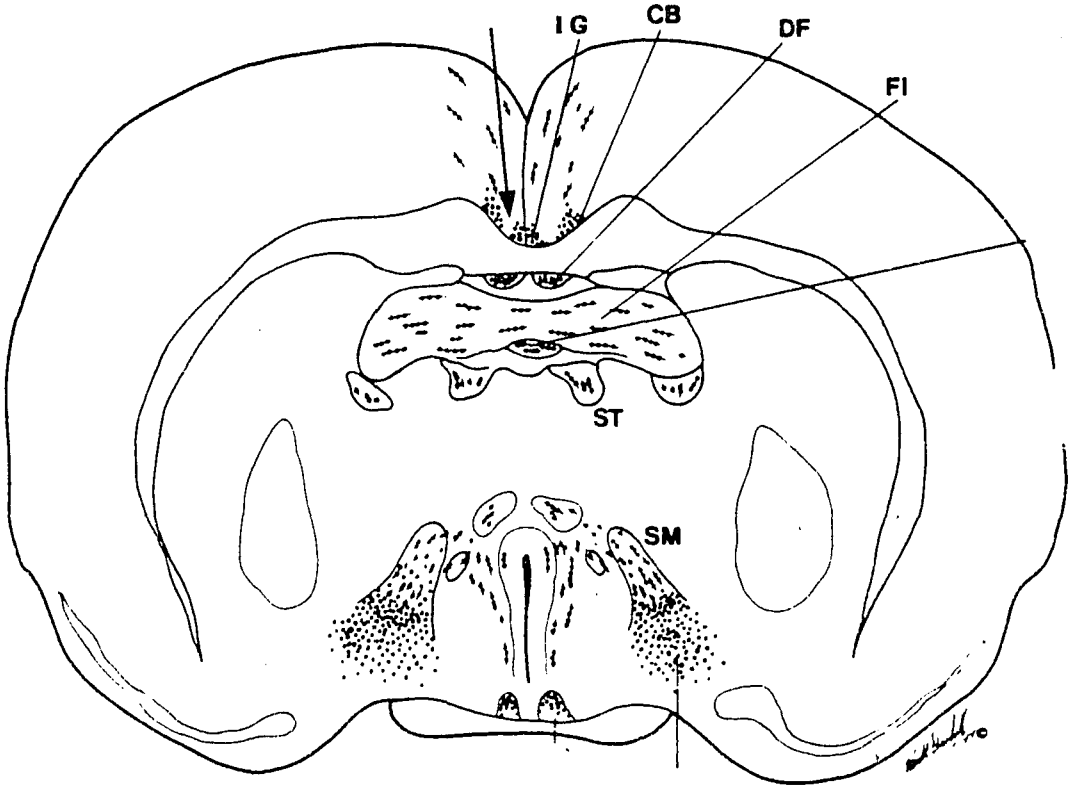
For injection of the neurotoxin, a hand-driven glass micropipet (80-120 um in diameter) was lowered at a lateral angle of 15^{o} to avoid damage to the sagittal sinus. Injections were made at coordinates relative to the bregma skull suture: 1.0 mm posterior, 1.3 mm lateral, and 2.4 mm ventral. Histological examination of brains of animals injected at these coordinates with trypan blue dye confirmed that the tip of the micropipet penetrated close to, but did not directly contact, the cingulum bundle. This was desirable, so as not to cause direct damage to cingulum bundle fibers.

Doses of 1 to 5 ug of the free base of 5,7-dihydroxytryptamine creatinine sulfate (5,7-DHT) dissolved in 0.9% saline containing 0.2 mg/ml ascorbic acid were injected unilaterally through the micropipet. 5,7-DHT was injected at a rate of 50 nl/min over a period of 8 minutes. Following the injection, the micropipet was left in place for 5 minutes and then slowly withdrawn from the brain parenchyma. The skin incision was closed with wound clips, sprayed with the antibacterial agent

Neosporin Aerosol, and swabbed with iodine. Animals were left to recover from anaesthesia under the warmth of a bright light.

Sham-lesioned animals were treated in the same manner, but were injected with the ascorbic acid-saline vehicle only.

Figure 6. Diagram of a coronal section through the rat brain at the level of the injection site. 5-HT fibers are shown in fine stippling. The arrow indicates the site of drug injection. CB, cingulum bundle; DF, dorsal fornix; FI, fimbria; IG, indiseum griseum. Adapted from Azmitia and Segal (1978).



A. Effect of dose and concentration of 5,7-DHT injected unilaterally into the cingulum bundle on TPOH activity in the hippocampus

In order to investigate the effects of the dose of 5,7-DHT injected into the cingulum bundle on TPOH activity in the hippocampus, rats were injected unilaterally with 0, 1, 2, 3, 4 or 5 ug (free base) 5,7-DHT. All doses were delivered in 400 nl vehicle. To study the effect of the concentration of 5,7-DHT on enzyme activity, 2 ug (free base) of the drug were injected in a volume of 160 nl (1.25% solution; injected at a rate of 40 nl/min) or in a volume of 400 nl (0.5% solution). Injections of vehicle alone were also performed. In all cases, the animals were sacrificed 7 days after injection, and the hippocampi were removed bilaterally and frozen. TPOH activity in hippocampi both ipsilateral and contralateral to the injection was assayed under standard conditions.

B. Time course of TPOH activity in the hippocampus following unilateral injection of 5 ug 5,7-DHT into the cingulum bundle

5 ug 5,7-DHT or vehicle alone were injected unilaterally into the cingulum bundle, and animals were sacrificed 3, 7, 14, 28, 42, 60, and 90 days later. Hippocampi were removed and frozen at -70°C. TPOH activity was assayed under standard conditions in hippocampi both ipsilateral and contralateral to the injection.

C. Effect of 5 ug 5,7-DHT injected into the cingulum bundle on the kinetics of TPOH in the hippocampus

To investigate the effects of a unilateral cingulum bundle

injection on the kinetic parameters of hippocampal TPOH, animals were injected with 5 ug 5,7-DHT or vehicle alone and sacrificed 7 or 60 days later. Hippocampi were removed and frozen at -70°C. At the time of assay, hippocampi ipsilateral to the injection were pooled from sham-lesioned animals and from 5,7-DHT-lesioned animals and TPOH was prepared from each group. The kinetic parameters of TPOH for tryptophan were determined for 5,7-DHT-lesioned and sham-lesioned animals by varying the concentration of tryptophan from 10 to 200 uM while maintaining the concentration of BH_4 at 400 uM. All other assay conditions were standard. The apparent Km and Vmax were calculated from equations obtained by the linear regression analysis of Lineweaver-Burk plots.

D. Time course of TPOH activity in the midbrain following unilateral injection of 5 ug 5,7-DHT into the cingulum bundle

TPOH activity was measured under standard conditions in whole midbrains removed and frozen from animals used for studies of hippocampal TPOH 3, 7, 14, 28, 42, 60, and 90 days after unilateral injection of 5 ug 5,7-DHT or vehicle into the cingulum bundle.

E. Effect of unilateral 5,7-DHT injection into the cingulum bundle on TPOH activity in the hypothalamus and SPOA

TPOH activity was measured under standard conditions in the ipsilateral and contralateral hypothalamus and SPOA removed and frozen from animals injected with 5 ug 5,7-DHT or vehicle alone 3, 7, and 14 days prior to sacrifice. The brain regions analyzed in these studies were taken from animals used in the previous two studies.

F. Time course of TPOH activity in the hippocampus following unilateral injection of 2 ug 5,7-DHT into the cingulum bundle

Animals were injected unilaterally with 2 ug 5,7-DHT or vehicle alone into the cingulum bundle and sacrificed 7, 28, and 42 days later. Hippocampi were removed and frozen for assay of TPOH. Enzyme activity was measured under standard conditions in the ipsilateral and contralateral hippocampus of each animal.

XII. Synaptosomal Uptake of ^{3}H-5-Hydroxytryptamine (^{3}H-5-HT)

Synaptosomes were prepared and the uptake of ^{3}H-5-HT was measured according to the method of Azmitia et al. (1983).

A. Synaptosomal preparation

Fresh tissue (hippocampus) was weighed and gently homogenized in 2 ml ice-cold 0.32 M sucrose (10 passes at 1000 rpm). Homogenate was centrifuged at 1000 x g for 10 minutes and the supernatant was carefully removed and saved. The pellet (P-1) was resuspended in 2 ml 0.32 M sucrose and centrifuged again at 1000 x g for 10 minutes. The supernatant from this spin was removed and combined with the supernatant from the previous spin, and the combined supernatants were centrifuged at 14,000 x g for 15 minutes. The resulting pellet (P-2) was resuspended in 10 volumes (volume/weight of the original wet tissue) ice-cold Krebs-Ringer-Bicarbonate (KRB) buffer supplemented with 10^{-4} M pargyline, 10^{-3} M ascorbic acid, and 10^{-2} M dextrose. The synaptosomal preparation was kept on ice until assayed for ^{3}H-5-HT uptake. All centrifugations were performed at 4^{o}C in an SM-24 rotor.

B. Synaptosomal uptake

1) 285 ul of supplemented KRB buffer was brought to 37°C in 6 wells of a multi-welled tissue culture plate.
2) The P-2 suspension was vortexed briefly and 15 ul was added to the KRB buffer.
3) 10 ul unlabelled 5-HT in KRB was delivered to half of the wells (3.2 nmol cold 5-HT; nonspecific uptake).
4) The reaction was initiated by the addition of 20 ul ^{3}H-5-HT in KRB to all 6 wells (17 pmol ^{3}H-5-HT; specific uptake).

After a 3 minute incubation at 37°C, the reaction was terminated by filtering the incubation medium through GF/B Whatman filters using a Titertek cell harvester. The synaptosomes, trapped on the surface of the filter paper, were washed for 15 seconds with ice-cold 0.9% NaCl in 0.1 M sodium phosphate buffer, pH 7.4. The filter paper was left to dry in a 45°C oven for 30-35 minutes and then the filters were punched into glass scintillation vials. Econofluor (10 ml) was added to the vials and radioactivity was counted as previously described. Counting efficiency was approximately 50%.

After subtracting the nonspecific from the specific uptake (in CPM), the number of moles of ^{3}H-5-HT taken up by one gram wet tissue weight during the 3 minute incubation period was calculated from the specific activity of the ^{3}H-5-HT (33.8 Ci/mmol):

pmol ^{3}H-5-HT/g wet tissue weight/3 min = DPM x Ci/2.22×10^{12} DPM x mmol/33.8 Ci x 1/1.5 mg wet weight x 1/3 min

C. Preparation of Krebs-Ringer-Bicarbonate buffer

Krebs-Ringer solution was composed of the following (final concentrations, following addition of sodium bicarbonate solution): 114 mM NaCl, 4.6 mM KCl, 1.2 mM $CaCl_2 \cdot 6H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$.

Krebs-Ringer solution was gassed with 95% oxygen/5% carbon dioxide for 20 minutes on ice. Then a solution of neutralized sodium bicarbonate in distilled water was added to the Krebs-Ringer (2.4 g $NaHCO_3$/liter final Krebs-Ringer-Bicarbonate buffer).

For synaptosomal uptake, Krebs-Ringer-Bicarbonate buffer was supplemented with pargyline, ascorbic acid, and dextrose as indicated previously, and bubbled with 95% oxygen/5% carbon dioxide for another 20 minutes on ice.

XIII. Effect of 2 ug 5,7-DHT Injected Unilaterally into the Cingulum Bundle on High Affinity Uptake of ^{3}H-5-HT in Synaptosomal Preparations of Hippocampus

Rats were injected unilaterally into the cingulum bundle with 2 ug 5,7-DHT in 400 nl vehicle or with vehicle alone and were sacrificed 7 and 42 days later. Hippocampi were removed bilaterally and prepared for synaptosomes. Uptake of ^{3}H-5-HT was measured in synaptosomes from hippocampi ipsilateral and contralateral to the injection.

XIV. Statistical Analysis

Statistical analysis was performed using a one-way or two-way analysis of variance (ANOVA) for groups of unequal number. Comparisons between individual groups were made using multiple Student's t-tests.

RESULTS AND DISCUSSION

Part I

Properties of the Tryptophan Hydroxylase Assay

Results

The method of Ichiyama et al. (1968; 1970) was selected for use in these studies because of its relative sensitivity and convenience. It allows one to perform assays of almost any desired magnitude.

Assay conditions for measuring TPOH activity vary widely from laboratory to laboratory. Therefore, using the Ichiyama method, it was necessary to establish conditions for measuring enzyme activity in this laboratory. In the process of modifying assay conditions, requirements for reducing agents and ferrous ion were investigated. Also, the characteristics of activation of TPOH activity by phosphorylating conditions were studied. In addition, an attempt was made to assay the enzyme under saturating conditions, keeping in mind the problem of direct decarboxylation of substrate.

I. Buffer and Molarity

Enzyme activity was found to be dependent on the nature of the buffer used in the reaction mixture. Tris-acetate, Tris-HCl, and sodium phosphate all produced good enzyme activity. However, sodium phosphate buffer resulted in blanks which were about 2 times greater than blanks from the other buffer systems. Overall, greatest levels of enzyme activity were achieved with Tris-HCl buffer.

Buffer molarity also affected the amount of $^{14}CO_2$ released

from tryptophan. Measurements of enzyme activity over a wide range of molarities (0.5 to 0.001 M) for Tris-acetate, Tris-HCl, and sodium phosphate revealed a profile of activity different for each buffer. With Tris-acetate, enzyme activity was greatest between 0.01 and 0.05 M. With Tris-HCl and phosphate buffers, highest levels of activity were reached when the molarity was 0.05 M.

II. pH and Temperature

In the presence of 0.05 M Tris-HCl, enzyme activity increased as the temperature was increased from 4° to 37°C. Furthermore, with the elevation in temperature, there was a gradual increase in the number of pmol $^{14}CO_2$ detected in blank tubes. The effect of temperature on enzyme activity may in part be due to temperature effects on the pH of the assay medium.

Enzyme activity was found to be dependent on the pH of the buffer used in the assay. When assayed under a range of pH's from 6.8 to 8.1, at 37°C, enzyme activity reached highest levels at pH 7.2-7.4. The number of pmol $^{14}CO_2$ released in blank tubes increased with increasing pH.

III. Linearity with Tissue Concentration

Figures 7 through 10 represent enzyme activity in various brain region preparations plotted as a function of the amount (ul) of supernatant used in the assay. The data in figures 7 and 8 were obtained by assaying for enzyme activity in the presence of 6-MPH_4. Linear regression analysis of the data in no case gave a y-intercept of 0, indicating that $^{14}CO_2$ was released in the absence of TPOH

preparation. In the presence of 10 uM tryptophan and 400 uM 6-MPH_4 (Figure 7), the average y-intercept of all brain regions was 944 CPM/30 min (21.7 pmol/30 min). When the concentration of tryptophan was increased to 400 uM, the average y-intercept was similarly 1003 CPM/30 min. When taking the specific activity of the substrate into account, 525.3 pmol $^{14}CO_2$ were released during the 30 minute incubation in the presence of 400 uM tryptophan. This indicates that nonenzymatic production of $^{14}CO_2$ was dependent on substrate concentration.

The y-intercepts of the data in Figures 9 and 10 are more difficult to evaluate because of their scatter around the origin. In the presence of 10 uM tryptophan, 116 uM BH_4 (Figure 9), the y-intercepts are all below 150 CPM/30 min (3.5 pmol/30 min), with 3 of these being negative. The 2 exceptions are caudate and midbrain plus Fe^{++}, which have values of 276.9 CPM (6.4 pmol)/30 min and 392.6 CPM (9.0 pmol)/30 min, respectively. On the other hand, in the presence of 200 uM tryptophan, 400 uM BH_4 (Figure 10), the y-intercepts are 189.4 CPM (99.2 pmol)/30 min and 210.4 CPM (110.2 pmol)/30 min for caudate and SPOA, respectively. (Because enzyme activity was measured only at two different tissue concentrations for midbrain, hippocampus, and hypothalamus, best fit lines were not determined for enzyme from these regions. However, these data are plotted in Figure 10.) It appears from these data that 6-MPH_4 produces higher rates of nonenzymatic hydroxylation than BH_4.

When nonenzymatic production of $^{14}CO_2$ is taken into account, linearity with tissue amount was found for all brain regions under all substrate/cofactor conditions within the range of volumes

tested. However, in the case of caudate, the nonenzymatic production of $^{14}CO_2$ proved to be too high under all assay conditions to allow reliable measurement of TPOH activity in this tissue.

IV. Linearity with Time

The standard assay (80 uM tryptophan, 116 uM BH_4) was linear for at least one hour for both midbrain and hippocampal enzyme preparations (Figure 11).

V. Effect of Freezing on Tryptophan Hydroxylase Activity

When enzyme activity was measured in supernatant from homogenate frozen at -70°C for 18 days, a 22-25% drop in activity was noted for both midbrain and hippocampal preparations when compared to fresh tissue preparations. Assay of TPOH in supernatant frozen as above revealed the same decrease in enzyme activity found for frozen homogenate. Contrary to these results, Vitto and Mandell (1981) found a 33% increase in the activity of enzyme supernatant stored for 13 days at -20°C. However, in the present study, freezing of whole tissue at -70°C did not appear to cause any decrease in enzyme activity when compared to fresh tissue and may, in fact, have resulted in a slight increase in activity.

VI. Assay of TPOH at High Substrate and Cofactor Concentrations

In view of the problems inherent in the use of high substrate concentrations in this coupled radiometric assay of TPOH, an effort was made to assay midbrain TPOH under saturating substrate and cofactor conditions (Table 2). TPOH activity was measured using 100 to 400 uM

tryptophan and 200 to 400 uM BH_4 or 6-MPH_4. By including blanks lacking both cofactor and enzyme preparation, an attempt was made to estimate the maximal amount of $^{14}CO_2$ released due to direct decarboxylation of tryptophan.

Substrate inhibition of TPOH has been reported to occur in the presence of BH_4 but not in the presence of $DMPH_4$ (Friedman et al., 1972; Tong and Kaufman, 1975). Peak activity is observed at about 200 uM tryptophan, with 50% inhibition occurring at 1.0 mM tryptophan. Similarly, in the present study, with 200 or 400 uM BH_4, maximal enzyme activity was reached when the concentration of tryptophan was 200 uM (Table 2). When the substrate concentration was further increased to 400 uM, TPOH activity slightly decreased, suggesting that substrate inhibition occurs when levels of tryptophan exceed 200 uM. However, substrate inhibition was not observed with 6-MPH_4; enzyme activity continued to increase as the substrate concentration was increased from 200 to 400 uM.

Jequier et al. (1969) reported that the use of BH_4 rather than $DMPH_4$ resulted in a higher rate of tryptophan hydroxylation. In their experiments, the concentration of tryptophan was 120 uM. Similarly, in the present study, in the presence of 100 uM tryptophan, BH_4 resulted in a higher level of enzyme activity than 6-MPH_4 (Table 2). The higher rate of tryptophan hydroxylation observed with BH_4 is likely due to the lower Km for substrate in the presence of this cofactor (see Table 1). On the other hand, at levels of substrate greater than 200 uM, the use of 6-MPH_4 lead to a higher rate of hydroxylation. This is probably the result of a lack of substrate

inhibition occurring in the presence of 6-MPH_4.

In the absence of cofactor, the number of nanomoles released in 30 minutes increased linearly as the concentration of tryptophan was increased from 100 to 400 uM (Table 2). Similarly, when enzyme preparation and cofactor were both omitted, there was a linear increase in the number of nmoles $^{14}CO_2$ released, suggesting that the $^{14}CO_2$ released was the result of direct decarboxylation of substrate.

VII. Blanks

To further investigate the source of $^{14}CO_2$ in blank tubes, a series of reaction tubes were incubated for 30 minutes in the presence and absence of AADC and/or TPOH and/or BH_4. In this experiment, the concentrations of substrate and cofactor were standard and enzyme activity was 986.2 pmol $^{14}CO_2$/50 ul midbrain/30 min.

The number of pmol $^{14}CO_2$ released in routinely used blanks (all assay components with the exception of cofactor) was 166.3. When TPOH preparation was omitted under these conditions, the number of pmol released was similarly 174.9, indicating that direct decarboxylation of substrate is the source of $^{14}CO_2$ in routinely used blanks. That 73.9 pmol $^{14}CO_2$ were found in the absence of both AADC and TPOH, suggests that almost half the number of pmol resulting from direct decarboxylation of tryptophan is produced by nonenzymatic mechanisms. Thus, approximately 50% of the pmol $^{14}CO_2$ in blank tubes is the result of decarboxylation of tryptophan by AADC.

When TPOH was omitted from the assay, there was a slightly greater

number of pmol than when both TPOH and BH_4 were omitted (208.9 versus 174.9 pmol). This indicates that with 116 uM BH_4, a relatively small amount of radioactivity (34 pmol) is produced by nonenzymatic hydroxylation of substrate.

Figure 7. Linearity of TPOH activity with tissue amount in the presence of 10 uM tryptophan, 400 uM 6-MPH_4. All other assay conditions were standard. Enzyme activity is expressed as CPM/30 min (83% efficiency). MB = midbrain, HYP = hypothalamus, SPOA = septum/preoptic area, HIP = hippocampus, CAU = caudate.

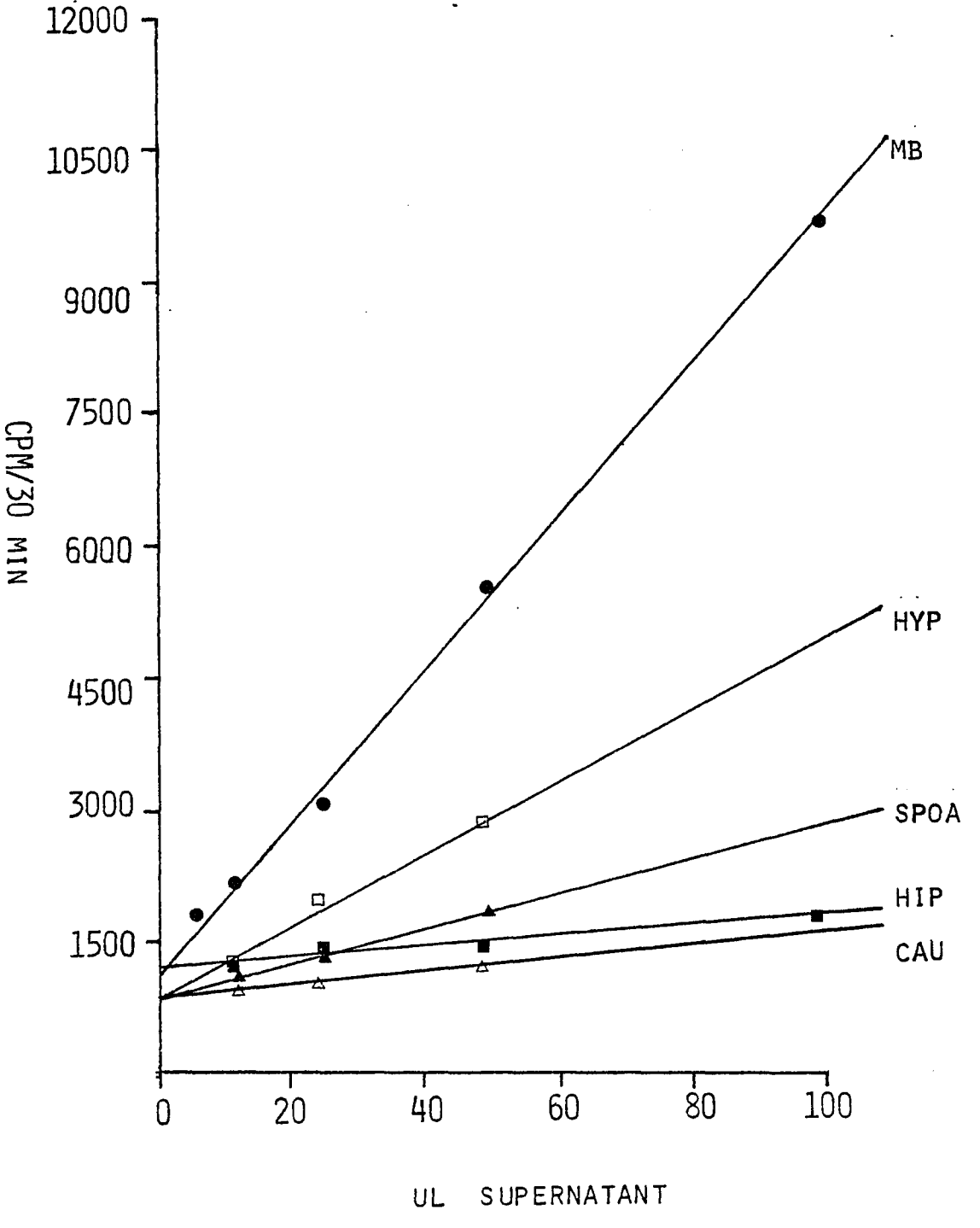


Figure 8. Linearity of TPOH activity with tissue amount in the presence of 400 uM tryptophan, 400 uM 6-MPH_4. All other assay conditions were standard. Enzyme activity is expressed as CPM/30 min (83% efficiency). All other abbreviations as in Figure 7.

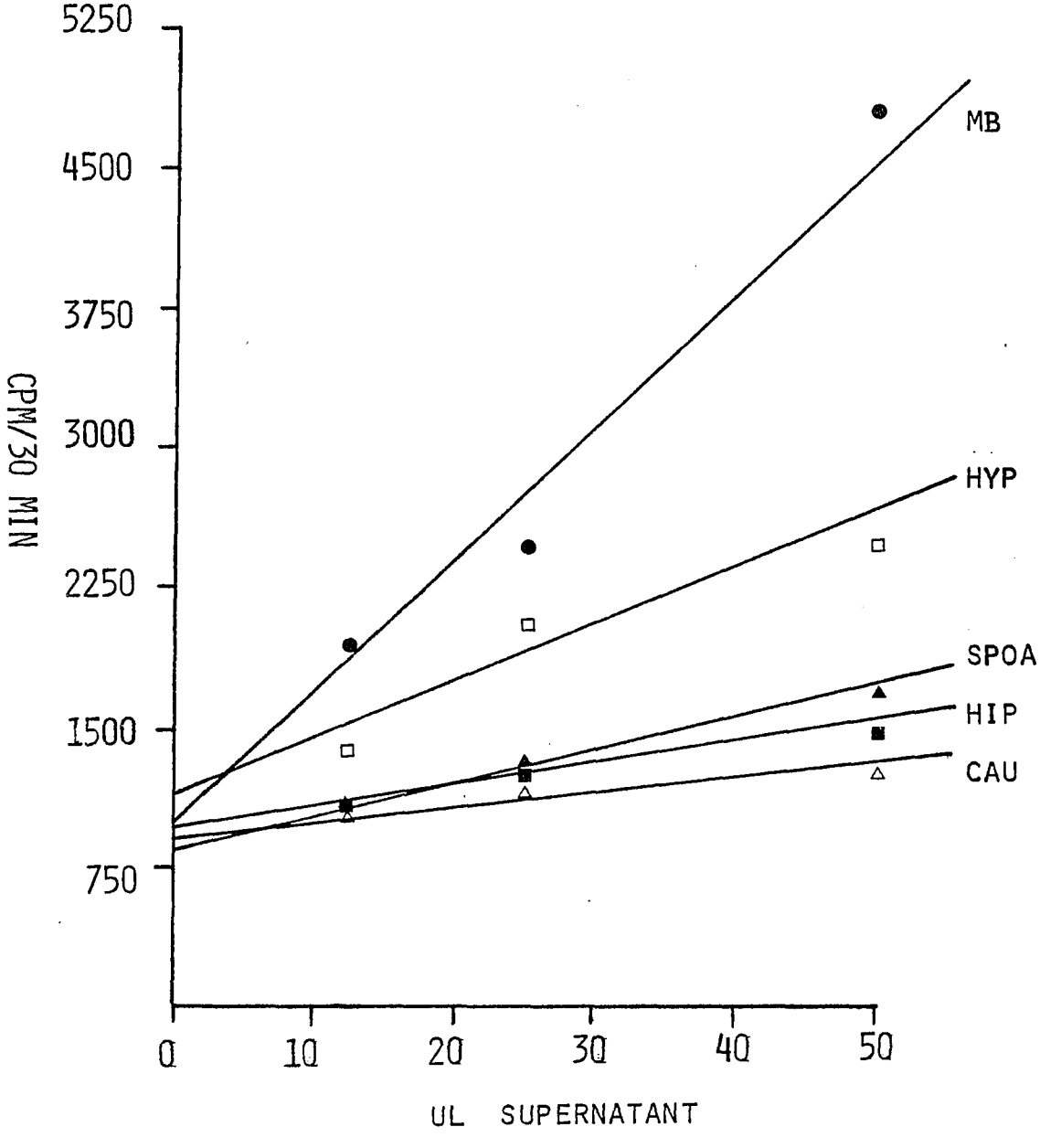


Figure 9. Linearity of TPOH activity with tissue amount in the presence of 10 uM tryptophan, 116 uM BH_4. All other assay conditions were standard with the exceptions of MB- and HIP- which lacked Fe^{++}. MB+, HIP+ = midbrain and hippocampus assayed in the presence of Fe^{++}. All other abbreviations as in Figure 7.

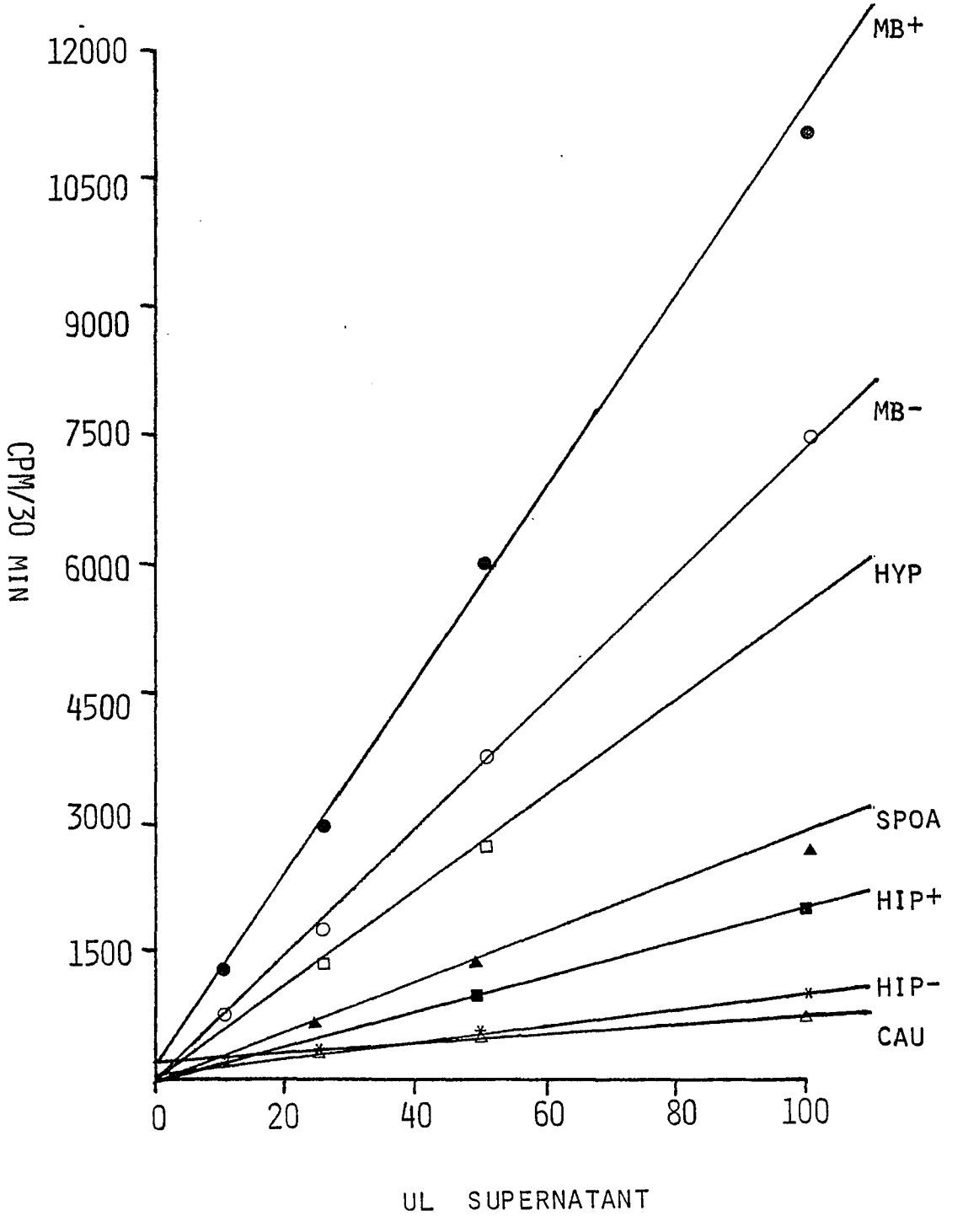


Figure 10. Linearity of TPOH activity with tissue amount in the presence of 200 uM tryptophan, 400 uM BH_4. All other assay conditions were standard. Enzyme activity and abbreviations as in Figure 7. Closed circles = MB, open squares = HYP, closed squares = HIP.

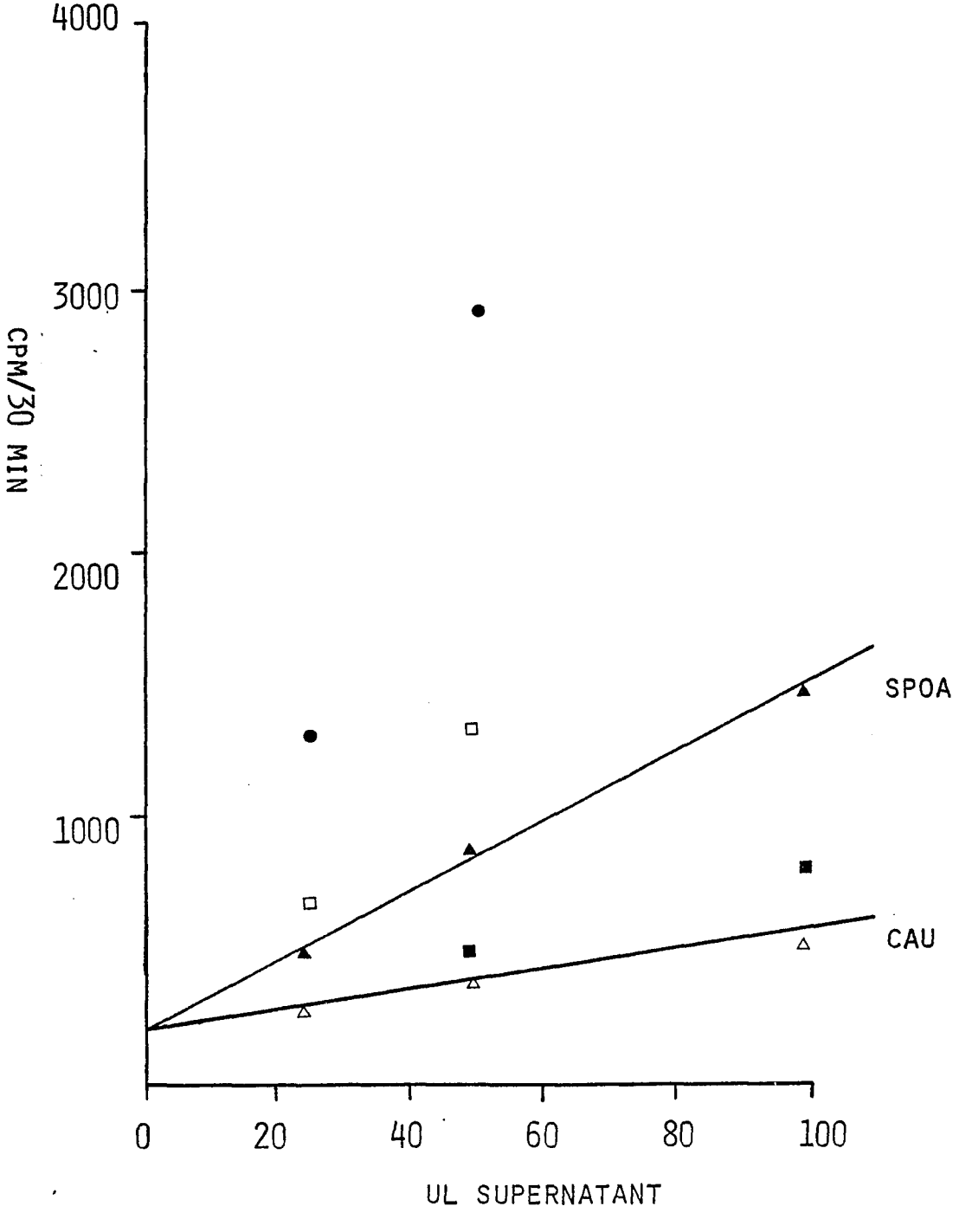


Figure 11. Linearity of midbrain (MB) and hippocampal (HIP) TPOH activity with time. Assay conditions were standard. Enzyme activity is expressed as CPM/50 ul MB or CPM/100 ul HIP (83% efficiency).

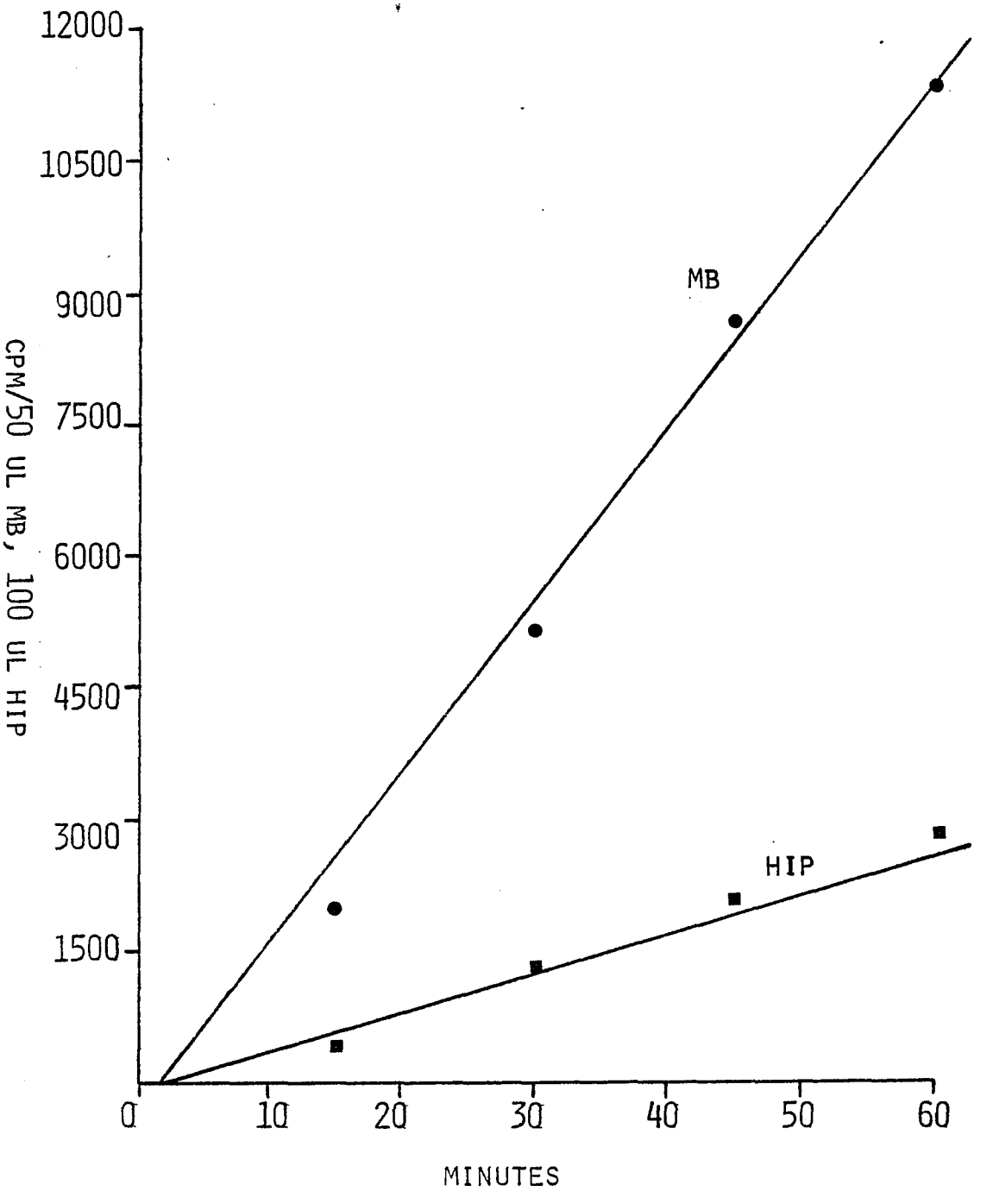


TABLE 2

ASSAY OF MIDBRAIN TPOH AT HIGH SUBSTRATE AND COFACTOR CONCENTRATIONS

	T=100	T=200	T=400
BH_4=200	1.75±0.04	2.13±0.02	2.07±0.03
BH_4=400	2.08±0.06	2.49±0.06	2.36±0.03
6-MPH_4=200	1.34±0.05	1.99±0.10	2.89±0.18
6-MPH_4=400	1.72±0.05	2.61±0.03	3.57±0.15
NO COFACTOR	0.16±0.00	0.31±0.02	0.57±0.01
NO COFACTOR, NO TPOH	0.16±0.01	0.30±0.00	0.64±0.10

Enzyme activity is expressed as nmol/50 ul/30 min (mean of triplicates± standard deviation). T = tryptophan concentration (uM). Cofactor concentrations are also in terms of uM.

Discussion

The basic characteristics of midbrain TPOH activity reported here are in keeping with those observed by other investigators. Enzyme activity is optimal using 0.05 M Tris-HCl, pH 7.4, at 37°C.

An important outcome of these studies was the finding of significant production of $^{14}CO_2$ in the absence of TPOH. The possibility exists that the radioactivity released in the absence of TPOH was the result of tryptophan hydroxylating activity present in the AADC preparation used in the assay. However, the observation that nonenzymatic production of $^{14}CO_2$ was less in the presence of BH_4 than in the presence of 6-MPH_4, suggests that nonenzymatic release of $^{14}CO_2$ from tryptophan is caused by mechanisms other than hydroxylating activity in the AADC preparation.

Aerobic incubation of tetrahydropterins leads to their inactivation as cofactors in the phenylalanine and tyrosine hydroxylating systems. Catalase can partially protect against this inactivation, indicating that a product of aerobic oxidation is hydrogen peroxide (H_2O_2) (Kaufman 1962). Kaufman and his colleagues have observed that in the absence of catalase, an H_2O_2-dependent nonenzymatic hydroxylation of tyrosine occurs when $DMPH_4$ and oxygen are present (Shiman et al., 1971). Thus, incubation of tyrosine with $DMPH_4$ results in the production of dopa. Moreover, in the presence of exogenous quinonoid dihydropterine reductase and NADPH (the enzymatic system for cofactor regeneration), addition of millimolar concentrations of Fe^{++} leads to very high rates of nonenzymatic hydroxylation. On the other hand, when

mercaptoethanol is used to maintain the cofactor in the reduced state, Fe^{++} does not have any effect on nonenzymatic hydroxylation (Shiman et al., 1971).

Similarly, in the present studies, hydrogen peroxide produced by oxidation of BH_4 and 6-MPH_4 may have resulted in nonenzymatic aerobic hydroxylation of tryptophan to 5-hydroxytryptophan, which was then decarboxylated, releasing $^{14}CO_2$. The idea of cofactor-dependent hydroxylation receives support from the data presented in VII. When TPOH was omitted from the assay in the presence of BH_4 (116 uM), the number of pmol $^{14}CO_2$ released was greater than when both TPOH and BH_4 were omitted, indicating that addition of cofactor to the nonenzymatic system results in some hydroxylation of tryptophan. (The number of pmol obtained in the absence of both TPOH and BH_4 is probably the result of direct decarboxylation of tryptophan, as discussed below.) It should be noted, however, that the radioactivity attributed to nonenzymatic hydroxylation is less than 10% of total hippocampal enzyme activity and less than 5% of total midbrain enzyme activity.

Although present in micromolar amounts, it is possible that in the present assay, Fe^{++} increased nonenzymatic hydroxylation of tryptophan. Conflicting results for the effects of Fe^{++} were found: in the case of midbrain, Fe^{++} increased the y-intercept, whereas in the case of hippocampus, Fe^{++} actually decreased the y-intercept (Figure 9). A valid explanation for this difference is lacking. The present assay system contained a mercaptan, dithiothreitol, in addition to the endogenous cofactor regenerating

system, further confounding the interpretation of the effects of Fe^{++} in light of the studies by Kaufman and his coworkers discussed above. Certainly, no conclusion can be made from these studies concerning the effect of micromolar Fe^{++} on nonenzymatic hydroxylation under the present assay conditions without further investigation of the problem.

Ichiyama et al. (1970) reported significant direct decarboxylation of tryptophan when the concentration of this substrate was raised beyond 10 uM. Quantitative analysis of reaction products showed that in the presence of 90 uM tryptophan, 18% of the total $^{14}CO_2$ evolved was due to direct decarboxylation of tryptophan to tryptamine. In the present study of midbrain TPOH, approximately 15% of the total $^{14}CO_2$ evolved in the presence of 80 uM tryptophan was found in blanks (VII). When the concentration of tryptophan was increased to 200 uM in the presence of either 200 or 400 uM BH_4, the proportion of blank to total pmol was essentially the same as when 80 uM tryptophan was used (11-13%).

The linear increase in the number of pmol $^{14}CO_2$ released in blanks observed as the concentration of tryptophan was increased from 100 to 400 uM indicates that essentially all the radioactivity detected in blanks is the result of direct decarboxylation of substrate. Unlike that discussed above, the release of $^{14}CO_2$ from substrate in the absence of cofactor can obviously not be cofactor dependent; in this case it is substrate dependent. However, the release of $^{14}CO_2$ in the absence of cofactor may involve both enzymatic and nonenzymatic mechanisms. As discussed in VII, the data

indicate that almost half the radioactivity present in routinely used blanks is the result of nonenzymatic decarboxylation of substrate. Ichiyama et al. (1970) reported 0.5% nonenzymatic decarboxylation of commercially available L-tryptophan-1-^{14}C. Upon further purification of the substrate using multiple column chromatography, nonenzymatic decarboxylation was reduced by 90% under their assay conditions. In the present studies using substrate from New England Nuclear prepared and stored as indicated in the methods, less than 0.3% of the total radioactivity present was evolved nonenzymatically as CO_2 under standard assay conditions (350 CPM out of a total of 145,000 CPM).

Part II

Homogenization, Preincubation, and Incubation of Tryptophan Hydroxylase Under Reducing Conditions

Results

A variety of reducing conditions have been employed in the preparation and assay of TPOH. Recently, several investigators have shown that this enzyme can be activated by preincubation with DTT and Fe^{++} under anaerobic conditions (Ichiyama et al., 1974; Hamon et al., 1978b; Kuhn et al., 1980). Some of the reducing conditions used in earlier work were repeated here and other conditions were tried.

I. Homogenization

Three reducing agents, dithiothreitol (DTT), ascorbic acid (AA), and metabisulfite (MBS), were tested for their effects on TPOH activity after homogenization of midbrain tissue in their presence (Table 3). Of the three, only DTT had a stimulatory effect on enzyme activity. AA and MBS both resulted in a profound decrease in the activity of TPOH when compared to enzyme prepared from homogenate containing no reducing agent. On the other hand, when 2 mM DTT was included in the homogenate, enzyme activity increased to 144% of control.

Whereas 2 mM DTT produced an increase in enzyme activity, 1 mM DTT and 5 mM DTT in the homogenate resulted in essentially no change in TPOH activity, indicating that DTT is useful in stimulating TPOH only over a narrow concentration range. The inclusion of 50 uM ferrous ammonium sulfate (Fe^{++}) in the homogenate with 5 mM DTT caused

enzyme activity to increase to 131% of control, whereas 50 uM Fe^{++} alone produced a greater than 50% drop in TPOH activity. The observation that Fe^{++} is only stimulatory in the presence of DTT suggests that the oxidation state of the iron is important in determining its effect on enzyme activity. When in aqueous solution, Fe^{++} is rapidly oxidized to Fe^{+++}. Upon addition of DTT, the solution turns pink-brown, indicating that Fe^{+++} has been reduced back to Fe^{++}. DTT maintains the iron in its reduced state (Fe^{++}). If, as has been suggested by Kuhn et al. (1980), a shift in the iron molecule to a higher oxidation status ($Fe^{++} \longrightarrow Fe^{+++}$) is associated with a decrease in the catalytic activity of TPOH, then it becomes apparent why Fe^{++} in aqueous solution in the absence of a reducing agent such as DTT, would produce a decrease in enzyme activity.

II. Preincubation

Preincubation of the 35,000 x g supernatant in air at room temperature for 45 minutes in the absence of reducing agent resulted in a greater than 50% decrease in enzyme activity (Table 4). Inclusion of 5 mM DTT and 50 uM Fe^{++} in the preincubation medium did not prevent the decline in enzyme activity at 25°C. However, when preincubation in the presence of DTT/Fe^{++} was performed at 4°C, TPOH activity increased to 122% of control. These observations suggest that the enzyme is oxidatively and thermally labile and that enzyme stability may be maintained by keeping the enzyme preparation on ice in the presence of DTT and Fe^{++}.

Activation of enzyme by DTT/Fe^{++} by preincubation under

anaerobic conditions was anticipated from the studies of other investigators (Hamon et al., 1978b; Ichiyama et al., 1975; Kuhn et al., 1980). In the present studies, preincubation of supernatant with 5 mM DTT/50 uM Fe^{++} under an atmosphere of 95% oxygen/5% carbon dioxide (O_2), 100% hydrogen (H_2), or 100% nitrogen (N_2) produced an increase in TPOH activity to 133-145% of control. The finding that enzyme activity was stimulated by DTT/Fe^{++} even in the presence of oxygen was unexpected. From this observation, it is apparent that the concentrations of DTT and Fe^{++} used in this study were protective against oxygen inactivation. However, it is difficult to reconcile this finding with that of a loss of activity following preincubation with DTT/Fe^{++} in air at the same temperature and for the same length of time.

Hamon et al. (1978b) found that TPOH activated by anaerobic preincubation with DTT and Fe^{++} possessed a pH optimum (pH 7.2) slightly lower than that of the native enzyme (pH 7.6). Also, contrary to reports of a pH optimum of 7.5 (Jequier et al., 1969), Hori (1975) reported a very low pH optimum (pH 6.5) of enzyme from bovine pineal activated by DTT/Fe^{++}. In the present study, when enzyme activated by preincubation with DTT/Fe^{++} was assayed at pH 7.4, pH 7.2, and pH 7.0, no such change in pH optimum was observed. Assay at the lower pH's actually resulted in a decrease in enzyme activity (Table 4). Similarly, when enzyme activated by DTT/Fe^{++} in the incubation medium was assayed at lower pH's, the increase observed was not as great as when the assay was performed at pH 7.4 (Table 5).

Hori (1975) observed that the pH optimum for activation of pineal

enzyme by DTT/Fe^{++} (pH 8.5) was different from that of the reaction (pH 6.5). Therefore, an attempt was made to activate the enzyme by preincubation at the higher pH. Contrary to the findings of Hori, this resulted in a decrease in enzyme activity (Table 4).

The importance of Fe^{++} in the activation of preincubated enzyme is evident from the following observations. When brain tissue was homogenized in 2 mM DTT alone, preincubated under N_2, and then assayed without addition of Fe^{++}, TPOH activity fell slightly to 89% of control. However, when enzyme homogenized in 2 mM DTT was preincubated in the presence of both DTT and Fe^{++} and assayed in the presence of 1 mM DTT/7 uM Fe^{++}, activity increased to 156% of control. Homogenization of tissue in 5 mM DTT/50 uM Fe^{++} and preincubation and assay without further addition of reducing agent also produced an increase in enzyme activity (to 152% of control). These results also indicate that Fe^{++} need not be present throughout the preparation of tissue (that is, from the point of homogenization) to exert its stimulatory effect.

III. Incubation

From Table 5, it can be seen that a further increase in the concentration of AA or MBS to 2 mM in the incubation mixture caused an even greater decrease in enzyme activity than that occurring in the presence of these reducing agents carried over from the homogenization procedure (0.3 mM). With 2 mM MBS in the incubation mixture, TPOH activity was less than 10% of control, whereas with 2 mM AA, enzyme activity was less than 5%. The finding of such extensive inhibition of enzyme activity by these reducing agents as opposed to stimulation by

DTT indicates that DTT may exert its activating effects on the enzyme specifically via the reduction of disulfide bonds and not merely via the maintenance of a general reducing environment. In support of this idea are the observations of Kuhn et al. (1980) that sulfhydryl compounds such as DTT are also capable of protecting TPOH from inhibition caused by agents which promote the formation of intraprotein disulfide bonds. On the other hand, Friedman et al. (1972) have suggested that the function of the DTT is to maintain the cofactor in the reduced form in the absence of exogenously added quinonoid dihydropterin reductase.

When the concentration of DTT was increased in the assay mixture to 2 mM, enzyme activity fell slightly to 130% of control (Table 5) from the high levels (144% of control, Table 3) reached in the presence of 0.3 mM DTT, the concentration carried over from homogenization. However, when the concentration of DTT was raised to 1 mM, enzyme activity remained unchanged from that measured in the presence of DTT carried over with the homogenate (144% of control). Thus, as previously noted, the concentration of DTT required for optimal enzyme activity is limited to within a narrow range.

Following homogenization in 2 mM DTT, when Fe^{++} was included in the assay together with 2 mM DTT or 1 mM DTT, TPOH activity increased to 164% and 183% of control, respectively. Enzyme homogenized in 5 mM DTT and supplemented with 7 uM Fe^{++} in the assay showed an increase to 125% of control. When homogenized in 5 mM DTT/50 uM Fe^{++} and further supplemented with 2 mM DTT in the assay, activity similarly rose to 118% of control. Homogenization in 50 uM

Fe^{++} and supplementation with 2 mM DTT in the assay resulted in a slight increase in enzyme activity (to 110% of control). From these results, it is clear that addition of DTT and Fe^{++} together to the incubation mixture produces greater increases in enzyme activity than when either DTT or Fe^{++} are added separately at various stages of the assay procedure (e.g. homogenization, preincubation, or incubation). Furthermore, higher levels of activity were achieved in experiments in which DTT/Fe^{++} were merely included in the incubation than in experiments in which enzyme was first preincubated with DTT/Fe^{++} for 45 minutes (Table 4).

In several experiments, TPOH was prepared in the absence but assayed in the presence of reducing agent. Enzyme activity in the presence of 0.7 mM DTT increased to 131% of control. As observed previously, the combination of DTT and Fe^{++} produced higher enzyme activity than DTT alone. When both DTT and 7 uM Fe^{++} were present in the incubation medium, enzyme activity rose further to 153% of control. Alone, 7 uM Fe^{++} caused no change in enzyme activity, suggesting as before, that Fe^{++} must be maintained in its reduced state in order to have any stimulatory effect. 2 mM DTT in the assay caused TPOH activity to increase to 139% of control, whereas a further increment in the DTT concentration to 4 mM resulted in little change in activity (92% of control), again demonstrating the narrow range of concentrations of DTT in the incubation medium required for optimal enzyme activity.

Taken together with the data presented above, these results indicate that enzyme activity is in general greater when DTT is

included at all steps of the assay procedure, beginning with homogenization, and when DTT/Fe^{++} are added in combination to the incubation medium. Thus, highest enzyme activity was achieved by homogenizing the tissue in 2 mM DTT and incubating the resulting 35,000 x g supernatant in the presence of 1 mM DTT and 7 uM Fe^{++}.

As discussed in detail in the introduction, cofactor regeneration is apparently accomplished within 5-HT neurons by the NADH-dependent quinonoid dihydropterin reductase (QDPR). Since this enzyme has been shown to be present in the same 35,000 x g supernatant used to measure TPOH activity (Bullard et al., 1978; Mandell et al., 1980), and Gal (1981) has emphasized that QDPR activity is probably great enough within the neuron so as not to be limiting in cofactor regeneration, it was decided not to include exogenous sheep liver QDPR in the assay as has been done by other investigators (Friedman et al., 1972). In order to determine whether NADH might be limiting in the assay, experiments were performed in the absence and presence of this compound. Enzyme activity in the absence of NADH was 86% of activity in the presence of 0.4 mM NADH using BH_4 as cofactor. With 6-MPH_4 as cofactor, a similar decrease was observed in the absence of NADH (Table 6). On the other hand, in the presence of 6-MPH_4, an increase in the concentration of NADH to 2 mM also produced a slight decline in enzyme activity, to 89% of control. These results reveal a narrow range of concentrations within which NADH stimulates TPOH activity under the conditions used.

Catalase was included in the original coupled radiometric assay developed by Ichiyama et al. (1968; 1970). Several years after these

studies, Friedman et al. (1972) suggested that the catalase was essential for the protection of a part of the hydroxylating system from H_2O_2 inactivation. In the present study, the use of catalase offered no advantage over standard assay conditions (Table 6). Simple addition of catalase in the amount (30 ug) used by Ichiyama et al. (1970) to the standard assay mixture (0.4 mM NADH, 1 mM DTT, 7 uM Fe^{++}) produced little change in enzyme activity (91% of control). Catalase could not replace NADH or DTT/Fe^{++} in the reaction mixture. Omission of DTT/Fe^{++} in the presence of catalase caused a decrease in enzyme activity to 77% of control. When NADH was replaced with catalase, enzyme activity fell slightly (87% of control), whereas omission of NADH and Fe^{++} produced a drop in enzyme activity to 80% of control. Omission of all three compounds (NADH, DTT, and Fe^{++}) in the presence of catalase resulted in a profound decrease in TPOH activity to 41% of control. (Note that in these experiments, because tissue was homogenized in 2 mM DTT, this reducing agent was always present in the assay. Therefore, when DTT is said to be omitted from the assay, this indicates that no further DTT was added to the incubation mixture as is done in the standard enzyme assay. In this case, then, the DTT concentration was 0.3 mM). These results provide further evidence against the suggestion that Fe^{++} functions to decompose H_2O_2 produced during the enzyme reaction.

Whereas Friedman et al. (1972) observed that 1 mM Fe^{++} produced an increase in TPOH activity in the absence of catalase and no change in enzyme activity in its presence, in the present study, increasing the Fe^{++} concentration to only 200 uM produced a decrease in enzyme activity under all conditions tested: in the

presence of 2 mM DTT plus 0.4 mM NADH (42% of control); 2 mM DTT plus 2 mM NADH (55% of control); 2 mM DTT, 0.4 mM NADH plus catalase (50% of control). When NADH was omitted in the presence of catalase and 200 uM Fe^{++}, enzyme activity fell below 20% of control (Table 6).

When TPOH was assayed with 1 mM DTT in the presence of 7, 14, or 68 uM Fe^{++}, highest levels of enzyme activity were reached when the concentration of Fe^{++} was 14 uM.

TABLE 3

EFFECT OF HOMOGENATE REDUCING CONDITIONS ON MIDBRAIN TPOH ACTIVITY

HOMOGENIZATION CONDITIONS	INCUBATION CONDITIONS*	% CONTROL
1 mM DTT	0.1 mM DTT	97 (1)
2 mM DTT	0.3 mM DTT	144 (4)
5 mM DTT	0.7 mM DTT	101 (1)
5 mM DTT, 50 uM Fe++	0.7 mM DTT, 7 uM Fe++	131 (1)
50 uM Fe++	7 uM Fe++	48 (1)
2 mM MBS	0.3 mM MBS	13 (1)
2 mM AA	0.3 mM AA	19 (1)

Enzyme activity was assayed in the presence of 10 uM tryptophan, 116 uM BH_4. Control samples were prepared and assayed in the absence of reducing agent. The number of separate experiments is indicated in parentheses; where more than one experiment was performed, the results are expressed as the mean % control. * indicates that there was no further addition of reducing agent. The concentration present in the incubation was carried over from homogenization.

TABLE 4

EFFECT OF PREINCUBATION OF ENZYME PREPARATION WITH REDUCING AGENTS ON MIDBRAIN TPOH ACTIVITY

HOMOGENIZATION CONDITIONS	PREINCUBATION CONDITIONS	TEMPERATURE (°C)	INCUBATION CONDITIONS	% CONTROL
0	air	25	0	47 (1)
0	air, 5 mM DTT, 50 uM Fe++	25	0.7 mM DTT, 7 uM Fe++	67 (1)
0	as above	4	as above	122 (1)
0	O2, 5 mM DTT, 50 uM Fe++	25	as above	133 (1)
0	H2, 5 mM DTT, 50 uM Fe++	25	as above	145 (1)
0	N2, 5 mM DTT, 50 uM Fe++	25	as above	136 (2)
0	as above	25	1 mM DTT, 7 uM Fe++	95 (1)
0	as above	25	1 mM DTT, 7 uM Fe++, pH 7.2	90 (1)
0	as above	25	as above, pH 7.0	73 (1)
0	as above, pH 8.5	25	as above, pH 7.4	70 (1)
2 mM DTT	N2, 2 mM DTT	25	0.3 mM DTT	89 (1)
5 mM DTT, 50 uM Fe++	N2, 5 mM DTT, 50 uM Fe++	25	0.7 mM DTT, 7 uM Fe++	152 (1)
2 mM DTT	as above	25	1 mM DTT 7 uM Fe++	156 (1)

Enzyme activity was assayed in the presence of 10 uM tryptophan, 116 uM BH_4. Controls were prepared and assayed in the absence of reducing agent and were maintained on ice until time of assay. The number of separate experiments is indicated in parentheses; where more than one experiment was performed, the results are expressed as the mean % control.

TABLE 5

EFFECT OF REDUCING AGENTS ADDED TO THE INCUBATION MEDIUM ON MIDBRAIN TPOH ACTIVITY

HOMOGENIZATION CONDITIONS	INCUBATION CONDITIONS	% CONTROL
0	2 mM DTT	139 (2)
0	4 mM DTT	92 (1)
0	0.7 mM DTT	131 (2)
0	0.7 mM DTT, 7 uM Fe^{++}	153 (1)
0	7 uM Fe^{++}	98 (1)
2 mM DTT	2 mM DTT	130 (3)
2 mM DTT	1 mM DTT	144 (2)
2 mM DTT	1 mM DTT, 7 uM Fe^{++}	183 (2)
2 mM DTT	2 mM DTT, 7 uM Fe^{++}	164 (1)
2 mM DTT	1 mM DTT, 7 uM Fe^{++}, pH 7.2	158 (1)
2 mM DTT	1 mM DTT, 7 uM Fe^{++}, pH 7.0	133 (1)
5 mM DTT	0.7 mM DTT, 7 uM Fe^{++}	125 (1)
5 mM DTT, 50 uM Fe^{++}	2 mM DTT, 7 uM Fe^{++}	118 (1)
50 uM Fe^{++}	2 mM DTT, 7 uM Fe^{++}	110 (1)
2 mM MBS	2 mM MBS	<10 (1)
2 mM AA	2 mM AA	<5 (1)

Enzyme activity was assayed in the presence of 10 uM tryptophan, 116 uM BH_4. Controls were prepared and assayed in the absence of reducing agents. The number of separate experiments is indicated in parentheses where more than one experiment was performed, the results are expressed as mean % control.

TABLE 6

EFFECT OF REDUCING AGENTS AND CATALASE ON MIDBRAIN TPOH ACTIVITY

INCUBATION CONDITIONS	% CONTROL
-NADH	86
+2 mM NADH	89
+catalase (30 ug), -NADH	87
+catalase	91
+catalase, -DTT, $-Fe^{++}$	77
+catalase, -NADH, $-Fe^{++}$	80
+catalase, -NADH, -DTT, $-Fe^{++}$	41
+2mM DTT, +200 uM Fe^{++}	42
+2 mM NADH, +2 mM DTT, +200 uM Fe^{++}	55
+catalase, +2 mM DTT, +200 uM Fe^{++}	50
+catalase, -NADH, +2 mM DTT, +200 uM Fe^{++}	17

Enzyme activity was assayed in the presence of 400 uM tryptophan, 400 uM 6-MPH_4. Control enzyme was measured using 0.4 mM NADH, 1 mM DTT, and 7 uM Fe^{++}. Variations from these conditions are indicated: + indicates that the compound was included in the assay; - indicates that it was excluded.

Discussion

In general, these results corroborate the findings of Kuhn et al. (1980) that brain TPOH is subject to inactivation by air and heat. A 45-minute preincubation in air at room temperature resulted in a greater than 50% loss of enzyme activity. DTT and Fe^{++} did not protect much against loss of activity at room temperature, but at 4°C, actually caused an increase in enzyme activity. Similarly, Kuhn et al. found that preincubation of TPOH in air at 37°C resulted in a greater than 50% drop in activity within one hour. When 2 mM DTT was included in the preincubation, activity fell about 40%. However, when the preincubation was performed at 0°C in the presence of 2 mM DTT, enzyme activity did not change significantly even after 6 hours of preincubation (Kuhn et al., 1980).

TPOH from midbrain can be activated by preincubation under anaerobic conditions in the presence of DTT and Fe^{++} (Table 4). However, the extent of activation was somewhat less than the 73-83% achieved by Hamon et al. (1978b) and much less than the 3-fold activation observed by Hori et al. (1976). In the present study, the effect of preincubation of TPOH with DTT and Fe^{++} under N_2 was dependent on both homogenization and assay conditions.

Whereas simple homogenization in 2 mM DTT produced an increase in TPOH activity (Table 3), preincubation of a similar preparation under N_2 actually caused a decrease in enzyme activity (Table 4). Thus, the conditions for activating TPOH are different depending on whether or not the enzyme is preincubated. Enzyme not preincubated can be activated by DTT alone, whereas preincubated enzyme requires the

presence of both DTT and Fe^{++} for activation to occur. Similarly, Hamon et al. (1978) found that TPOH preincubated under anaerobic conditions or in air could only be activated by a combination of DTT and Fe^{++}. Alternatively, the decrease observed with enzyme preincubated for 45 minutes at room temperature under N_2 as compared to enzyme homogenized in 2 mM DTT and kept on ice until assay suggests the existence of a temperature-dependent inactivating mechanism occurring with time. This inactivating mechanism is different from that of oxygen, since preincubation was supposedly carried out under anaerobic conditions. Loss of enzyme activity may be the result of the action of endogenous proteases on the enzyme or the consequence of thermal denaturation of enzyme molecules.

Of interest is the observation that even when enzyme was preincubated in the presence of DTT/Fe^{++} under oxygen, activity was increased. Kuhn et al. (1980) reported that 2 mM DTT could partially protect against inactivation of TPOH resulting from incubation under oxygen for 20 minutes. In the present case, the concentration of DTT/Fe^{++} used (5 mM/50 uM) apparently afforded the enzyme a more complete protection against the effects of oxygen.

A possible explanation of the apparent increase in enzyme activity under oxygen is that the preincubated enzyme was fully saturated with its substrate (O_2), whereas enzyme kept in air on ice for 45 minutes was not. Thus, when whole homogenate (Green and Sawyer, 1966) and partially purified TPOH (Youdim et al., 1975) were incubated under 95-100% O_2, enzyme activity increased 400% and 40% respectively. On the other hand, the reported Km of purified TPOH for O_2 in the

presence of BH_4 is 2.5% (Friedman et al., 1972), suggesting that even in air, the enzyme is saturated with O_2. It is likely that the Km of the enzyme for O_2 is dependent on the state of purity of the enzyme.

The extent of activation of TPOH by preincubation in the presence of DTT/Fe^{++} under anaerobic conditions was never as great as when 1 mM DTT and 7 uM Fe^{++} were merely included in the incubation medium of the enzyme assay. Recently, Nakata and Fujisawa (1982) purified TPOH to homogeneity and found that pure TPOH was stimulated 3.5-fold by the addition of 20 uM Fe^{++} to the enzyme assay. Their assay also included 2 mM DTT. Contrary to these results, Hamon et al. (1978b) found that the inclusion of 3 mM DTT and 5 uM Fe^{++} in the assay resulted in a 25% decrease in enzyme activity of the 35,000 x g supernatant. In the present experiments, DTT alone in the incubation, when present in concentrations less than 4 mM, was able to activate TPOH to some degree. Kuhn et al. (1980) observed extensive inhibition of enzyme activity when the concentration of DTT in the assay was raised to 5 mM. These observations are in marked contrast to those noted with the enzyme from bovine pineal. Pineal TPOH activity is not inhibited by DTT concentrations as high as 20-30 mM, and assays of pineal enzyme routinely use these high concentrations of DTT (Ichiyama et al., 1974; Hori, 1975; Hori and Ohotani, 1978; 1981). The requirement of bovine pineal TPOH for higher DTT concentrations may be a consequence of unavoidable exposure of the pineal tissue to air during transport from the slaughterhouse to the laboratory. As a result of exposure to air, pineal TPOH has probably lost much of its activity and requires higher levels of DTT to be reactivated. Kuhn et

al. (1980) have shown that rat midbrain TPOH inactivated by exposure to oxygen can be reactivated by anaerobic preincubation with 5 mM DTT/50 uM Fe^{++}.

Based on the observation that in the presence of a sheep liver dihydropteridine reductase system for cofactor regeneration, DTT produced an inhibition of TPOH, Friedman et al. (1972) suggested that the stimulation of TPOH activity caused by the inclusion of a mercaptan in the assay seen by many other investigators was due to its reducing effect on the cofactor. The present studies cannot absolutely rule out this possibility, as stimulation of enzyme activity was greatest when 1 mM DTT was included in the assay. Moreover, in all cases of stimulation, DTT was present in the incubation medium. However, work by Hamon et al. (1978b) has demonstrated that following preincubation with DTT, the presence of DTT in the assay is not necessary to produce enzyme activation.

Friedman et al. (1972) reported a lack of stimulation of TPOH activity by Fe^{++} in the presence of catalase. It should be noted, however, that the concentration of Fe^{++} used by these investigators was 1 mM. In the present study, an Fe^{++} concentration of only 0.2 mM caused a marked inhibition of enzyme activity (Table 6). Because in their study, catalase markedly stimulated TPOH activity and Fe^{++} stimulated activity only in the absence of catalase, Friedman et al. suggested that, as with tyrosine hydroxylation, Fe^{++} could substitute for catalase in protecting TPOH from H_2O_2-mediated inactivation.

In this study, the use of catalase offered no advantage over the

standard conditions used in the assay for TPOH. The slight, but possibly insignificant decrease in enzyme activity occurring when catalase was included in the assay, may have been the result of the ability of catalase to protect against H_2O_2-mediated nonenzymatic tryptophan hydroxylation (see discussion, Part I). Ichiyama et al. (1970) had observed that in the presence of catalase, enzyme activity increased 3.5 times when DTT and Fe^{++} were included in the assay. When only DTT was included, activity increased 2.5 times. On the other hand, Friedman et al. (1972) observed that in the presence of catalase, 1.4 mM DTT actually produced a drop in enzyme activity. These latter observations are more consistent with those of the present study. However, it should be emphasized that catalase could not completely substitute for DTT and Fe^{++}, as enzyme activity dropped to 77% of control when the DTT concentration was reduced to that carried over from homogenization and Fe^{++} was replaced by catalase.

Recent studies by Kuhn et al. (1980) have complicated the question of whether H_2O_2-mediated inactivation of TPOH really occurs in the absence of catalase as suggested by Friedman et al. (1972). As mentioned previously, Kuhn et al. demonstrated that TPOH can be inactivated by exposure to oxygen. The finding that oxygen inactivation could not be prevented by scavengers of oxygen radicals and H_2O_2 suggests that molecular oxygen and not an oxygen radical is repsonsible for enzyme inactivation.

Although the results of the present experiments, when considered together with those of other studies, indicate that TPOH requires

Fe^{++} and a sulfhydryl-reducing agent for full activity, the roles of these agents in the enzyme reaction are far from clear. The ability of DTT to (re)activate TPOH and to protect against O_2 inactivation and inactivation by sulfhydryl blocking agents (Kuhn et al., 1980) suggests that -SH groups may be critical in maintaining enzyme stability and catalytic activity. Similarly, iron might be involved in maintaining the structure of the active enzyme and may have a catalytic function in the enzyme reaction. Studies on purified phenylalanine hydroxylase suggest that Fe^{++} is involved in the catalytic functioning of this enzyme. The metal may play a role in the activation of oxygen, facilitating the transfer of electrons between BH_4 and O_2 (Fisher et al., 1972). Recent work on purified TPOH showing an increase in enzyme activity and a significant change in the pH profile for enzyme activity in the presence of Fe^{++}, suggests that the effect of Fe^{++} is related to a conformational change in active enzyme (Nakata and Fujisawa, 1982). Thus, neither a structural role nor a functional role for Fe^{++} in TPOH can be ruled out at the present. On the contrary, it seems likely that Fe^{++} would be involved in both the structure and function of the enzyme.

Part III

Preincubation and Incubation of Tryptophan Hydroxylase Under Phosphorylating Conditions

Results

Several groups of investigators (Hamon et al., 1978c; Kuhn et al., 1978; Lysz and Sze, 1978; Yamauchi and Fujisawa, 1979a; Vitto and Mandell, 1981) have reported activation of TPOH by phosphorylating conditions. The requirement of calcium ion and calmodulin in this activation has been recently demonstrated directly by Yamauchi and Fujisawa (1979b; 1980; 1981) and by Kuhn et al. (1980). Similarly, tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis, has been shown to be activated by phosphorylating conditions. Activation of tyrosine hydroxylase by ATP and Mg^{++} is cyclic AMP dependent (Goldstein et al., 1976; Lovenberg et al., 1975; Morgenroth et al., 1975).

Acheson and Zigmond (1981) have provided evidence for the existence of an activated form of tyrosine hydroxylase in residual noradrenergic terminals in the hippocampus following 6-hydroxydopamine (6-OHDA) damage to the region. Thus, 5 to 7 days after an intraventricular injection of 6-OHDA, hippocampal tyrosine hydroxylase cannot be activated by phosphorylating conditions, but possesses the pH optimum and Km of the activated form of the enzyme. By 3 weeks after the injection, the characteristics of the enzyme have returned to normal and the enzyme can again be activated by ATP and Mg^{++}.

In view of these observations, the following studies of activation

of TPOH by phosphorylating conditions were undertaken with the aim of investigating the state of TPOH in 5-HT terminals remaining in the hippocampus after 5,7-DHT lesions of the cingulum bundle.

I. Effect of Phosphorylating Conditions on Midbrain and Hippocampal TPOH Activity

Midbrain TPOH assayed in the presence of $MgCl_2$ or $CaCl_2$ alone showed a slight increase in activity when compared to enzyme assayed in the absence of these compounds. A 14% increase was observed in the presence of $MgCl_2$, whereas $CaCl_2$ produced an 11% increase. However, when assayed in the presence of ATP alone, enzyme activity slightly decreased 10% from control. When all 3 compounds (ATP, $MgCl_2$, $CaCl_2$) were included in the assay in various combinations of concentrations from 0.5 to 2 mM for ATP, 5 to 10 mM for $MgCl_2$, and 10 uM for $CaCl_2$, enzyme activity increased 13 to 24% above control. In the absence of $CaCl_2$, ATP and $MgCl_2$ together produced an increase in TPOH activity: 17% above control for 1 mM ATP/0.5 mM $MgCl_2$ and 20% above control for 1 mM ATP/10 mM $MgCl_2$. Thus, in these experiments Ca^{++} had little or no stimulatory effect beyond that of ATP/Mg^{++} alone. In general, activation of midbrain TPOH by ATP and $MgCl_2$ with and without $CaCl_2$ as observed in these studies was much less than that observed by other investigators (see discussion).

Phosphorylating conditions produced a larger percent increase in TPOH activity in the hippocampus. 0.5 to 2 mM ATP/10 mM $MgCl_2$/10 uM $CaCl_2$ caused hippocampal TPOH activity to rise 46 to 50% above control. ATP plus $MgCl_2$ alone in concentrations of 1 and 10 mM,

respectively, produced a 48% increase in TPOH activity. Again, Ca^{++} had no effect on activation of TPOH by ATP/Mg^{++} in these experiments, and the activation observed in the hippocampus was still less than that observed in the midbrain by other investigators. The following series of experiments were performed in an attempt to establish conditions under which greater levels of activation of TPOH by ATP/Mg^{++} could be achieved.

II. Effect of DTT/Fe^{++} on Activation of TPOH by Phosphorylating Conditions

In order to determine whether the inclusion of DTT and Fe^{++} in the assay might prevent full activation of TPOH by phosphorylating conditions, the amount of DTT in the assay was varied 100-fold (0.01 mM to 1 mM) and Fe^{++} was omitted or varied 10-fold (0.7 uM to 7 uM). Enzyme activity was assayed in the absence and presence of phosphorylating conditions. In all but one case, 1 mM ATP and 10 mM $MgCl_2$ produced an increase (from 7 to 23 %) in TPOH activity. However, when enzyme was assayed in the presence of 0.01 mM DTT alone, ATP/$MgCl_2$ actually resulted in a 19% decrease in activity. The reason for this decrease is unknown. It is possible that very low concentrations of DTT in the absence of Fe^{++} produce a form of the enzyme which is more susceptible to deactivation processes. These results indicate that, in general, activation of TPOH is probably not inhibited by the presence of DTT and Fe^{++} in the assay. On the contrary, it appears that a minimal DTT concentration is necessary to maintain the enzyme in a form stable enough to be activated.

III. Efect of pH on Activation of TPOH by Phosphorylating Conditions

(1)

Hamon and his colleagues (1978c) had previously observed a progressive increase in ATP/Mg^{++} induced activation of TPOH from almost zero at pH 7.3 to 80% at pH 8.3. These investigators also observed a shift in the optimal pH for TPOH activity from pH 7.6 to pH 7.9 when ATP and Mg^{++} were included in the assay. Thus, the pH optimum was determined for enzyme assayed under phosphorylating conditions. Since 6-MPH_4 is the cofactor which has been used most often in studies of TPOH activation, a pH profile was also determined for enzyme assayed with this cofactor. Also, a lower concentration of BH_4 was included in these experiments, since activation is most apparent when the enzyme is assayed near its cofactor Km (Kuhn et al., 1978). Therefore, a pH profile of activation by phosphorylating conditions was established for enzyme assayed with 30 and 116 uM BH_4 and with 116 uM 6-MPH_4.

When the concentration of BH_4 was 30 uM, activation by 1 mM ATP/10 mM $MgCl_2$ occurred to different degrees as the pH of the assay medium was varied from 7.2 to 7.8. At pH 7.2 and 7.6, maximal activation was 20%. As the pH was increased above 8.0, phosphorylating conditions produced essentially no change in enzyme activity. A similar situation was observed when TPOH activity was assayed at 116 uM BH_4. However, maximal activation under this cofactor concentration was 13% at pH 7.4 and a fall in enzyme activity with ATP/$MgCl_2$ occurred at pH 8.0-8.2. The pattern of activation observed by Hamon et al., whereby the percent activation was seen to increase with increasing pH, was not found in this experiment.

6-MPH_4 (116 uM) gave a very different pH profile of activation by phosphorylating conditions. Unlike with BH_4, in the presence of 6-MPH_4, ATP/$MgCl_2$ produced an increase in TPOH activity at every pH. Maximal activation was 32% at pH 7.6. Again, in contrast to the observations of Hamon et al., there was no increase in activation by phosphorylating conditions beyond pH 7.6.

In these studies, there was a slight shift in the pH optimum of enzyme assayed with 116 uM 6-MPH_4, from pH 7.4 under control conditions to pH 7.6 under phosphorylating conditions. With 30 uM BH_4, the pH optimum was 7.6 under both sets of conditions; and with 116 uM BH_4, the pH optimum was 7.4 under both sets of conditions. In further experiments on activation of TPOH by phosphorylating conditions (with the exception of IV, below), the pH of the assay medium was kept at 7.4 when measuring both control and activated enzyme activity.

IV. Effect of pH on Activation of TPOH by Phosphorylating Conditions (2)

Hamon et al. (1978c) used a spectrofluorometric method to measure the activity of TPOH in the presence of 150 uM tryptophan and 160 uM 6-MPH_4. In their assay, addition of AADC plus PLP was unnecessary. An attempt was made here to reproduce as closely as possible the conditions of the assay used by Hamon et al. The differences to be noted are the use of Tris-HCl as opposed to Tris-acetate, the addition of 50 ul AADC plus 0.1 mM PLP, and a substrate concentration of 10 uM. All other conditions (pH of homogenization, use of mercaptoethanol and catalase) were those used by these other investigators.

Again, the pH profile of activation of TPOH observed by Hamon et al. was not found with the assay conditions used here. Maximal activation (32%) was found at pH 7.2. Activation at other pH's varied from 4 to 17%. Moreover, under these assay conditions, the pH optimum of TPOH activity actually decreased from pH 7.8 under control conditions to pH 7.6 under phosphorylating conditions. The differences between the results of the present study and that of Hamon et al. might be the result of the inclusion of AADC or the use of a lower concentration of substrate in the present assay (see discussion).

V. Effect of Preincubation on Activation of TPOH by Phosphorylating Conditions

In one experiment, it was noted that when TPOH was preincubated for 7 minutes at 37°C in the presence of all reagents with the exception of substrate and cofactor (no phosphorylating conditions), enzyme activity fell 24% from that measured when no preincubation was carried out. Unexpectedly, inclusion of BH_4 in the preincubation resulted in a slight (10%) increase in enzyme activity when compared to enzyme not preincubated. The level of enzyme activity found when preincubation was performed in the presence of BH_4 was almost equal to that found under phosphorylating conditions, which in this experiment increased enzyme activity 13%. It was hypothesized that the continuous presence of BH_4 in the incubation mixture might be partially masking the activation effect. Thus, a series of experiments was performed to determine the effects of preincubation of TPOH under various cofactor/substrate conditions on the activation of enzyme activity by 1 mM ATP/10 mM $MgCl_2$ (Table 7). All preincubations

were performed in the presence of NADH, DTT, Fe^{++}, AADC, and PLP.

When TPOH was preincubated with ATP/$MgCl_2$ for 10 minutes at 37°C in the absence of cofactor and substrate and assayed at 116 uM BH_4, there was little activation of enzyme activity. Moreover, when cofactor was included in the preincubation, activation by phosphorylating conditions did not occur. This is in marked contrast to the observations of Vitto and Mandell (1981). These investigators found that when BH_4 was included in the preincubation, enzyme activity in the presence of ATP/Mg^{++} was essentially twice that of control enzyme activity (no ATP/Mg^{++}), whereas in the absence of BH_4, enzyme activity in the presence of ATP/Mg^{++} declined rapidly to almost control levels within 20 minutes of preincubation.

Activation of enzyme not preincubated at all was 16%. When the preincubation mixture included tryptophan (10 uM), activation increased to 25%. However, with both BH_4 and tryptophan in the preincubation mixture, again activation did not occur. These results suggest that the presence of tryptophan was necessary for activation by ATP/Mg^{++}, whereas the presence of BH_4 prevented activation from occurring.

When ATP/$MgCl_2$ were not included in the preincubation but were added just prior to assay, a very different pattern of activation of preincubated enzyme was observed. Phosphorylating conditions produced a 13% decrease in the activity of enzyme preincubated with tryptophan. There was no change with ATP/$MgCl_2$ when enzyme was preincubated in the presence of BH_4 or tryptophan plus BH_4. Under these conditions, when ATP/Mg^{++} were not added until after the

preincubation was complete, it appears that preincubation with tryptophan resulted in a form of the enzyme which was susceptible to inactivation by ATP/Mg^{++}. Yamauchi and Fujisawa (1979a) have provided evidence to show that TPOH is inactivated by an endogenous phosphoprotein phosphatase in the presence of Mg^{++}. The possibility exists that TPOH in the presence of its substrate is more susceptible to the phosphatase than to the phosphorylating enzyme.

Control enzyme activity was also found to vary depending on the conditions of the preincubation mixture. In these experiments, activity of preincubated enzyme was essentially unchanged from that of non-preincubated enzyme. However, when BH_4 was included in the preincubation, enzyme activity increased 33% above the activity of non-preincubated enzyme (275.4 pmol vs 207.5 pmol), indicating that cofactor alone had an activating effect on the enzyme. However, it appears from the above results, that cofactor activated enzyme is less susceptible to both activation by ATP/Mg^{++} and inactivation by Mg^{++}.

On the other hand, inclusion of tryptophan in the preincubation mixture produced a decrease (16%) in enzyme activity when compared to non-preincubated enzyme (173.3 pmol vs 207.5 pmol), suggesting that substrate had the opposite effect of cofactor. Tryptophan appears to cause inactivation of the enzyme, rendering it more susceptible to activation and inactivation in the presence of phosphorylating conditions.

When enzyme was assayed in the presence of 200 uM 6-MPH_4, overall activation by ATP/Mg^{++} was greater than in the presence of

BH_4 (Table 7). Activation of enzyme preincubated alone with $ATP/MgCl_2$ was only 14% - a decrease from the 30% activation observed when enzyme was not preincubated. When 6-MPH_4 was included in the preincubation, activation was 19%. An increase in activation to 32% was observed when enzyme was preincubated in the presence of tryptophan alone. Inclusion of both 6-MPH_4 and tryptophan in the preincubation mixture resulted in a 17% activation of TPOH. These findings parallel those obtained using BH_4.

Again, using 6-MPH_4, a very different pattern of activation was observed when $ATP/MgCl_2$ were not included in the preincubation medium. Addition of $ATP/MgCl_2$ to the assay of enzyme preincubated in the absence of cofactor and substrate produced essentially no change in enzyme activity. However, when enzyme was preincubated in the presence of 6-MPH_4, a 16% activation was again observed. Preincubation of enzyme in the presence of tryptophan alone or tryptophan plus 6-MPH_4 and assay in the presence of $ATP/MgCl_2$, resulted in a 19% increase in activity.

As in the case of BH_4, control enzyme activity assayed with 200 uM 6-MPH_4 depended on the conditions of the preincubation. Activity of enzyme preincubated alone was 35% greater than activity of enzyme not preincubated at all (96.7 pmol vs 71.6 pmol). When 6-MPH_4 was included in the preincubation mixture, enzyme activity rose 55% above non-preincubated enzyme activity (110.0 pmol vs 71.6 pmol). Enzyme preincubated with tryptophan alone showed a 21% increase in activity when compared to non-preincubated enzyme (86.6 pmol vs 71.6 pmol). From these data, it is apparent that the characteristics of

enzyme activation/ inactivation by phosphorylating conditions and by cofactor/substrate are dependent on the cofactor used.

VI. Effect of Order of Addition of Assay Components on Activation of TPOH by Phosphorylating Conditions

All previous preincubations had been carried out in the presence of NADH, DTT, Fe^{++}, AADC, and PLP. The possibility that inclusion of these reagents in the preincubation mixture was hindering the full expression of activation by phosphorylating conditions was tested by adding all the above reagents after preincubation (Table 8).

Major changes in the pattern of activation observed in the presence and absence of cofactor and/or substrate were seen when all other assay components were excluded from the preincubation. Enzyme not preincubated at all showed 23% activation in the presence of both ATP/$MgCl_2$ and ATP/$MgCl_2$/$CaCl_2$. However, when enzyme was first preincubated for 8 minutes at 37° in the presence of ATP/$MgCl_2$ or ATP/$MgCl_2$/$CaCl_2$, enzyme activity fell 13% and 25%, respectively. Inclusion of BH_4 in the preincubation mixture resulted in little change in enzyme activity in the presence of ATP/$MgCl_2$ and a 13% activation in the presence of ATP/$MgCl_2$/$CaCl_2$. On the other hand, when preincubation was carried out in the presence of tryptophan, ATP/$MgCl_2$ caused a less than 10% decrease in activity, whereas ATP/$MgCl_2$/$CaCl_2$ resulted in a 13% decrease in activity. Thus, under these conditions, in contrast to preincubation in the presence of all assay components, BH_4 seemed to exert a permissive effect on phosphorylation by ATP/Mg^{++}/Ca^{++}, whereas tryptophan had the opposite effect.

When enzyme preincubation was carried out in the absence of all other assay components with the exception of substrate and/or cofactor, preincubated control enzyme activity was always less than non-preincubated control enzyme activity (Table 8). When compared to that of the preceeding section, these data suggest that one of the other assay components (AADC, PLP, DTT/Fe^{++}) acts to stabilize the enzyme and possibly to protect it from inactivating mechanisms. Relevant to these observations is the finding discussed previously (II) of a decrease in enzyme activity in the presence of ATP/Mg^{++} when the concentration of DTT was very low (0.01 mM). Thus, as concluded earlier, a minimal concentration of DTT may be required to stabilize the enzyme and enable it to be activated by phosphorylating conditions.

VII. Effect of an ATP-Regenerating System on Activation of TPOH by Phosphorylating Conditions

In order to assure optimal utilization of ATP, an ATP-regenerating system was included in the assay of TPOH under phosphorylating conditions. The ATP-regenerating system employed here consisted of creatine kinase (CK, 1 mg/ml), creatine phosphate (20 mM), and ATP (1 mM). Enzyme activity was assayed in the presence and absence of 7 uM ferrous ammonium sulfate (Fe^{++}).

In the presence of 1 mM ATP/10 mM $MgCl_2$, the ATP-regenerating system (CK-CP) had essentially no effect on activation both in the presence and absence of Fe^{++}. In all 4 cases ($\pm Fe^{++}/\pm$CK-CP), activation ranged between 18 and 21%. When 10 uM $CaCl_2$ was included with ATP/$MgCl_2$, CK-CP actually produced a decrease in activation from 32% (no CK-CP) to 24% and 22% in the presence and

absence of Fe^{++}, respectively. Because the ATP-regenerating system did not increase the degree of activation by ATP/Mg^{++}, it was concluded that rapid hydrolysis of ATP did not play a significant role in limiting the activating effects of ATP/Mg^{++}.

VIII. Effect of EDTA on Activation of TPOH by Phosphorylating Conditions

The presence of heavy metals results in an inhibition of phosphorylation reactions. EDTA chelates heavy metals, and low concentrations of this compound have been shown to activate phosphotransfer reactions (Milstein, 1961; O'Sullivan and Morrison, 1963). Thus, to protect against the possible presence of heavy metals in the assay, enzyme activity was measured under phosphorylating conditions in the presence of 10^{-3}, 10^{-4}, and 10^{-5} M EDTA.

In the first experiment, enzyme activity was assayed with 1 mM ATP/10 mM $MgCl_2$ and with 1 mM ATP/10 mM $MgCl_2$/10 uM $CaCl_2$. In the first case, enzyme activity was increased 25% above control. Inclusion of 10^{-3} M EDTA in the assay both decreased control enzyme activity by 20% and resulted in essentially no activation in the presence of ATP/$MgCl_2$. The decrease in control enzyme activity is likely due to the iron-chelating activity of EDTA, whereas the lack of activation observed with ATP/Mg^{++} might be the result of magnesium and calcium chelation by EDTA. 10^{-4} M EDTA produced a similar decrease in control enzyme activity, although activation was greater than for 10^{-3} M EDTA (20%). With 10^{-5} M EDTA, control enzyme activity decreased less than 10% and activation was similar to that observed with 10^{-4} M EDTA (19%). These data suggest that

activation by ATP/Mg^{++} was not being inhibited by heavy metals present in the assay.

In the presence of ATP/$MgCl_2$ plus $CaCl_2$, TPOH activity increased to 32% above control. With 10^{-3} M EDTA in the assay mixture, again there was essentially no activation. 10^{-4} M EDTA resulted in a 26% activation, whereas 10^{-5} M EDTA produced a 19% activation of TPOH under these conditions. These results are similar to those obtained in the absence of Ca^{++}.

Because EDTA also chelates calcium ion (Ca^{++}), the concentration of $CaCl_2$ in the assay was increased to 50 and 100 uM when EDTA was present with phosphorylating conditions. Without EDTA, activation rose from 31% in the presence of ATP/$MgCl_2$ alone, to 41-42% when 50 or 100 uM $CaCl_2$ was included in the assay. In the presence of 10^{-4} M EDTA, activation increased from 25% with ATP/$MgCl_2$ alone, to 48% when 100 uM $CaCl_2$ was included in the assay. Surprisingly, when 10^{-5} M EDTA was present, activation observed with ATP/$MgCl_2$ was the same in the presence and absence of up to 100 uM $CaCl_2$ (34-37%). These results indicate that EDTA had little effect on calcium-dependent activation of TPOH.

An increase in the calcium ion concentration in these experiments to 50-100 uM resulted in a large increment in the extent of enzyme activation (with the exception of 10^{-5} M EDTA), suggesting that Ca^{++} may have been limiting activation in previous experiments. However, results obtained with the use of Ca^{++} concentrations greater than 100 uM must be interpreted with caution. Knapp et al. (1975) and Boadle-Biber (1975) reported that TPOH activity was

stimulated by high, unphysiological levels of Ca^{++} (0.5-10 mM). Hamon et al. (1977) demonstrated that activation of TPOH by these high concentrations of Ca^{++} was the result of partial enzyme proteolysis by a Ca^{++}-dependent protease. Recently, Vitto and Mandell (1981) showed that the effects of ATP/Mg^{++} and 0.5 mM Ca^{++} were additive. Thus, the activation effect observed with 50-100 uM Ca^{++} may not be identical to that observed in the presence of ATP/Mg^{++} alone or with lower Ca^{++} concentrations.

In this last experiment, activation by phosphorylating conditions in the absence and presence of Ca^{++} was greater than that seen in previous experiments. The reason for this difference is unknown. However, it is apparent from all these experiments that the magnitude of activation by phosphorylating conditions is quite variable. The extent of activation observed in any one experiment may depend on the state of activation of the enzyme in vivo. There is evidence to show that endogenous levels of activated enzyme exist and may persist in vitro (see discussion).

TABLE 7

EFFECT OF PREINCUBATION CONDITIONS ON THE ACTIVATION OF MIDBRAIN TPOH BY PHOSPHORYLATING CONDITIONS

PREINCUBATION	CONTROL	ACTIVATED	% CHANGE (before preinc.)	% CHANGE (after preinc.)
116 uM BH4				
none	207.5±3.7	241.1±5.6*	↑ 16 (3)	-
Enzyme (E)	202.3±6.1	213.2±5.3*	↑ 5 (3)	↓ 6 (1)
E + BH4	275.4±6.1	274.1±4.5	no change (3)	no change
E + TRP	173.3±4.3	216.0±5.0***	↑ 25 (3)	↓ 13 (1)
E + BH4 + TRP	318.9±1.9	324.6±6.3	↑ 2 (3)	no change
200 uM 6-MPH4				
none	71.6±6.7	93.4±5.4*	↑ 30 (2)	-
Enzyme (E)	96.7±19.8	110.7±19.1*	↑ 14 (3)	↓ 8 (1)
E + 6-MPH4	111.0±23.1	132.0±24.1**	↑ 19 (3)	↑ 16 (1)
E + TRP	86.6±16.0	113.9±19.3*	↑ 32 (3)	↑ 19 (1)
E+6-MPH4+TRP	124.9±23.1	146.2±26.8*	↑ 17 (3)	↑ 16 (1)

Phosphorylating conditions were 1 mM ATP, 10 mM $MgCl_2$. Before preinc. indicates that ATP/$MgCl_2$ were present during the preincubation (10 min at 37°C); after preinc. indicates that ATP/$MgCl_2$ were added to the assay after the preincubation period. Enzyme activity was assayed in the presence of 10 uM tryptophan; cofactor concentrations are indicated. Activity is expressed as pmol $^{14}CO_2$/50 ul/30 min. The number of separate experiments is indicated in parentheses; where more than one experiment was performed, the results represent the mean±SEM. Preincubation was carried out in the presence of DTT, Fe^{++}, AADC, and PLP. Asterisks indicate significant increase above control by paired t-test; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

TABLE 8

EFFECT OF THE ORDER OF ADDITION OF ASSAY COMPONENTS ON THE ACTIVATION OF TPOH BY PHOSPHORYLATING CONDITIONS

	CONTROL	ATP/Mg++	% CHANGE	ATP/Mg++/Ca++	% CHANGE
I	283.0±3.8	347.7±11.9	↑ 23	347.2±15.5	↑ 23
II	239.6±2.7	207.7±4.9	↓ 13	178.7±8.6	↓ 25
III	257.0±3.8	271.3±6.0	↑ 6	291.4±8.5	↑ 13
IV	239.7±1.8	223.8±13.1	↓ 7	208.9±8.2	↓ 13

Phosphorylating conditions were 1 mM ATP, 10 mM $MgCl_2$ with and without 10 uM $CaCl_2$. Activity was assayed in the presence of 10 uM tryptophan, 116 uM BH_4 and is expressed as pmol/50 ul/30 min (mean of triplicates ± S.D.). I = no preincubation; II = preincubation without substrate and cofactor; III = preincubation with cofactor only; IV = preincubation with substrate only. When phosphorylating conditions were tested, the appropriate components were included in the preincubation mixture. All other reactants were added after preincubation. Preincubation was carried out for 10 min at 37°C.

Discussion

The extent of activation of midbrain TPOH activity by phosphorylating conditions in these experiments was, in general, less than that observed by other investigators: Hamon et al. (1978c) observed a 45-55% increase in enzyme activity with ATP and Mg^{++} in the assay and a further 15-20% increase when Ca^{++} (10 uM) was included. Yamauchi and Fujisawa (1979a), Kuhn et al. (1978), and Vitto and Mandell (1981) reported a 2 to 2.5-fold increase in TPOH activity with phosphorylating conditions. Using the method of Ichiyama et al. for assaying TPOH activity, Lysz and Sze (1978) reported a 50-70% increase in enzyme activity from mouse midbrain when the assay included ATP and Mg^{++}.

Activation of hippocampal TPOH by phosphorylating conditions was more than twice that of midbrain TPOH. Of the reports of TPOH activation by phosphorylating conditions, only two have indicated that enzyme from brain regions other than midbrain or brainstem could also be activated by these conditions. Although Kuhn et al. (1978) reported that stimulation of TPOH from hypothalamus, septum, pineal, and mesencephalic tectum occurred with ATP and Mg^{++}, they did not present the data to substantiate their claims. Moreover, no mention was made of any differences in the extent of activation between the various regions tested. However, Hamon et al. (1979) found that the percent increase in TPOH activity from cerebral cortex was more than twice the percent increase in enzyme activity from brainstem when enzyme was assayed under phosphorylating conditions. The difference in activation observed in the study of Hamon et al. between brainstem and

cortex and that in the present study observed between midbrain and hippocampus might reflect the physiological role of these regions in 5-HT synthesis. Serotonin cell bodies, the site of TPOH synthesis, are concentrated in the midbrain and brainstem. Cortex and hippocampus are terminal areas in which 5-HT synthesis and release occur. In the nerve terminal, a calcium-dependent activating process such as the phosphorylating/dephosphorylating mechanism under discussion would allow the neuron to adapt the rate of 5-HT synthesis to the rate of action potentials arriving at the terminal and thus to consequent changes in intraterminal calcium. On the other hand, such a mechanism is not crucial to the functioning of the cell body, where 5-HT release does not appear to occur.

The cofactors BH_4 and 6-MPH_4 give distinct patterns of activation of midbrain TPOH under phosphorylating conditions. In general, 6-MPH_4 produced greater enzyme activation (32%) than BH_4 (about 20%). Moreover, with 6-MPH_4, phosphorylating conditions resulted in enzyme activation at all pH's tested and under all conditions with one exception (see Table 7, enzyme preincubated alone, ATP/Mg^{++} added after preincubation). On the other hand, with BH_4 as cofactor, phosphorylating conditions produced a decrease or no change in enzyme activity at high pH's and when ATP/Mg^{++} were added to the assay after preincubation of the enzyme under a variety of conditions (Table 7). These differences between cofactors must be considered in light of the fact that BH_4, not 6-MPH_4, is the natural cofactor for TPOH. All studies on TPOH activation by phosphorylating conditions have used 6-MPH_4, with the exception of that of Vitto and Mandell (1981). Further studies of TPOH

activation by phosphorylating conditions should utilize the natural cofactor if any conclusions concerning the physiological significance of the phosphorylating process are to be made.

The observation that activation of TPOH by ATP and Mg^{++} in the presence of BH_4 (100 uM) is 100% (Vitto and Mandell, 1981), lends greater support to the notion that a phosphorylation process regulates TPOH activity in vivo. The reason for the difference in activation observed by Vitto and Mandell and that in the present experiments is unknown. Vitto and Mandell used HEPES buffer in their activation studies; HEPES may be a more suitable buffer for phosphorylating reactions than Tris-HCl. Also, Vitto and Mandell used a fluorometric assay in their studies. The Ichiyama method used here requires the addition of AADC to the assay. It is possible that the AADC preparation used in these experiments contains an endogenous inhibitor of phosphorylation.

An important difference between the experiments of Vitto and Mandell and the present experiments lies in the substrate concentration used. The former investigators assayed TPOH using 100 uM tryptophan, whereas in the present studies, the concentration of tryptophan was 10 uM. (In the experiments of Lysz and Sze (1978), the concentration of tryptophan was 10 uM; however, because these investigators used 6-MPH_4 as cofactor, a comparison here is not relevant.) If, as discussed below, tryptophan renders the enzyme more susceptible to activation by ATP and Mg^{++}, then activation in the presence of 100 uM tryptophan would likely be greater than activation in the presence of 10 uM tryptophan. Thus, this difference in substrate concentration

might account for the generally lower activation observed in these studies.

Finally, the possibility exists that the enzyme used in these studies was already present in a more activated state than that used in other studies. Boadle-Biber (1978; 1979a) and Hamon et al. (1979) have shown that enzyme extracted from brainstem slices depolarized by high K^+ or treated with agents to promote Ca^{++} uptake, is present in an activated state. Moreover, incubation of slices in a calcium-free, manganese-substituted medium results in a 25% decrease in the activity of enzyme prepared from these slices as compared to enzyme prepared from control slices (Boadle-Biber, 1979b). These observations suggest that TPOH may normally exist in a partially activated state in the intact neuron.

The series of experiments investigating the effects of enzyme preincubation conditions on the extent of activation by phosphorylating conditions reveal some interesting characteristics of this activation. When cofactor (BH_4) was included in the preincubation of TPOH together with ATP and Mg^{++}, no activation was observed. However, greatest levels of enzyme activity were achieved when the enzyme was preincubated in the presence of BH_4. When tryptophan was included in the preincubation, the extent of activation observed was even greater than when enzyme was not preincubated at all. Moreover, in this case, absolute levels of enzyme activity were about 10-15% lower than nonpreincubated enzyme activity both in the control and activated condition. When both BH_4 and tryptophan were included in the preincubation or when enzyme was preincubated alone, again very little

activation occurred.

These data indicate that TPOH can be activated by its cofactor (BH_4) and further suggest that activation by cofactor prevents activation by phosphorylating conditions. On the other hand, the presence of tryptophan appears to be necessary for activation to occur; preincubation in the absence of tryptophan results in essentially no change in enzyme activity when ATP and Mg^{++} are present (Table 7). Conformational changes in the enzyme molecule caused by the binding of cofactor and/or substrate may be responsible for exposing or concealing activating sites on the enzyme molecule.

It was found that preincubation of TPOH at 37°C for 7 minutes resulted in a decline in enzyme activity. When BH_4 was included in the preincubation, enzyme activity increased slightly above non-preincubated enzyme activity. Enzyme preincubation for 10 minutes at 37°C resulted in essentially no change in enzyme activity (Table 7). But when BH_4 was included in the preincubation, activity rose substantially. On the other hand, in the experiments of Table 8, preincubation of enzyme in both the presence and absence of BH_4 caused a decline in enzyme activity, although the decrease was less with BH_4. In these latter experiments, the preincubation mixture did not include DTT, Fe^{++}, AADC, and PLP. The exclusion of DTT and Fe^{++} from the preincubation mixture may have allowed enzyme inactivation to occur and this inactivation was not completely reversed by the inclusion of these agents in the assay. Alternatively, the AADC preparation may have contained a factor which protected the enzyme against inactivation during preincubation in the experiments of Table

7.

The reason for the difference between the effect of preincubation for 7 minutes and the effect of preincubation for 10 minutes is unknown. However, the observation that preincubation with BH_4 resulted in an increase in enzyme activity in both cases suggests that the cofactor may have been protecting the enzyme against inactivating processes occurring over time. On the other hand, the finding that inclusion of the substrate tryptophan in the preincubation medium resulted in a slight decrease in activity (when assayed with BH_4), indicates that substrate binding destabilizes the enzyme and renders it more susceptible to inactivating processes. Degradation of enzyme by endogenous proteases and thermal denaturation of enzyme molecules could account for a loss of activity observed even in the presence of DTT and Fe^{++}. Both labilization and stabilization of enzymes by their cofactors, substrates, and a variety of other ligands against proteolytic inactivation and thermal denaturation have been demonstrated in a variety of enzyme systems (Citri, 1973).

Contrary to the results of the present studies, Fisher et al. (1972) found that phenylalanine could partially stabilize phenylalanine hydroxylase against heat inactivation. In this case, the enzyme had been treated to remove Fe^{++}. Moreover, untreated enzyme preincubated with phenylalanine for 20 minutes had slightly greater activity than enzyme not preincubated at all.

Very different characteristics of activation were observed depending on whether ATP/Mg^{++} were included in the preincubation mixture in the presence of other assay components or in their absence,

or whether ATP/Mg^{++} were added to the assay mixture following preincubation. It is apparent that in vitro activation by phosphorylating conditions is highly influenced by the enzyme environment. The stability of the enzyme molecule resulting from its environment may affect the enzyme's ability to be activated by phosphorylating conditions. Conversely, activation of TPOH by ATP/Mg^{++} has been shown to affect enzyme stability (Vitto and Mandell, 1981). Activated enzyme incubated in air at 37°C loses activity at a faster rate than untreated enzyme.

The dependence of activation on preincubation and assay conditions makes it very difficult to extrapolate in vitro results to the in vivo situation, but suggests that the phosphorylation process is highly regulated by both substrate and cofactor. The conformational state of the enzyme molecule, as determined by substrate and cofactor binding, may dictate whether or not the molecule can be phosphorylated. However, it should be emphasized that it has not yet been determined whether TPOH itself is phosphorylated by ATP/Mg^{++}, or whether these conditions result in the phosphorylation of another protein which then acts to stimulate TPOH. In the case of tyrosine hydroxylase, which has been shown to be regulated by a c-AMP-dependent phosphorylation/dephosphorylation process, phosphorylation results in the incorporation of phosphate into the enzyme molecule itself (Joh et al., 1978; Yamauchi and Fujisawa, 1979c). Phosphorylation of purified tyrosine hydroxylase produces an enzyme species distinctly different from the non-phosphorylated enzyme. Phosphorylated tyrosine hydroxylase has a different pH optimum, cofactor affinity, salt and polyanion dependence, and thermal stability from that of

non-phosphorylated enzyme (Lazar et al., 1981; 1982).

Because of its complexity, variability, and lack of ultimate characterization, activation of TPOH by phosphorylating conditions does not lend itself well to the proposed biochemical characterization of the response of 5-HT neurons in the hippocampus to neurotoxic damage. Therefore, further study of this aspect of TPOH regulation with respect to 5-HT neuron plasticity was not undertaken. Additional work is required to determine the product of phosphorylation and its mechanism of activation of the enzyme molecule and, further, to establish the normal physiological role of this process in 5-HT neurons. Once a physiological role is established, then it will be of interest to study this phenomenon in pathologic states, such as CNS injury.

PART IV

5,7-DHT Lesions of the Cingulum Bundle

Results

I. Effect of Dose and Concentration of 5,7-DHT Injected Unilaterally into the Cingulum Bundle on TPOH Activity in the Hippocampus

To examine the effect of the dose of 5,7-DHT injected into the cingulum bundle on TPOH activity in the hippocampus, an analysis of variance (ANOVA) was performed on data obtained from animals injected unilaterally with 0 to 5 ug of the drug in a volume of 400 nl (Figure 12). Analysis of the side ipsilateral to the injection revealed a significant effect of dose on hippocampal TPOH activity [$F(5,33)=18.39$, $p<0.0001$]. Comparisons made between individual dosage groups indicated that animals receiving 1 to 5 ug of the drug all had significantly lower hippocampal enzyme activity than animals receiving 0 ug. However, no significant differences were found between animals receiving 1, 2, 3, 4, or 5 ug of the drug.

Analysis of the hippocampus contralateral to the injection also revealed a significant dose effect [$F(5,33)=7.90$, $p<0.0001$]. Individual t-tests showed no significant differences between animals receiving 0 ug and those receiving 1 or 2 ug of the drug. In contrast, injections of 3, 4, and 5 ug of 5,7-DHT produced significant decreases in TPOH activity of the contralateral side (Figure 12).

The effect of concentration of 5,7-DHT was investigated by comparing data obtained from animals injected with 2 ug 5,7-DHT in 160

nl of vehicle (1.25% solution), 2 ug in 400 nl of vehicle (0.5% solution), and 5 ug in 400 nl (1.25% solution) (Figure 13). ANOVA of the ipsilateral side showed a significant overall effect [$F(3,37)=33.71$, $p<0.0001$] of concentration. However, individual comparisons revealed that although 2 ug in 400 nl was not significantly different from 5 ug in 400 nl, 2 ug in 160 ul was significantly different from 5 ug in 400 nl.

On the contralateral side, there was also a significant effect of drug concentration on hippocampal TPOH activity [$F(3,37)=9.86$, $p<0.0001$]. 2 ug in 160 or 400 nl did not cause a significant change in TPOH activity, whereas, as previously observed, 5 ug produced a highly significant decrease in enzyme activity (Figure 13).

II. Time Course of TPOH Activity in the Hippocampus Following Unilateral Injection of 5 ug 5,7-DHT into the Cingulum Bundle

In order to investigate possible changes in hippocampal TPOH activity occurring over time as a result of unilateral injection of 5 ug 5,7-DHT into the cingulum bundle, ANOVA was performed on data obtained from animals sacrificed 3 to 90 days post-lesion (Table 9). Analysis of the ipsilateral side revealed a significant effect of treatment (the lesion) [$F(1,49)=221.28$, $p<0.0001$] and of the day of sacrifice [$F(6,49)=11.69$, $p<0.0001$]. Moreover, a significant interaction between treatment and day was found [$F(6,49)=4.43$, $p<0.0012$], indicating that the effect of the lesion was dependent on the number of days following treatment. Individual comparisons showed that at 3 days after the lesion, TPOH activity in the ipsilateral hippocampus had not changed significantly from sham. However, at all

other times after the lesion a signficant decrease from sham occurred. From Table 9, it can be seen that lowest levels of enzyme activity were reached by 7 days post-lesion. By 28 days after the lesion, TPOH activity began to increase significantly from that observed at 7 days. Enzyme activity continued to climb, peaking 60 days post-lesion, although at this time, activity was still significantly below sham. By 90 days post-lesion, enzyme activity had fallen substantially from the high levels seen at 60 days, although activity remained significantly greater than that observed 7 days post-lesion. These changes in enzyme activity, expressed as percent of sham, are summarized in Figure 15.

Analysis of the contralateral hippocampus also demonstrated significant treatment [$F(1,49)=38.54$, $p<0.0001$] and day [$F(6,49)=21.49$, $p<0.0001$] effects. However, no significant interaction between treatment and day was observed. Individual comparisons revealed a pattern of changes similar to those observed in the ipsilateral hippocampus (Table 9). Again, lowest levels of enzyme activity were reached by 7 days after the lesion and by 28 days, activity was beginning to increase significantly. The reason for the decrease in activity observed from 28 to 42 days, although not significant, is unclear. However, at this time (42 days), enzyme activity did not differ from sham. Similarly, at 60, days enzyme activity in the contralateral side was not different from sham. By 90 days, activity had dropped slightly from that observed in sham animals, although it remained significantly higher than activity seen in animals 7 days post-lesion. These results, expressed as percent of sham, are also summarized in Figure 15.

III. Effect of 5 ug 5,7-DHT Injected into the Cingulum Bundle on the Kinetics of TPOH in the Hippocampus

In an attempt to determine whether the changes in hippocampal TPOH activity observed after 5,7-DHT lesions were the result of changes in the amount of active enzyme (Vmax) or in the apparent affinity of the enzyme for its substrate tryptophan (Km), the kinetic parameters of TPOH from lesioned and sham-lesioned animals were studied 7 and 60 days after treatment. In two separate experiments, A and B, the apparent Km's for tryptophan of TPOH from hippocampi of sham-lesioned animals were 44.7 and 49.5 uM, respectively (Table 10). Seven days after 5,7-DHT lesion of the cingulum bundle, an increase (with respect to sham) in the apparent Km for tryptophan (to 63.9 and 65.7 uM for experiments A and B, respectively) was observed. Thus, enzyme remaining in the hippocampus following neurotoxic damage seems to possess a decreased affinity for its substrate.

Injections of 5,7-DHT produced a decrease in the apparent Vmax of hippocampal TPOH for tryptophan when compared to sham-injected animals 7 days after treatment. In experiment A, the apparent Vmax declined from 54.6 to 23.6 pmol $^{14}CO_2$/mg wet weight/30 min (43% of sham), whereas in experiment B, the Vmax decreased from 80.0 to 29.5 pmol $^{14}CO_2$/mg wet weight/30 min (37% of sham). Note that, expressed as percent sham, the change in Vmax found in these experiments is similar to the change in enzyme activity observed 7 days post-lesion (Table 9, 37% sham), indicating that the decrease in enzyme activity can be attributed mainly to a decrease in Vmax.

TPOH from hippocampi of animals sacrificed 60 days after sham

injections had an apparent Km similar to that observed in animals sacrificed 7 days after sham injections (45.0 and 42.5 uM for experiments C and D, respectively). The results obtained in experiments C and D for animals injected with 5,7-DHT and sacrificed 60 days later are conflicting. In experiment C, the apparent Km for 5,7-DHT-lesioned animals was the same as that for shams (49.5 uM). On the other hand, in experiment D, the Km was greater for the lesioned group (62.6 uM) and similar to that observed for animals sacrificed 7 days after 5,7-DHT lesion (see above).

When compared to 7 days post-lesion, the apparent Vmax obtained for animals sacrificed 60 days after the lesion remained low in both experiments C and D (Table 10). In experiment C, the apparent Vmax fell from 88.6 to 33.3 pmol $^{14}CO_2$/mg wet weight/30 min (38% of sham), whereas in experiment D, the apparent Vmax decreased from 62.9 to 30.0 pmol $^{14}CO_2$/mg wet weight/30 min (48% of sham). Thus, in these experiments, it appears that the increase in enzyme activity previously observed 60 days post-lesion (Table 9) did not occur.

IV. Time Course of TPOH Activity in the Midbrain Following Unilateral Injection of 5 ug 5,7-DHT into the Cingulum Bundle

In order to investigate a possible biochemical response of the median raphe cell bodies whose axons had been damaged by injection of 5,7-DHT into the cingulum bundle, TPOH activity was measured in the midbrains of those animals used for the studies of hippocampal TPOH. Enzyme activity was measured in whole midbrain, rather than in each side separately, to avoid damage to raphe neurons lying along the midline, and because of recently demonstrated bilateral projections

from the raphe nuclei to the hippocampus (Azmitia, 1981).

Analysis of variance of the data revealed a significant effect of treatment [$F(1,49)=6.17$, $p<0.016$] and day [$F(6,49)=18.26$, $p<0.0001$], with no significant interaction between treatment and day. Although at all times after the lesion (with the exception of 90 days), midbrain TPOH activity in 5,7-DHT-lesioned animals was greater than that in sham-lesioned animals, a significant increase above sham was observed only at 28 days (Table 11). At this time after the lesion, a significant increase in TPOH activity in the hippocampus also first appeared. Thus, the increase in enzyme activity in the hippocampus may be accompanied by increased synthesis of active enzyme molecules in the cell body region. These results, expressed as percent of sham, are summarized in Figure 15.

V. Effect of Unilateral 5,7-DHT Injection into the Cingulum Bundle on TPOH Activity in the Hypothalamus and Septum/Preoptic Area

The main bundle of ascending 5-HT fibers travels in the medial forebrain bundle through the hypothalamus. From the MFB, 5-HT fibers branch into the diagonal band to innervate the septal nuclei. Fibers continue dorsally from the septum to join the cingulum bundle at the level of the genu of the corpus callosum (Azmitia, 1978). Because 5-HT fibers carried in the cingulum bundle have passed through the hypothalamus and the septal area, the extent of retrograde degeneration of 5,7-DHT-lesioned fibers can be assessed by measuring TPOH activity in these two regions. The preoptic area, which receives 5-HT fibers also via the MFB, was combined with the septal area in order to have enough tissue for assay. Enzyme activity was measured in ipsilateral

and contralateral sides separately, 3, 7, and 14 days after unilateral injection of 5 ug 5,7-DHT. Statistical analysis of the data showed no significant effect of treatment or day on enzyme activity in the SPOA or in the hypothalamus (Tables 12 and 13), indicating that 5-HT fibers in these regions were unaffected by the cingulum bundle lesion.

VI. Time Course of TPOH Activity in the Hippocampus Following Unilateral Injection of 2 ug 5,7-DHT into the Cingulum Bundle

A dose of 2 ug was chosen for repetition of the time course of enzyme activity in the hippocampus following 5,7-DHT injections because, as previously shown, this dose caused a significant depletion of ipsilateral TPOH activity without affecting enzyme activity in the contralateral side. TPOH activity was measured in ipsilateral and contralateral hippocampi 7, 28, and 42 days after a unilateral lesion of the cingulum bundle with 2 ug 5,7-DHT in 400 nl of vehicle. Each time point was repeated twice, and if no difference was observed between experiments, the data for the two experiments was combined. The results from this series of experiments are shown graphically in Figure 16. When all the data together were analyzed by ANOVA, a significant effect of treatment only was found [$F(1,46)=95.04$, $p<0.0001$] for the ipsilateral hippocampus. Thus, as individual comparisons indicated, TPOH activity fell significantly by 7 days post-lesion and remained unchanged at 28 and 42 days after the lesion.

Because experiment A (28 days post-lesion, Figure 16) gave significantly different results from experiment B (28 days post-lesion, Figure 16), an analysis of the data was performed excluding the data of experiment B. In this case, significant effects of both treatment

[F(1,35)=66.33, p<0.0001] and day [F(2,35)=4.39, p<0.02] were revealed for the ipsilateral hippocampus. Comparisons between various groups indicated that enzyme activity rose significantly between 7 and 28 days. Enzyme activity 42 days after the lesion was not significantly different from either 28-day activity or 7-day activity, making interpretation of these data difficult. From an evaluation of the data from individual animals, however, one gains the overall impression that enzyme activity at 42 days was greater than activity at 7 days, but less than activity at 28 days, even though statistical analysis did not demonstrate such differences.

VII. Effect of 2 ug 5,7-DHT Injected Unilaterally into the Cingulum Bundle on High Affinity Uptake of ^{3}H-5-HT in Synaptosomal Preparations of Hippocampus

In vitro measurements of high affinity ^{3}H-5-HT uptake into slices or synaptosomal preparations of brain tissue have been used by many investigators to assess the effects of neurotoxic lesions on the 5-HT innervation of different brain regions (Bjorklund et al., 1973c; Daly et al., 1973; Gerson and Baldessarini, 1975; Nygren et al., 1974). Changes in high affinity uptake have been shown to parallel changes in 5-HT innervation patterns as detected by fluorescence histochemistry (Bjorklund et al., 1973c; Nygren et al., 1974) and autoradiography (Azmitia and Marovitz, 1980). The present studies of ^{3}H-5-HT uptake were undertaken in order to further characterize the response of intact 5-HT fibers in the hippocampus to 5,7-DHT lesions of the cingulum bundle. The method used to measure synaptosomal high affinity uptake has been characterized in detail by Azmitia et al. (1983).

A dose of 2 ug 5,7-DHT in 400 nl vehicle was injected unilaterally into the cingulum bundle and high affinity uptake of ^{3}H-5-HT was measured in synaptosomes prepared from ipsilateral and contralateral hippocampi 7 and 42 days later. The results of these experiments are shown graphically in Figure 17. ANOVA of the ipsilateral side showed a significant effect of treatment [$F(1,29)=34.92$, $p<0.0001$] and a significant interaction between treatment and day [$F(1,29)=10.73$, $p<0.0027$]. Whereas at 7 days post-lesion there was no significant difference between uptake in sham-lesioned and 5,7-DHT-lesioned animals, at 42 days post-lesion there was a highly significant decrease.

Analysis of uptake in the contralateral hippocampus revealed no significant effect of either treatment or day alone. However, a significant interaction between treatment and day was found [$F(1,29)=15.36$, $p<0.0005$], indicating that the effect of the lesion was dependent on the length of time elapsing between surgery and sacrifice. Thus, although at 7 days, the lesion produced no significant change in uptake in the contralateral side, by 42 days after the lesion, there was a significant decrease in uptake from that seen in shams. These changes in hippocampal uptake of ^{3}H-5-HT do not closely parallel the changes observed in hippocampal TPOH activity.

Figure 12. Effect of dose 5,7-DHT injected unilaterally into the cingulum bundle on TPOH activity in the hippocampus (HIP). Enzyme activity was assayed under standard conditions and is expressed as pmol $^{14}CO_2$/mg wet weight/30 min, mean ± standard error of the least square mean. Number of animals is indicated in parentheses. Asterisks indicate significant difference with respect to corresponding 0 ug group, * $p<0.01$, ** $p<0.001$. ■, contralateral HIP; ●, ipsilateral HIP.

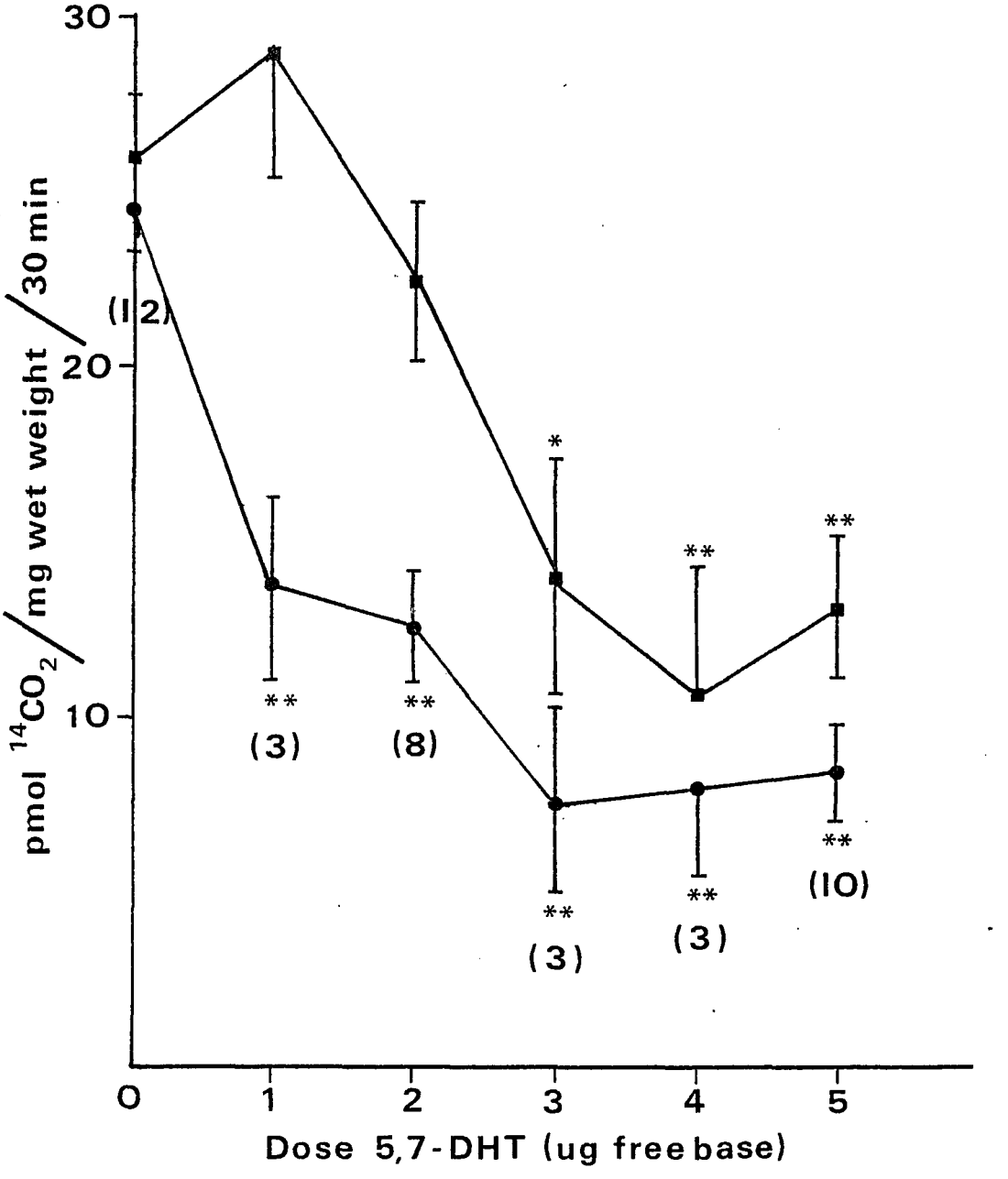


Figure 13. Effect of concentration 5,7-DHT injected unilaterally into the cingulum bundle on TPOH activity in the HIP. Enzyme activity was assayed under standard conditions and is expressed as pmol $^{14}CO_2$/mg wet weight/30 min, mean ± standard error of the least square mean. Number of animals in each group is indicated within each column. Asterisks indicate significant difference with respect to corresponding 5 ug group, * $p<0.05$, ** $p<0.005$, *** $p<0.001$. C, contralateral HIP; I, ipsilateral HIP.

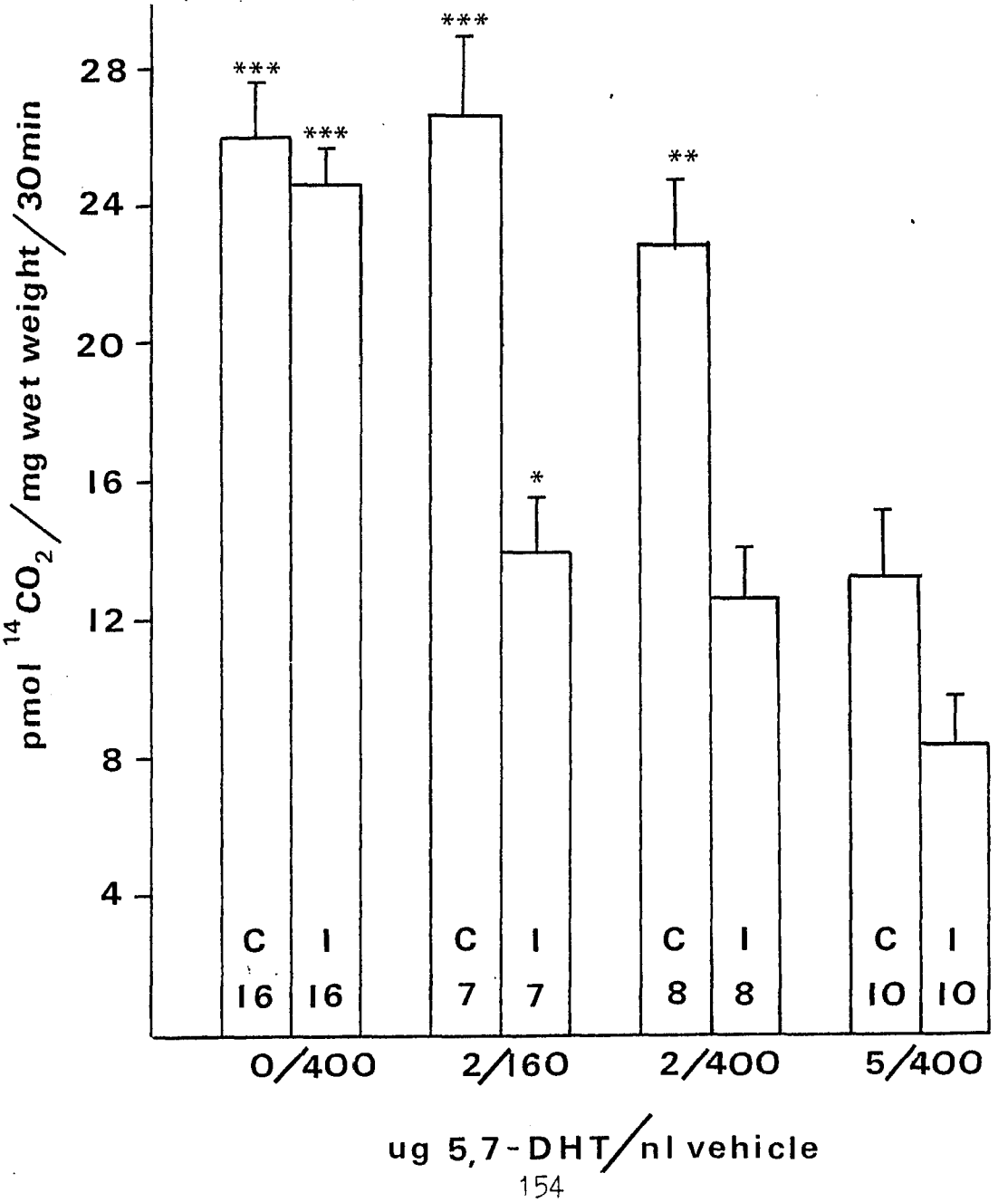


TABLE 9

TIME COURSE OF TPOH ACTIVITY IN THE HIPPOCAMPUS FOLLOWING UNILATERAL INJECTION OF 5 UG 5,7-DHT INTO THE CINGULUM BUNDLE

DAYS POST-LESION	SIDE	SHAM	(N)	5,7-DHT	(N)
3	I	16.5 ± 1.6	(4)	12.2 ± 1.6+	(4)
	C	19.8 ± 1.9	(4)	15.1 ± 1.9	(4)
7	I	21.4 ± 1.6	(4)	8.1 ± 1.2***	(7)
	C	23.5 ± 1.9	(4)	12.2 ± 1.4***	(7)
14	I	22.3 ± 1.4	(5)	8.2 ± 1.8***	(3)
	C	21.1 ± 1.7	(5)	12.0 ± 2.2**	(3)
28	I	28.5 ± 1.4	(5)	13.1 ± 1.4***++	(5)
	C	30.3 ± 1.7	(5)	24.8 ± 1.7*+++	(5)
42	I	23.6 ± 1.8	(3)	13.9 ± 1.4***++	(5)
	C	22.8 ± 2.2	(3)	17.5 ± 1.7+	(5)
60	I	28.2 ± 1.6	(4)	17.6 ± 1.4***+++	(5)
	C	29.4 ± 1.9	(4)	29.0 ± 1.7+++	(5)
90	I	31.7 ± 1.4	(5)	13.0 ± 1.6***+	(4)
	C	33.5 ± 1.7	(5)	26.9 ± 1.9*+++	(4)

Enzyme activity was assayed under standard conditions and is expressed as pmol $^{14}CO_2$/mg wet weight/30 min, mean ± standard error of the least square mean. The number of animals in each group (N) is indicated in parentheses. Asterisks indicate significant difference with respect to the corresponding sham group, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Daggers indicate significant difference with respect to the corresponding 7-day group, + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$. I, ipsilateral hippocampus; C, contralateral hippocampus.

TABLE 10

EFFECT OF 5 UG 5,7-DHT INJECTED INTO THE CINGULUM BUNDLE ON THE KINETICS OF TPOH IN THE HIPPOCAMPUS

DAYS POST-LESION		SHAM		5,7-DHT	
7	Km	A	44.7	A	63.9
		B	49.5	B	65.7
7	Vmax	A	54.6	A	23.6
		B	80.0	B	29.5
60	Km	C	45.0	C	49.5
		D	42.5	D	62.6
60	Vmax	C	88.6	C	33.3
		D	62.9	D	30.0

The concentration of BH_4 was held constant at 400 uM while the concentration of tryptophan was varied from 10 to 200 uM. Km is expressed as uM, Vmax is expressed as pmol $^{14}CO_2$/mg wet weight/30 min. A, B, C, and D are four separate experiments.

Figure 14. Lineweaver-Burk plots of kinetic data from experiments A and B in Table 10.

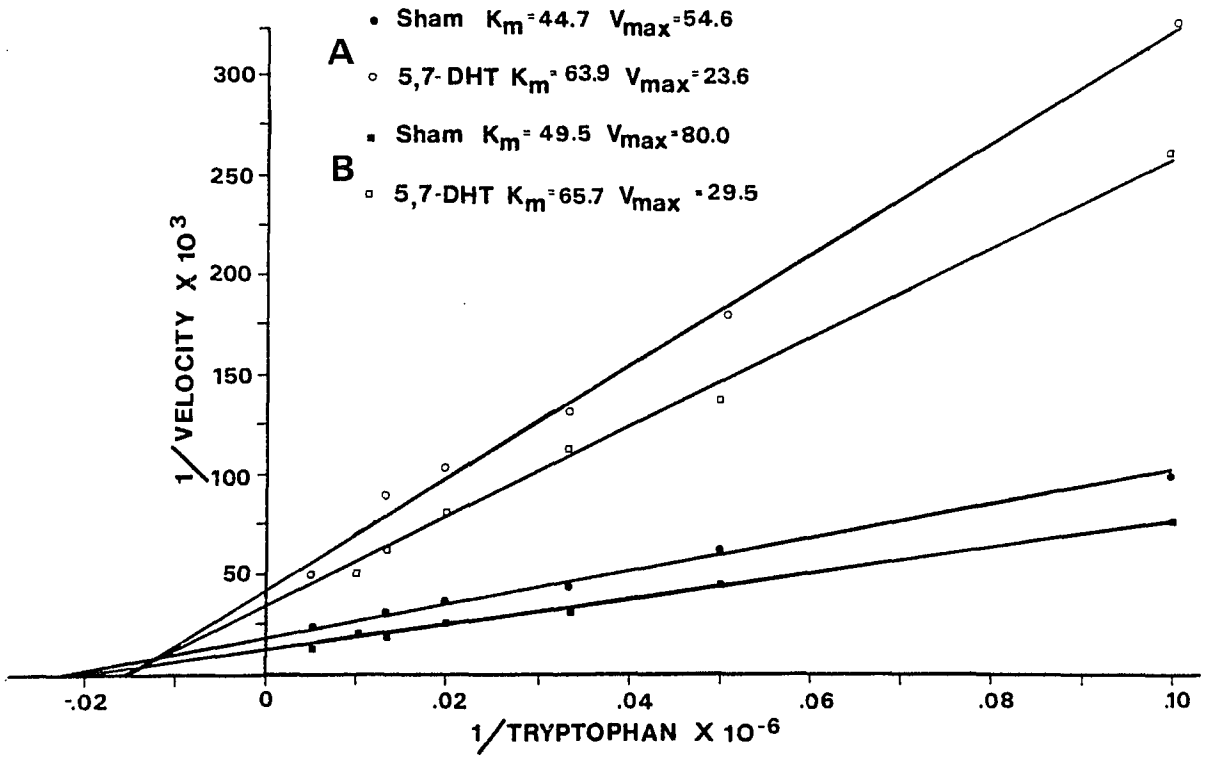


TABLE 11

TIME COURSE OF TPOH ACTIVITY IN THE MIDBRAIN FOLLOWING UNILATERAL INJECTION OF 5 UG 5,7-DHT INTO THE CINGULUM BUNDLE

DAYS POST-LESION	SHAM	(N)	5,7-DHT	(N)
3	144.9 ± 12.0	(4)	151.1 ± 12.0	(4)
7	134.9 ± 12.0	(4)	147.7 ± 9.1	(7)
14	111.6 ± 10.7	(5)	129.0 ± 13.9	(3)
28	182.1 ± 10.7	(5)	221.0 ± 10.7*	(5)
42	117.7 ± 13.9	(3)	142.6 ± 10.7	(5)
60	160.8 ± 12.0	(4)	182.4 ± 10.7	(5)
90	216.5 ± 10.7	(5)	202.4 ± 12.0	(4)

Enzyme activity was assayed under standard conditions and is expressed as pmol $^{14}CO_2$/mg wet weight/30 min, mean ± standard error of the least square mean. The number of animals in each group (N) is indicated in parentheses. Asterisk indicates significant difference with respect to sham group, $p<0.02$.

Figure 15. Data in Tables 9 and 11 expressed as percent of corresponding sham group. Circles = ipsilateral hippocampus; squares = contralateral hippocampus; triangles = midbrain.

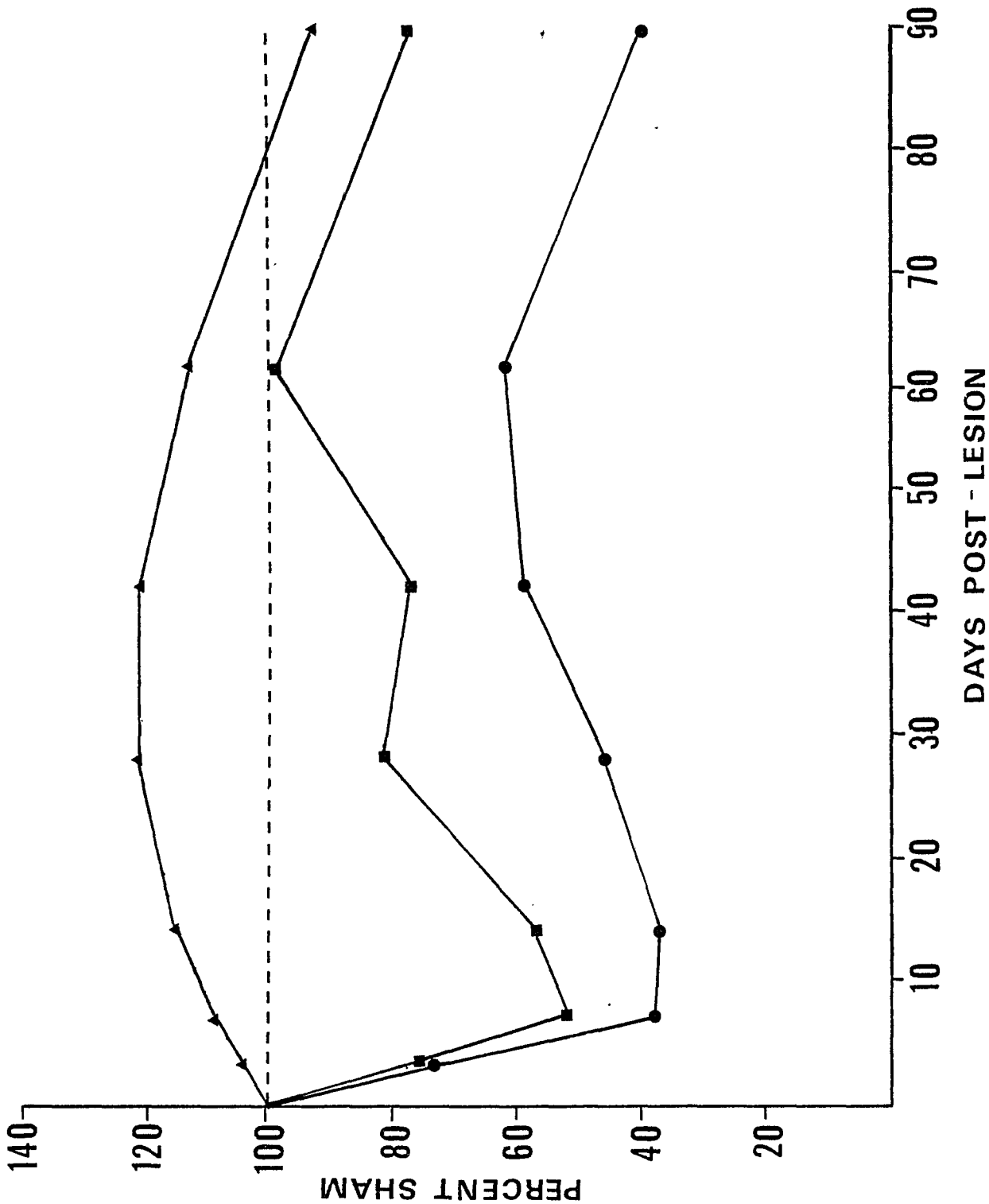


TABLE 12

EFFECT OF UNILATERAL 5,7-DHT INJECTION INTO THE CINGULUM BUNDLE ON TPOH ACTIVITY IN THE SEPTUM/PREOPTIC AREA

DAYS POST-LESION	SIDE	SHAM	(N)	5,7-DHT	(N)
3	I	33.8 ± 3.7	(4)	36.3 ± 2.1	(4)
	C	36.3 ± 1.7	(4)	40.6 ± 2.1	(4)
7	I	39.7 ± 1.4	(4)	44.6 ± 2.7	(7)
	C	36.7 ± 2.4	(4)	50.5 ± 3.6	(7)
14	I	39.6 ± 2.9	(5)	41.3 ± 3.5	(3)
	C	45.5 ± 3.4	(5)	43.8 ± 3.3	(3)

Enzyme activity was assayed under standard conditions and is expressed as pmol $^{14}CO_2$/mg wet weight/30 min, mean ± SEM. The number of animals in each group (N) is indicated in parentheses. ANOVA indicated no significant effect of treatment or day. C, contralateral SPOA; I, ipsilateral SPOA.

TABLE 13

EFFECT OF UNILATERAL 5,7-DHT INJECTION INTO THE CINGULUM BUNDLE ON TPOH ACTIVITY IN THE HYPOTHALAMUS

DAYS POST-LESION	SIDE	SHAM	(N)	5,7-DHT	(N)
3	I	61.4 ± 4.1	(4)	61.3 ± 5.0	(4)
	C	55.1 ± 6.2	(4)	58.5 ± 5.0	(4)
7	I	53.5 ± 7.8	(4)	54.2 ± 6.0	(6)
	C	60.2 ± 4.8	(4)	66.3 ± 6.4	(6)
14	I	67.5 ± 8.1	(5)	62.5 ± 4.6	(3)
	C	57.5 ± 7.3	(5)	77.5 ± 9.3	(3)

Enzyme activity was assayed under standard conditions and is expressed as pmol $^{14}CO_2$/mg wet weight/30 min, mean ± SEM. The number of animals in each group (N) is indicated in parentheses. ANOVA indicated no significant effect of treatment or day. C, contralateral hypothalamus; I, ipsilateral hypothalamus.

Figure 16. Time course of TPOH activity in the hippocampus following unilateral injection of 2 ug 5,7-DHT into the cingulum bundle. Enzyme activity was assayed under standard conditions and is expressed as pmol $^{14}CO_2$/mg wet weight/30 min, mean ± standard error of the least square mean. The number of animals in each group is indicated within each column. A and B represent data from 2 experiments giving significantly different results. Asterisk indicates significant difference with respect to corresponding 7-day group, $p<0.005$. S, sham-injected group; X, 5,7-DHT-injected group; C, contralateral hippocampus; I, ipsilateral hippocampus.

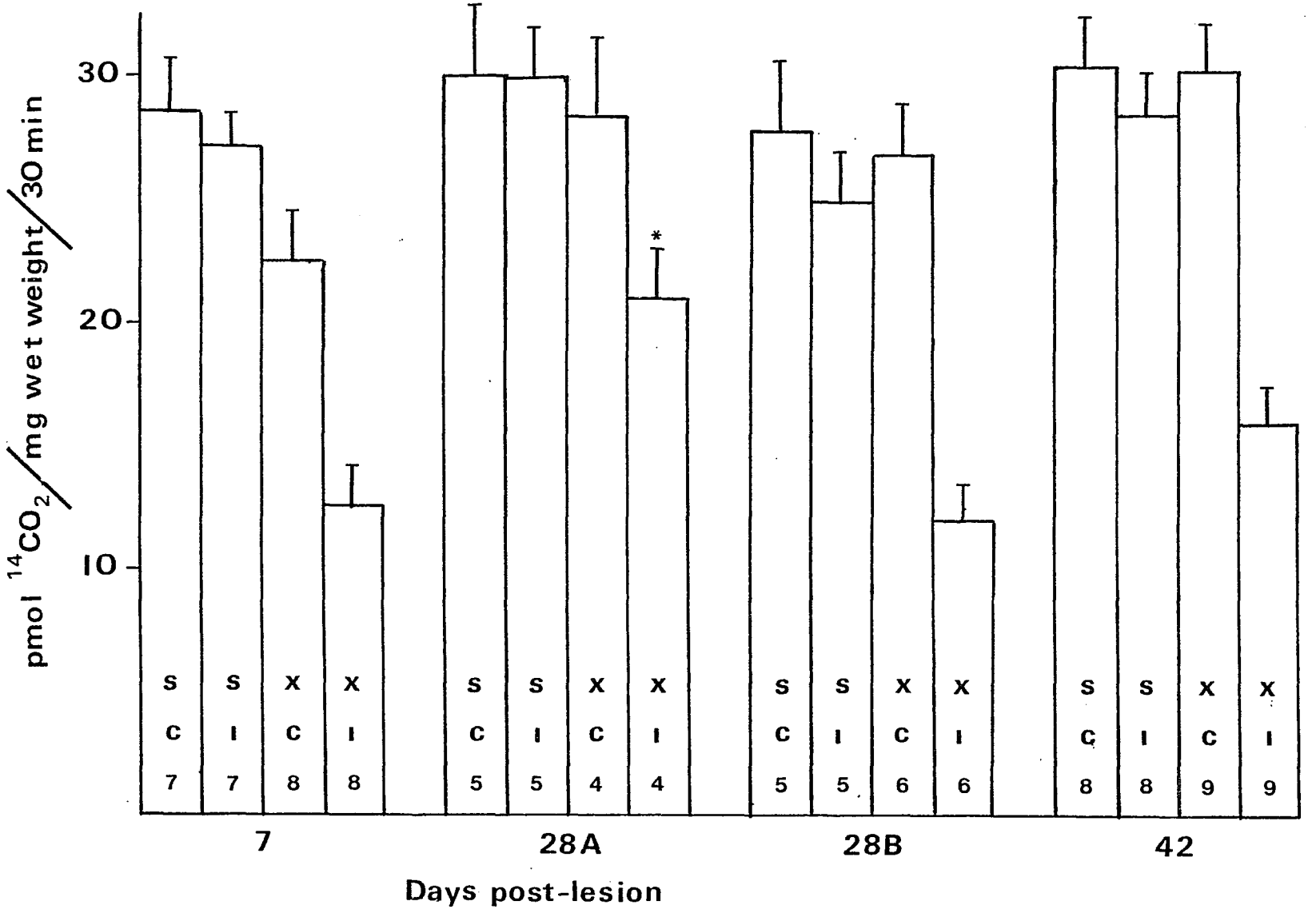
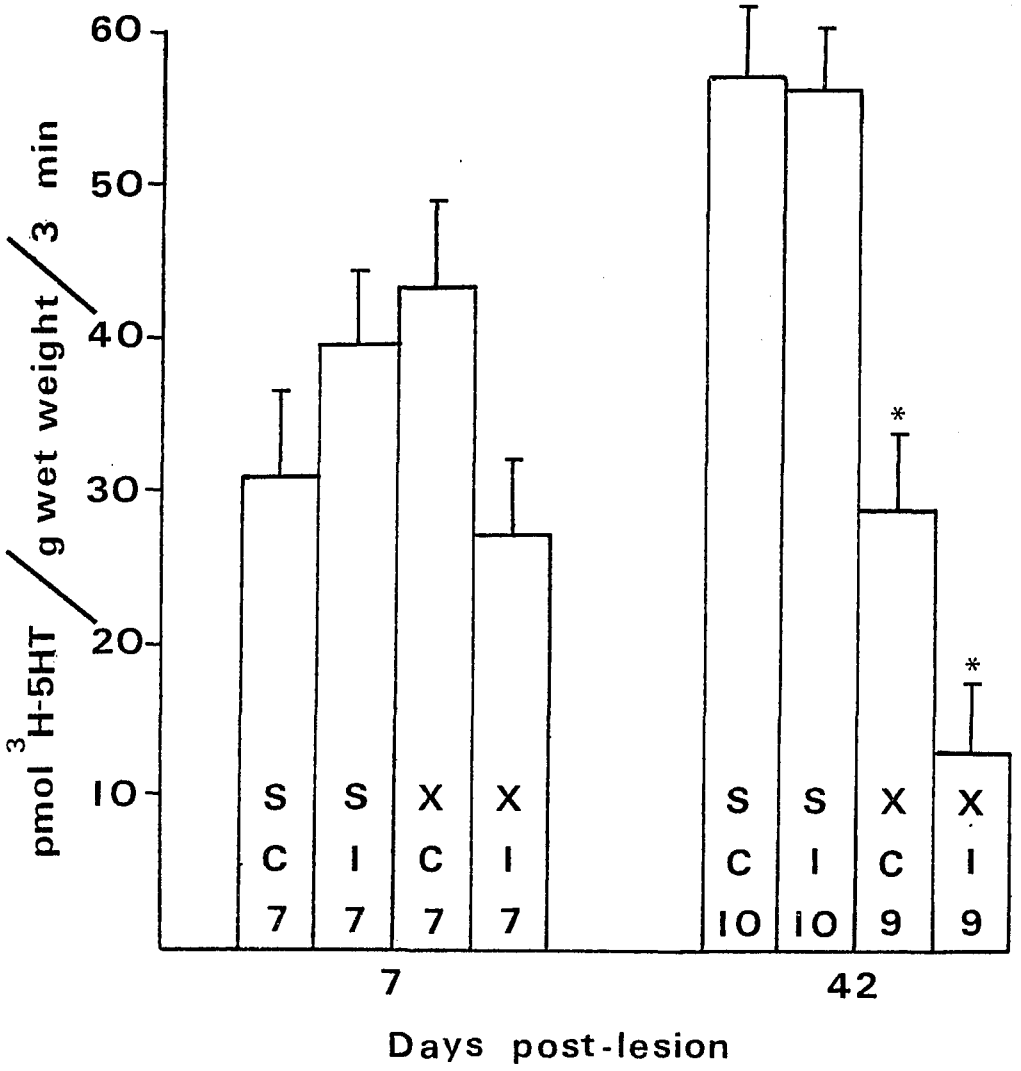

pmol $^{14}CO_2$/mg wet weight/30 min
30
20
10
*
S C 7
S I 7
X C 8
X I 8
S C 5
S I 5
X C 4
X I 4
S C 5
S I 5
X C 6
X I 6
S C 8
S I 8
X C 9
X I 9
7
28A
28B
42
Days post-lesion

Figure 17. Time course of ^{3}H-5-HT uptake in the hippocampus following unilateral injection of 2 ug 5,7-DHT into the cingulum bundle. Uptake is expressed as pmol ^{3}H-5-HT/g weight/3 min, mean ± standard error of the least square mean. The number of animals in each group is indicated within each column. Asterisks indicate significant difference with respect to corresponding sham group, $p<0.001$. S, sham-injected group; X, 5,7-DHT-injected group; C, contralateral hippocampus; I, ipsilateral hippocampus.



Discussion

The studies of TPOH presented here were undertaken in an effort to biochemically characterize plasticity of hippocampal 5-HT input as observed by Azmitia and colleagues (1978). Following injection of 5 ug 5,7-DHT into the cingulum bundle, these investigators observed a decrease in the transport of ^{3}H-proline from the median raphe nucleus to the hippocampus for as long as 14 days after the lesion. Autoradiography of sections through the dentate gyrus indicated that the lesion produced a loss of 5-HT terminals in the hippocampus during this time. Twenty-eight to 90 days post-lesion, the pattern of terminals in the hippocampus had returned to normal despite the failure of regeneration of 5-HT fibers in the lesioned cingulum bundle. This latter observation suggested that new 5-HT terminals in the hippocampus arose from fibers in a pathway other than the cingulum bundle, e.g. the fornix-fimbria. Zhou and Azmitia (1981) have recently confirmed the idea that sprouting of fornix-fimbria fibers occurs in response to cingulum bundle lesions using retrograde transport of horseradish peroxidase.

In the present study, an injection of 5 ug 5,7-DHT unilaterally into the cingulum bundle resulted in a bilateral decline in TPOH activity in the hippocampus. Two possibilities to account for this bilateral decrease are readily apparent: 1) 5-HT fibers in the cingulum bundle cross posterior to the injection site to innervate the contralateral hippocampus. Thus, the lesion would destroy these fibers and produce a drop in contralateral hippocampal enzyme activity. However, there is no evidence to suggest that 5-HT fibers cross in this

region. 2) Diffusion of the toxin might produce damage to 5-HT fibers of the contralateral side. Because of the bilateral presence of 5-HT fibers along the midline and the location of the injection site (Figure 6), the possibility of damage to contralateral fibers by diffusion of the toxin certainly exists. This possibility prompted the examination of the effect of the dose of the drug on TPOH activity in hippocampi both ipsilateral and contralateral to the injection.

An injection of 3 to 5 ug of the neurotoxin was clearly too high a dose to limit damage to the ipsilateral hippocampus. Fibers in the contralateral indiseum griseum lying along the midline probably received enough of the toxin by diffusion to result in degeneration of their terminals in the hippocampus. On the other hand, 2 ug 5,7-DHT resulted in an insignificant change in contralateral enzyme activity. The results of this dose-response study indicate that the dose of the neurotoxin must be carefully regulated in order to damage all the 5-HT fibers ipsilateral to the injection without affecting fibers of the contralateral side. These results further suggest that the decrease in enzyme activity of the contralateral hippocampus is probably not due to damage of 5-HT fibers crossing distal to the injection site.

Following injection of 5 ug 5,7-DHT, partially reversible changes in TPOH activity of hippocampi both ipsilateral and contralateral to the injection were observed. Changes in the ipsilateral hippocampus were generally paralleled by changes in the contralateral hippocampus. In both cases, TPOH activity declined gradually, reaching a minimum 7 to 14 days after the lesion. A decrease of similar magnitude in the apparent Vmax for tryptophan of TPOH from hippocampi ipsilateral to the

lesion suggests that the observed decrease in enzyme activity is largely the result of a loss of active enzyme protein. However, 5,7-DHT also produced a decrease in the catalytic efficiency of the enzyme remaining in the hippocampus 7 days post-lesion. Because enzyme activity was assayed at subsaturating levels of substrate, this increase in the apparent Km for tryptophan may also have contributed to the lower enzyme activity observed.

The physiological significance of a decrease in the apparent affinity of TPOH for its substrate after neurotoxic damage is not clear. A change in Km of enzyme in intact fibers may be the consequence of an altered terminal environment. Thus, in an attempt to sprout, the neuron may increase its synthesis of proteins and lipids required to expand its axonal membrane, resulting in profound changes in the enzyme environment both in vivo and in vitro. It is also possible that residual products of degenerating terminals in the area might act as enzyme inhibitors in vitro. In contrast to the finding of a lower substrate affinity form of TPOH in lesioned animals, Acheson and Zigmond (1981) found that 5 days after intraventricular administration of 6-hydroxydopamine, hippocampal tyrosine hydroxylase showed a more than 2-fold increase in the affinity for cofactor (6-MPH_4).

The reduction in hippocampal TPOH observed following damage to 5-HT fibers in the cingulum bundle resulted from the disconnection of the source of enzyme molecules in midbrain raphe neurons from terminal sites in the hippocampus and subsequent degeneration of isolated 5-HT terminals. Azmitia and Conrad (1976) reported a similar time course of

reduction in hippocampal TPOH activity following fimbrial transection. Maximal reduction was observed by 8 days post-transection. Similarly, following electrolytic lesions of the midbrain raphe, minimum levels of TPOH activity in the forebrain are reached 8 days post-lesion (Kuhar et al., 1971). Intraventricular injections of 5,6-DHT produce a maximum loss of TPOH activity in the hypothalamus, tectum, and forebrain 9 to 12 days after the injection (Victor et al., 1973). Thus, the time course for anterograde degeneration of damaged terminals may reflect an instrinsic metabolic property of 5-HT neurons. It occurs within the same time interval irrespective of whether the lesion damages the cell body or transects its axons in midpassage and whether the lesion is mechanically, electrolytically, or neurotoxically induced. Moreover, the time course of terminal degeneration following neuronal damage might conceivably reflect that of terminals undergoing natural turnover in the absence of injury.

Following an initial decline, there was a partial return in ipsilateral hippocampal TPOH activity and a complete return in contralateral hippocampal TPOH activity. In light of previous observations by Azmitia and coworkers (Azmitia et al., 1978; Zhou and Azmitia, 1981), these data suggest that the return in enzyme activity can be attributed to the presence of new enzyme molecules in collateral sprouts of intact 5-HT fibers reaching the hippocampus by way of the fornix-fimbria. The time course of decline and return of anterograde transport of ^{3}H-proline from the median raphe to the hippocampus correlates well with that of enzyme activity. Both are minimal 14 days after the lesion and have returned significantly by 28 days. This suggests that the reappearance of TPOH activity in the hippocampus

depends on the restoration of normal levels of axonal transport.

Preliminary immunocytochemical studies using an antibody to 5-HT support the proposal that sprouting of intact fibers occurred in response to the cingulum bundle lesion (Zhou and Azmitia, 1983a). Between 3 and 14 days following cingulum bundle lesion, a decrease in the density of 5-HT-immunoreactive fibers in the dorsal hippocampus was observed. By 42 days after the lesion, the distribution of 5-HT-immunoreactive fibers was almost normal, indicating that intact 5-HT fibers had sprouted new terminals to fill areas vacated by degenerated cingulum bundle terminals in the dorsal hippocampus. No fibers regenerating in the cingulum bundle could be observed reaching the hippocampus at this time. These observations again suggest that the increase in hippocampal TPOH activity is probably the result of additional enzyme present in new 5-HT terminals of collateral sprouts. However, although it has been shown that sprouting of fornix-fimbria fibers occurs in the dorsal hippocampus, the present studies using preparations of whole hippocampus cannot rule out the possibility of sprouting of 5-HT fibers reaching the hippocampus by the ventral pathway.

Acheson et al. (1980) observed an early decline in hippocampal tyrosine hydroxylase activity followed by a partial return 21 days after intraventricular administration of 6-hydroxydopamine. Concomitant with the decrease in enzyme activity, there was a decrease in synaptosomal high affinity uptake of ^{3}H-norepinephrine. At 21 days, when enzyme activity had partially returned, synaptosomal uptake remained low. Without anatomical evidence to the contrary, these

investigators interpreted their observations to be an indication of increased enzyme activity in intact terminals in the absence of collateral or regenerative sprouting. The autoradiographic and immunocytochemical evidence discussed above indicates that in the present study, the secondary increase in hippocampal TPOH activity was the result of additional enzyme in new sprouts of intact 5-HT axons and not merely an increase in enzyme in residual terminals.

Based on the similarity observed between the change in hippocampal enzyme activity 7 days post-lesion and the change in Vmax for tryptophan found at this time, the Vmax of TPOH obtained from animals 60 days after the lesion was unexpected. It was anticipated that an increase in the Vmax would occur between 7 and 60 days, paralleling the increase in enzyme activity observed. However, there was essentially no change in the Vmax from 7 to 60 days. The reason for this is not known.

Conflicting data for the Km of 5,7-DHT-lesioned animals sacrificed 60 days post-lesion were obtained in two separate experiments. The reason for this difference in the Km's of experiments C and D is not apparent. However, the kinetic data do suggest that different forms of TPOH exist in the hippocampi of normal and 5,7-DHT-lesioned animals and that in lesioned animals, there may be a conversion of one form to the other. Thus, a lower affinity form seems to be characteristic of the early response of TPOH in intact 5-HT terminals after degeneration of most of the lesioned terminals. This form of the enzyme might reflect a change in the terminal environment caused by the attempt of an intact axon to expand its terminal territory. Alternatively, there may be

synthesis of a new enzyme form in neuronal cell bodies in response to damage of cingulum bundle fibers and subsequent transport to intact teminals in the hippocampus.

The partial return in TPOH activity after 5,7-DHT lesion was temporary; by 90 days after the lesion, enzyme activity had fallen substantially from the peak levels reached at 60 days, suggesting that the newly sprouted fibers were not permanent. Similarly, in the rat spinal cord, after intraventricular injection of 5,6-DHT, the number of 5-HT varicosities in the cervical ventral horn as detected by fluorescence histochemistry, decreases to a minimum 8 to 14 days after the injection, and then increases to a maximum by 30 days post-lesion, only to decline again from 30 to 60 days post-lesion (Nygren et al., 1974). However, by 90 days, the number of varicosities increases again, reaching 50-70% of normal. A similar cycle of synaptic turnover has been described by Bernstein and Bernstein (1977) in the spinal cord following hemisection. These investigators observed that when the number of synapses on interneuron somata has peaked 30 days post-lesion, the frequency of specific bouton types is abnormal, whereas by the time the second peak is reached at 90 days, the proportion of each type has returned to normal. This suggests that cyclic turnover of terminals may be required to establish a normal pattern of innervation. In light of these studies, the decrease in TPOH activity observed at 90 days might be a biochemical manifestation of the cyclic turnover of sprouting 5-HT terminals. More work is required to determine whether enzyme activity undergoes further changes beyond 90 days post-lesion.

The pattern of changes in TPOH activity in the ipsilateral hippocampus obtained after injection of 2 ug 5,7-DHT differs from that obtained after injection of 5 ug 5,7-DHT in several respects. Injection of 2 ug resulted in a significant decrease in TPOH activity. Although there was no statistically significant difference between the low levels of enzyme activity resulting from injection of 2 or 5 ug of the drug, examination of Figure 12 suggests that 2 ug may not have produced as extensive a denervation as 5 ug (38% of sham for 5 ug, 46% of sham for 2 ug). This small difference in the degree of denervation may explain the difference in the dynamics of TPOH following lesions using 2 or 5 ug of the drug.

When 2 ug 5,7-DHT was used, the percent increase in TPOH activity from 7 days to 28 days (46% of sham to 70% of sham) was similar to that observed for 5 ug from 7 days to 60 days post-lesion (38% of sham to 62% of sham). For both doses, the increase in enzyme activity was essentially the same when expressed as percentage (24%) or in absolute terms (8.5 pmol for 2 ug and 9.5 pmol for 5 ug), but the time course of return was different. In the case of 2 ug, the rate of return of TPOH activity was apparently faster and maximal levels of activity were reached sooner (28 days, A) than for 5 ug. With 5 ug of the drug, maximal enzyme levels were not reached until 42-60 days post-lesion. The decrease observed between 28 and 42 days after injection of 2 ug may correspond to the decrease observed between 60 and 90 days after 5 ug. Thus, it appears that the time course of enzyme return may be affected by small differences in the extent of damage to 5-HT terminals.

The data obtained for two groups of animals lesioned with 2 ug 5,7-DHT and sacrificed 28 days later (Figure 16, 28A and B) suggest that sprouting does not inevitably occur in response to the 5,7-DHT lesion. Moreover, a lack of sprouting may account for the absence of an increase in the Vmax of TPOH observed between 7 and 60 days after lesion. As a growth phenomenon, sprouting may be dependent on the animal's physiological state, and changes in hormonal environment are likely to affect its response to neuronal damage. Adrenal steroids have been shown to be necessary for the occurrence of sprouting of 5-HT fibers in the hippocampus (Zhou and Azmitia, 1982) and, at high levels, to inhibit axonal sprouting in the hippocampus after entorhinal cortex lesions (Scheff and Cotman, 1982).

The cell bodies of axons travelling in the cingulum bundle are located in the median raphe nucleus of the midbrain. 5,7-DHT damage to these axons produced an increase in midbrain TPOH activity, peaking simultaneously with the earliest increase in hippocampal TPOH activity observed 28 days post-lesion. This change in midbrain TPOH activity is likely not the consequence of accumulation of enzyme molecules in raphe neurons whose axons were damaged by injection of the neurotoxin. Studies on central catecholaminergic neurons have indicated that both the size of the lesion and its proximity to cell bodies are important factors in determining the magnitude and duration of accumulation of transmitter and synthetic enzymes in the cell body and regions of the axon proximal to the lesion (Reis and Ross, 1973; Reis et al., 1978; Gilad and Reis, 1978). The temporary increase in 5-HT fluorescence of raphe cell bodies occurring 1 to 2 days following intraventricular injection of 5,6- or 5,7-DHT is probably produced by the accumulation

of 5-HT molecules proximal to the point of axonal damage. This increased fluorescence is largely dissipated by 4 days after treatment (Nobin et al., 1973; Baumgarten and Lachenmayer, 1972). In the present study, the lack of significant change in SPOA and hypothalamic TPOH activity for at least 2 weeks after 5,7-DHT lesion of the cingulum bundle suggests that there was no pile-up of enzyme in damaged fibers as they passed through these regions and, further, that the lesion was too distal to elicit retrograde changes in the midbrain raphe nuclei of the kind noted above.

The relatively late rise in midbrain TPOH activity implies an ongoing metabolic process different from that of retrograde accumulation. The simultaneous increase in midbrain and hippocampal TPOH activity suggests that the secondary recovery of hippocampal enzyme activity was dependent on elevated synthesis of TPOH in raphe cell bodies. New molecules of TPOH may be synthesized in the raphe nuclei at an increased rate in order to supply terminals sprouting in the hippocampus. Similarly, Gilad and Reis (1979) demonstrated a 21% rise in tyrosine hydroxylase activity in the A-10 dopaminergic neurons of the midbrain 4 to 10 days following unilateral olfactory bulbectomy. This temporary increase in enzyme activity was considered by these investigators to be uniquely related to the appearance of collateral sprouts from dopaminergic axons in the olfactory tubercle in response to removal of olfactory bulb afferents.

The time course of synaptosomal ^{3}H-5-HT uptake in 5,7-DHT-lesioned animals did not parallel that of TPOH activity. In this study, synaptosomal uptake apparently reached minimal levels at

some point between 7 and 42 days. Kuhar et al. (1972) found that following midbrain raphe lesions, maximal reduction of forebrain synaptosomal uptake activity did not occur until 14 days post-lesion, whereas maximal reduction of TPOH activity was observed by 8 days post-lesion. It is possible that in the present study, minimal levels of uptake were similarly reached by 2 weeks after the lesion, but the choice of time points for investigation did not allow observation of this.

The lack of increase in the uptake capacity of hippocampal synaptosomes from 7 days to 42 days post-lesion was unexpected. The low uptake observed 42 days post-lesion suggests that the development of uptake capacity in new sprouts lags behind the development of the ability to synthesize 5-HT. It is also possible that the sprouted terminals never acquire the capacity to take up 5-HT via the high affinity mechanism or that synaptosomes of newly sprouted fibers possess characteristics different from those of normal fibers so that they were not isolated by the method used.

In contrast to these observations, sprouting of dopaminergic axons in the olfactory tubercle is accompanied by an increase in synaptosomal uptake of ^{3}H-dopamine (Gilad and Reis, 1979). The increase in uptake occurs rapidly, reaching maximal levels by 10 days after bulbectomy, while tyrosine hydroxylase in the tubercle continues to increase for up to 28 days. In addition, in a study of 5-HT fibers regenerating after intraventricular injection of 5,6-DHT, Nygren et al. (1974) demonstrated a return of ^{3}H-5-HT uptake in the spinal cord which closely paralleled the return of 5-HT terminals as observed by

fluoresence histochemistry. Similarly, Frankfurt and Azmitia (in preparation) have observed that following intrahypothalamic injection of 5,7-DHT, a decrease in the 5-HT innervation of the area, as observed by 5-HT immunocytochemistry, is followed by an increase which parallels changes in synaptosomal ^{3}H-5-HT uptake.

The biochemical characteristics of sprouting 5-HT fibers appear to be quite different from those of regenerating 5-HT fibers. They are also different from those observed by Gilad and Reis (1979) in collateral sprouts of intact dopaminergic axons. In this latter study, sprouting of dopaminergic terminals appeared to be a response to the removal of non-dopaminergic input to the olfactory tubercle. In the present experimental paradigm, partial removal of serotonergic input induced sprouting of intact serotonergic fibers.

Certainly several mechanisms can be proposed to account for the growth of intact 5-HT fibers observed in the studies of Azmitia and colleagues. As discussed in the introduction, removal of a segment of the axonal tree of a single neuron results in sprouting of intact collaterals of that neuron (pruning). Zhou and Azmitia (1983b) have reported that about one-quarter of the total median raphe neurons projecting to the hippocampus send fibers via both the cingulum bundle and fornix-fimbria. Damage to these fibers by injection of 5,7-DHT into the cingulum bundle might result in sprouting of fornix-fimbria collaterals in the hippocampus. In this case, the damaged and sprouting axon are collaterals of the same neuron. However, sprouting might also occur from fornix-fimbria fibers arising from median raphe neurons different from those damaged by the lesion. This mechanism may

involve transneuronal communication at the level of the median raphe nucleus. Damage to 5-HT terminals in the hippocampus may elicit a change in the cell body which is transmitted to neighboring cell bodies, such as observed in the spinal cord by Rotshenker (1982). Finally, if there is any overlap of the terminal fields of 5-HT fibers carried in the cingulum bundle, fornix-fimbria, and the ventral pathway, then transneuronal induction of sprouting could also occur in the terminal field. Thus, removal of cingulum bundle 5-HT input to a hippocampal neuron may produce changes in that neuron which are transmitted to other 5-HT fibers presynaptic to the neuron. In this manner, the growth capacity of the intact 5-HT afferent could be mobilized. Sprouting of non-serotonergic afferents in response to the lesion might be induced in the same way.

In light of the work by Azmitia and colleagues, the studies presented in this thesis indicate that 5-HT fibers sprouting in response to 5,7-DHT lesions of the cingulum bundle possess the capacity to synthesize 5-HT. McNaughton et al. (1980) observed that cingulum bundle and fornix-fimbria 5-HT fibers are functionally equivalent with respect to their ability to affect hippocampal theta rhythm. That changes in theta rhythm are dependent on the extent of depletion of hippocampal 5-HT suggests that the return of biosynthetic capacity in hippocampal 5-HT axons after cingulum bundle lesion might result in functional restoration. In fact, behavioral studies by Azmitia et al. (1978) indicate that this is the case. Fourteen to 28 days after unilateral cingulum bundle lesion, rats show abnormal turning behavior after intraperitoneal injection of the 5-HT precursor 5-HTP. By 42 to 56 days post-lesion, rats no longer exhibit this abnormal behavior

unless a second 5,7-DHT injection is made into the fornix-fimbria. Thus, 5-HT fibers in the fornix-fimbria appear to be able to assume the anatomical, biochemical, and behavioral function of 5-HT fibers in the cingulum bundle following removal of the latter.

GENERAL DISCUSSION

Regulation of 5-HT synthesis within the neuron is highly complex, and many aspects of this regulation remain controversial. 5-HT synthesis seems to be dependent on both the availability of tryptophan and the intrinsic activity of tryptophan hydroxylase. In turn, availability of tryptophan depends on a variety of mechanisms inside and outside the CNS affecting changes in plasma tryptophan, binding to serum albumin, transport across the blood-brain barrier, and transport into 5-HT neurons. These mechanisms appear to exert a longterm or tonic control of 5-HT synthesis, as suggested by Hamon et al. (1981). For example, the normal circadian variations in 5-HT synthesis observed in vivo and in brain slices are related to fluctuations in the transport of tryptophan into 5-HT neurons (Hery et al., 1972).

In contrast, rapid and reversible changes in the activity of tryptophan hydroxylase would enable the neuron to respond to sudden changes in nerve impulse flow. Thus, it is possible that the influx of calcium resulting from the arrival of action potentials at the terminal could activate TPOH by a Ca^{++}-dependent phosphorylation process.

It might be hypothesized that the in vivo oxidation state of TPOH would also play a significant role in the regulation of 5-HT synthesis. The activity of TPOH might be altered by intraneuronal fluctuations in oxygen tension in conjunction with an enzymatic reducing system. It is interesting to note that in the process of purifying TPOH from pineal, Hori and Ohotani (1978) have isolated a non-dialyzable, protease-sensitive substance which activates the enzyme in the presence of DTT. These investigators suggest that the substance activates TPOH

by interacting with the enzyme itself. Thus, the existence of an endogenous protein involved in the maintenance of TPOH in its reduced, active state is not unlikely. Such a mechanism would control the level of active enzyme present in the neuron and thus the overall capacity of the neuron to synthesize 5-HT.

It is apparent from the studies presented in this thesis that changes in TPOH occur in intact 5-HT axons in response to damage of other 5-HT axons. The biochemical nature of these changes and their physiological consequences are unclear. The properties of the enzyme in collateral sprouting or regenerating axons are unknown. It is highly possible that any of the processes discussed above could be altered in growing neurons. Among these processes may be the calcium-, ATP-dependent and DTT/Fe^{++}-dependent mechanisms described and examined in this thesis. Changes in these mechanisms could result from changes in the immediate intracellular environment of the enzyme or from changes in the enzyme molecule itself.

In conclusion, the studies presented in this thesis indicate that intact 5-HT axons in the hippocampus are capable of undergoing compensatory biochemical changes in response to partial removal of hippocampal 5-HT input. These biochemical changes parallel anatomical changes previously observed which are indicative of new growth from undamaged 5-HT fibers (Azmitia et al., 1978; Zhou and Azmitia, 1981; 1983a). Sprouting of biochemically functional terminals may result in the restoration of normal 5-HT function in the hippocampus.

APPENDIX

TABLE 14

SAMPLE OF RAW DATA

CH 1	2S%	CH 2	2S%	H=	2/1	TIME
MB						
5539.6	1.4	3101.2	1.9	77	.559	3.23
5560.6	1.4	3085.8	1.9	80	.554	3.25
5474.3	1.4	2992.2	1.9	84	.546	3.35
779.2	3.2	418.0	4.3	81	.536	5.00
766.0	3.2	418.0	4.3	81	.545	5.00
799.2	3.1	438.4	4.2	78	.548	5.00
5700.6	1.4	3168.3	1.9	83	.555	3.16
5600.6	1.4	3101.8	1.9	80	.553	3.23
5684.7	1.4	3121.4	1.9	80	.549	3.21
752.2	3.2	403.0	4.4	82	.535	5.00
750.2	3.2	429.2	4.3	78	.572	5.00
755.8	3.2	414.6	4.3	77	.548	5.00
5324.4	1.4	2952.8	1.9	81	.554	3.39
4997.7	1.4	2760.6	1.9	78	.552	3.63
5312.2	1.4	2931.5	1.9	80	.551	3.42
~~1293.6~~	2.4	698.6	3.3	81	.540	5.00
797.0	3.1	428.8	4.3	82	.538	5.00
753.0	3.2	404.2	4.4	79	.536	5.00
4455.1	1.4	2454.4	1.9	79	.550	4.08
4492.8	1.4	2474.5	1.9	79	.550	4.05
HIP						
2976.6	1.6	1557.6	2.2	82	.523	5.00
2900.8	1.6	1521.2	2.2	87	.524	5.00
3096.2	1.6	1641.6	2.2	83	.530	5.00
700.8	3.3	359.6	4.7	86	.513	5.00
836.4	3.0	443.2	4.2	86	.529	5.00
728.4	3.3	380.6	4.5	85	.522	5.00
2349.6	1.8	1259.2	2.5	85	.535	5.00
2305.6	1.8	1224.4	2.5	86	.531	5.00
2471.0	1.7	1309.2	2.4	85	.529	5.00
742.8	3.2	410.8	4.4	82	.553	5.00
~~1441.0~~	2.7	554.4	3.7	81	.532	5.00
704.6	3.3	374.0	4.6	83	.530	5.00
1599.0	2.2	872.2	3.0	83	.545	5.00
1540.0	2.2	809.6	3.1	83	.525	5.00
1588.8	2.2	843.2	3.0	82	.530	5.00
661.8	3.4	351.8	4.7	85	.531	5.00
719.4	3.3	380.0	4.5	83	.528	5.00
726.6	3.3	371.8	4.6	85	.511	5.00

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