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Rapid Alterations in Rat Brain Tryptophan Hydroxylase Activity; Effects of Adrenal Steroids, Drugs and Stressors

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RAPID ALTERATIONS IN RAT BRAIN TRYPTOPHAN HYDROXYLASE ACTIVITY; EFFECTS OF ADRENAL STEROIDS, DRUGS AND STRESSORS

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by

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PREFACE

The experimental method is characterized by being dependent only on itself, because it includes within itself its criterion,--experience. It recognizes no authority other than that of facts and is free from personal authority. When Descartes said that we must trust only to evidence or to what is sufficiently proved, he meant that we must no longer defer to authority, as scholasticism did, but must rely only on facts firmly established by experience.

The result of this is that when we have put forward an idea or a theory in science, our object must not be to preserve it by seeking everything that may support it and setting aside everything that may weaken it. On the contrary, we ought to examine with the greatest care the facts which apparently would overthrow it, because real progress always consists in exchanging an old theory which includes fewer facts for a new one which includes more. This proves that we have advanced, for in science the best precept is to alter and exchange our ideas as fast as science moves ahead. Our ideas are only intellectual instruments which we use to break into phenomena; we must change them when they have served their purpose, as we change a blunt lancet that we have used long enough.

The ideas and theories of our predecessors must be preserved only in so far as they represent the present state of science, but they are obviously destined to change, unless we admit that science is to make no further progress, and that is impossible. In this connection, we should perhaps make a distinction between mathematical sciences and experimental sciences. As mathematical truths are immutable and absolute, the science of mathematics grows by simple successive juxtaposition of all acquired truths. As truths in the experimental sciences, on the contrary, are only relative, these sciences can move forward only by revolution and by recasting old truths in a new scientific form.

Claude Bernard, 1927

ABSTRACT

Tryptophan (TP) hydroxylase activity was measured using a modification of the method developed by Ichiyama *et al*. (1968). The distribution of the enzyme in the rat brain was determined and found to correlate with serotonin (5-HT) distribution. Bilateral adrenalectomy resulted in a decrease enzyme activity in most areas examined with the largest drop occurring in the midbrain. A similar decrease was found in 5-HT *in vivo* turnover. Injection of corticosterone led to an increase both in normal and adrenalectomized rats. This increase was blocked by cycloheximide, a protein synthesis inhibitor. The midbrain enzyme activity was increased in normal rats by short term stressors--cold (5° C), ether, and electric foot-shock--which resulted in increase plasma corticosterone levels. Adrenalectomized rats showed no increase when subjected to similar stressors. 5-HT brain levels of normal but not adrenalectomized rats were also elevated following short-term cold stress. These changes in TP-hydroxylase activity were not a result of changes in endogenous L-TP levels or L-aromatic amino acid decarboxylase activity.

The rapidity of the changes after cold stress, corticosterone or cycloheximide injection do not appear to result from a change in enzyme amount. This is supported by the failure of a second protein synthesis inhibitor, puromycin, from producing the same results found with cycloheximide. Evidence was found to suggest that the availability of reduced cofactors may be the controlling factor in regulating rapid changes in TP-hydroxylase activity.

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INTRODUCTION

A. Stress-Pituitary-Adrenal Axis

All biological systems have physiological processes for maintaining homeostasis. Assaults on these homeostatic balances are sometimes described as "stresses" to the organism. The ability of the organism as a whole, and each tissue in particular, to react and counterbalance that stress is crucial for survival. One of the best known and reproducible indicators that an animal has been exposed to a stressor is the plasma concentration of adrenocortical hormones. The release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary is believed to be mediated by the central nervous sytem (Ganong and Hume, 1954). A transplanted, denervated pituitary gland will secrete only very low levels of adrenocorticotrophic hormone (Ganong, 1959). Therefore, the close relationship between the pituitary gland and the hypothalamus through the median eminence and portal blood system is necessary for normal functioning of the pituitary. The specific releasing factor of ACTH is believed to be a polypeptide, corticotropin-releasing factor, although vasopressin (a polypeptide stored in the posterior pituitary) can also cause release of ACTH from anterior pituitary (Guillemin, 1967; McCann and Porter, 1969). The localization of the neurosecretory cells for CRF is believed to be in the periventricular and supraoptic region of the hypothalamus (Yuwiler, 1971), although evidence from a lesion study suggest a more diffuse distribution of these cells throughout the hypothalamus (Mess, 1969). There are numerous nerve terminals on these blood vessels and capillary beds in the median eminence (Adams et al., 1965) which provide a local site for release of CRF (Figure 1). The figure also shows that there are abundant terminals on the cell bodies of the releasing-factor producing cells of the hypothalamus where neuronal control of CRF synthesis, transport, or release may occur (Szentágothai, 1964; Hedge and Smelik, 1969; Schadé, 1969). Once released the CRF is transported by the hypothalamo-hypophyseal portal blood system from the median eminence to the anterior pituitary (Popa and Fielding, 1930, 1933; Harris and George, 1969).

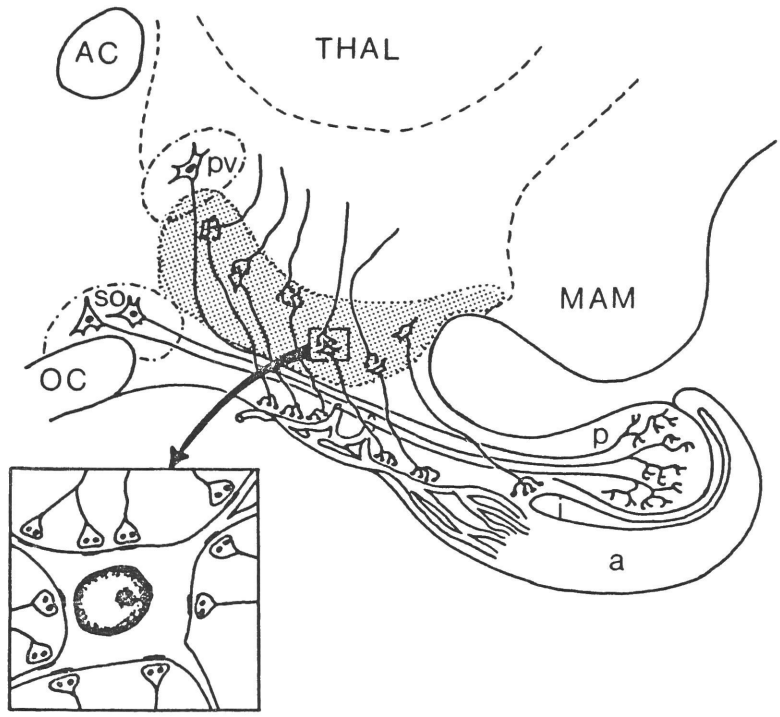


Figure 1. Figure modified from J. Szentágothai (1964) illustrating the system of small neurons, the axons of which terminate in the superficial zone of the median eminence and the proximal stalk. Rectangle indicates situation of the inset where nerve terminals surround a CRF neuron, the projection ends on the hypothalomo-hypophyseal portal blood system. Hatched zone in the diagram indicates "hypophysiotrophic region" of the hypothalamus as found in experiments by Halasz et al. (1962). It corresponds to the localization of the nerve cells giving rise to the tubero-infundibular tract. AC = anterior commissure, a = anterior lobe, OC = optic chiasm, I = intermediate lobe, MAM = mammillary body, P = posterior lobe, PV = paraventricular nucleus, SO = supraoptic nucleus, dashed line indicates outlines of 3rd ventricle.

The primary site of ACTH action is the adrenal cortex, although ACTH has known effects on epididymal fat pads (White and Engel, 1959), on melanophores (Hu and Chavin, 1956) and on brain electroencephalographic (EEG) activity (Glasser et al., 1955) and neuronal firing (Ruf and Steiner, 1967; Pfaff et al., 1971). The action on the adrenal cortex in stimulating the release of steroids is generally believed to depend on de novo protein synthesis of some enzyme needed for corticosterone biosynthesis from cholesterol. Evidence for this is based on reports that protein synthesis inhibitors (puromycin, Ferguson, 1963; cycloheximide and chloramphenicol, Ferguson, 1967; Farese, 1964) block the ACTH induced production of corticosterone. Adenyl cyclase activity may also be involved in this stimulation by ACTH in the adrenal cortex (Reddy and Streeto, 1967). Whatever the mechanism, the effect of ACTH is known to be extremely rapid. Levels of corticosterone (the principle adrenal steroid in the rat) reach a maximum within 15 minutes after stress (Yuwiler, 1971).

Adrenocortical steroids are bound in serum to a high affinity globular binding protein called transcortin (Slaunwhite and Sandberg, 1959) or corticosterone binding globulin (CBG, Seal and Doe, 1962; Daughaday, 1958). The amount of this CBG can fluctuate widely in an animal (Westphal, 1971). The resting values of circulating corticosterone and CBG binding capacity are similar at the peak of the circadian cycle (Seal and Doe, 1966). Cold stress, however, leads to a decrease in CBG binding capacity and an increase in corticosterone in male rats (McDonagh, 1966). The free form of the steroid is believed to be the active form.

Plasma glucocorticoids readily enter most tissues and have effects on brain, liver, adipose tissue, muscle and lymphatic tissue (Yuwiler, 1971). The steroid can easily pass the blood-brain barrier and enter the cerebral tissue. Corticosterone has been shown to be specifically retained in nervous tissue with highest concentrations in the cell nuclei of the hippocampus (McEwen et al., 1972), and in the pituitary (Schapiro and Katz, 1960). The functional significance of this binding may be related to the feedback inhibition which adrenal

steroids exert on ACTH release. Elevation of plasma corticoids by stress or exogenous steroid injection depress subsequent basal circulating corticosterone levels for 2-4 hours (Zimmermann and Critchlow, 1969). However, circulating steroids do not suppress the responsiveness of the pituitary to repeated stressors (Hodges and Jones, 1963; Stockham, 1964; Bohus, 1968; Zimmermann and Critchlow, 1969). Nevertheless, some workers have found that steroids do inhibit for brief periods immediately following the elevation of steroid plasma levels (Yates et al., 1961; Hodges and Jones, 1965; Dallman and Yates, 1967). Supraphysiological doses of corticosterone can lead to a prolonged suppression lasting as long as 16 hours (Smelick, 1963; Hodges and Jones, 1964). Yates (1967) has classified stressors as to their ability to cause ACTH release despite prior injection of dexamethasone, a potent, long lasting synthetic steroid. Class I stressors (ether, burns, and electric shock) are blocked whereas Class II (Hemorrhages, intestinal traction and anoxia) are not blocked. These results are interpreted to mean that ACTH secretion is controlled by a number of neural pathways impinging on the hypothalamus some of which are sensitive to steroid feedback. It is accepted that a large number of brain regions are involved in the control of ACTH release from the anterior pituitary (Mangili et al., 1966).

A diurnal rhythm of circulating adrenal hormones is found in the rat, with the trough in the early morning, while the rat is active, and the peak in the late afternoon, while the rat is normally asleep (Critchlow, 1963). This rhythm can be abolished by hippocampal fornix section, midbrain ráphe lesions, and injection of the serotonin (5-HT) synthesis inhibitor, para-chlorophenylalanine (Mason, 1959; Scapagnini and Preziosi, 1972). Furthermore cerebral 5-HT has a diurnal rhythm similar to that for plasma corticosterone (Chapter I G). These results suggest that a presumptive neuronal transmitter system (5-HT) may be interrelated with the pituitary adrenal axis. Other evidence supporting such a relationship is provided from various lines of research. (1) Serotonin can directly inhibit ACTH release if injected in the hypothalamic region (Naumenko, 1969); (2) Serotonin containing neurons have endings and axons in all areas which are known to influence ACTH release

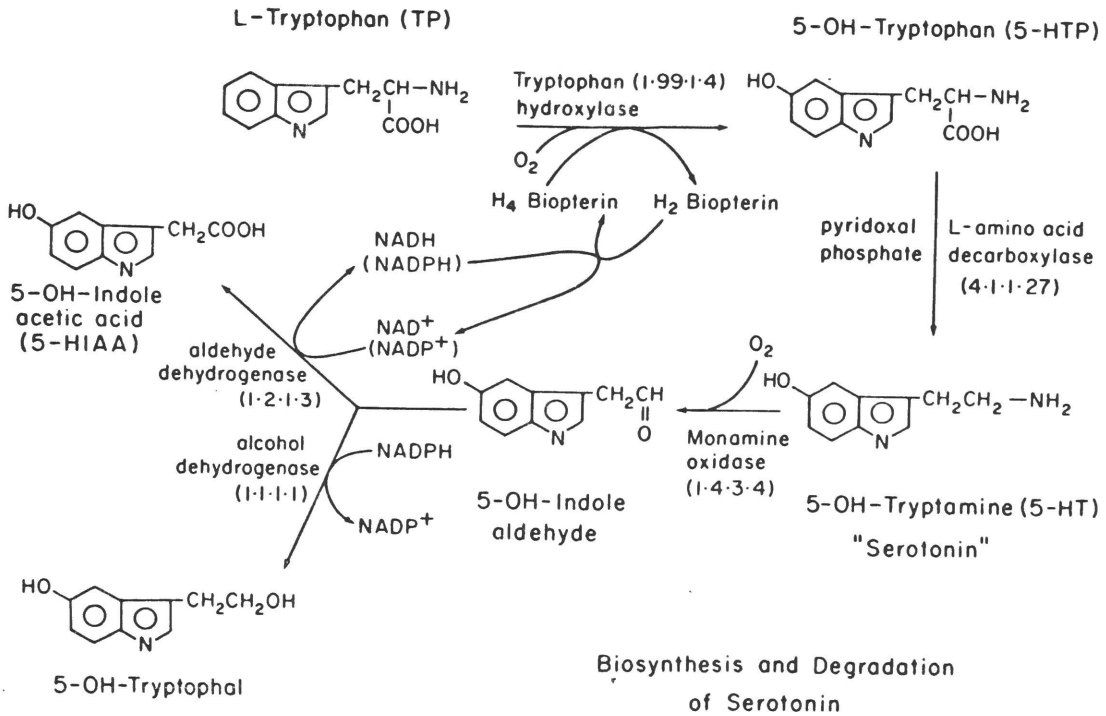
(Brodal et al., 1960; Dahlstrom and Fuxe, 1964). The highest concentration of 5-HT terminals are found in the suprachiasmatic nucleus, destruction of which abolishes the diurnal rhythm of corticosterone secretion in the rat (Moore and Eichler, 1972); (3) The affective states in which serotonin has been implicated (Woolley, 1962) and which are influenced by adrenal steroids (Cleghorn, 1951, 1957) are similar. Both substances can produce changes in mood, personality and activity.

I began in 1968 a study of the influences of adrenal steroids on brain enzymes involved in 5-HT biosynthesis with Dr. Bruce McEwen. Glucocorticoids are known to induce a number of adrenal and hepatic enzymes (Yuwiler, 1971). Therefore a precedent exists for adrenal steroid involvement in enzyme regulation. In the brain it has been established that corticosterone can regulate brain enzymes found in glial cells (De Vellis and Inglish, 1968; De Vellis et al., 1971). α-glycerophosphate dehydrogenase activity falls dramatically in all areas of the brain after adrenalectomy and increases after adrenal steroids treatment over several days. Other dehydrogenases such as isocitrate, malate, and lactate dehydrogenase are unaffected.

In the following sections of the introduction I will discuss certain aspects of serotonin relevant to understanding its function and synthesis in the brain. My results presented in Chapter III show a correlation between increased tryptophan hydroxylase activity measured in vitro and increased levels of adrenal steroids. Additional data indicates that there are in vivo changes in brain 5-HT levels and turnover consistent with such a correlation. The mechanism by which this occurs appears to be quite rapid and not related to de novo enzyme synthesis.

B. Source and Metabolism of Tryptophan

The biosynthesis of serotonin begins with hydroxylation of an amino acidL-tryptophan (Fig. 2). The mammalian body is unable to synthesize this amino acid from any internal precursor and the only source is from the diet (Mahler and Cordes, 1966). This essential amino acid is limiting in most foods. The adult rat daily consumes only 500-600 μmoles of



Biosynthesis and Degradation of Serotonin

Figure 2

L-tryptophan/Kg body weight if standard laboratory chow is provided (Wurtman and Fernstrom, 1972). The amount of serotonin byproducts daily excreted in urine is 0.9-1.25 μmole/Kg body weight (Bertaccini, 1960). Therefore approximately 0.2% of the total L-tryptophan consumed is converted to serotonin in the adult rat. In humans it is estimated that 1% is converted to serotonin after an injection of L-tryptophan (Sjoerdsma et al., 1956).

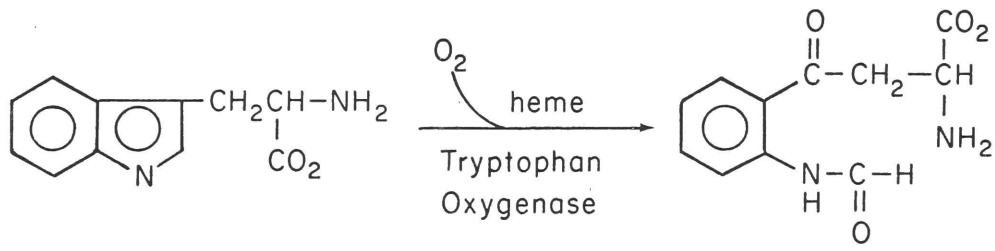
The major catabolic pathway of plasma L-tryptophan occurs in the liver. Here tryptophan oxygenase (1.99.2c) converts L-TP to N-formyl kynurenine (Guroff and Lovenberg, 1970). This is the first step in the synthesis of the important cofactor nicotinamide-adenine-dinucleotide (NAD) and nicotinamide-adenine-dinucleotide phosphate (NADP) (Nishizuka and Hayaiski, 1963; Gholson et al., 1964). There is some evidence that NAD levels may regulate its rate of biosynthesis by feedback inhibition on tryptophan oxygenase (Yamaguchi et al., 1967). Nicotinic acid can bypass many of these steps in its conversion to NAD (Mahler and Cordes, 1966). The phosphorylation of NAD produces a cofactor (NADP) important for many cytoplasmic reducing enzymes. The reduced form of NAD^+ is required for normal activity of tryptophan hydroxylase since the cofactor, dihydro-biopterin, must be maintained in a reduced form for the hydroxylation reaction to occur (Craine et al., 1972).

$$H_4\text{-Biopterin} + \text{tryptophan} + O_2 \rightleftharpoons \text{5-HTP} + H_2\text{-Biopterin}$$

$$H_2\text{-Biopterin} + \text{NADH} + H^+ \rightleftharpoons H_4\text{-Biopterin} + NAD^+$$

Further information concerning the importance of these cofactors is found in the review by Krebs (1967).

The regulation of the enzyme, tryptophan oxygenase, has been shown to be quite sensitive and complex (Greengard, 1963). A flow chart of some of the major points of control is outlined in Figure 5. Figure 5 illustrates that both hormone and substrate are necessary for maximum enzyme activity. This additive effect has been convincingly demonstrated (Civen and Knox, 1959). In the rat injection of L-tryptophan (50 mg/100 gm) or hydrocortisone (1.5 mg/100 gm) caused a 4-fold



L-tryptophan

N-formyl
kynurenine

Figure 3. The oxygenation of tryptophan to N-formyl kynurenine in the liver.

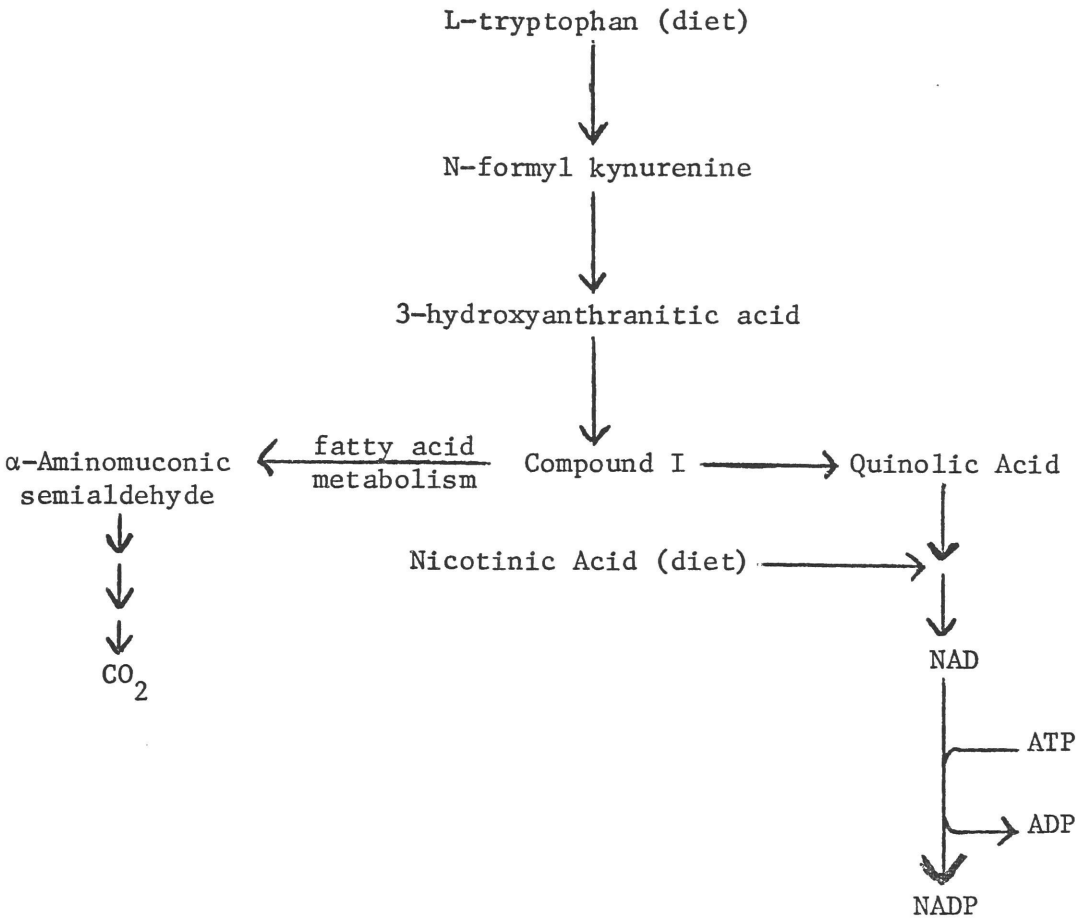

L-tryptophan (diet)
N-formyl kynurenine
3-hydroxyanthranitic acid
α-Aminomuconic semialdehyde
fatty acid metabolism
Compound I
Quinolic Acid
Nicotinic Acid (diet)
CO_2
NAD
ATP
ADP
NADP

Figure 4

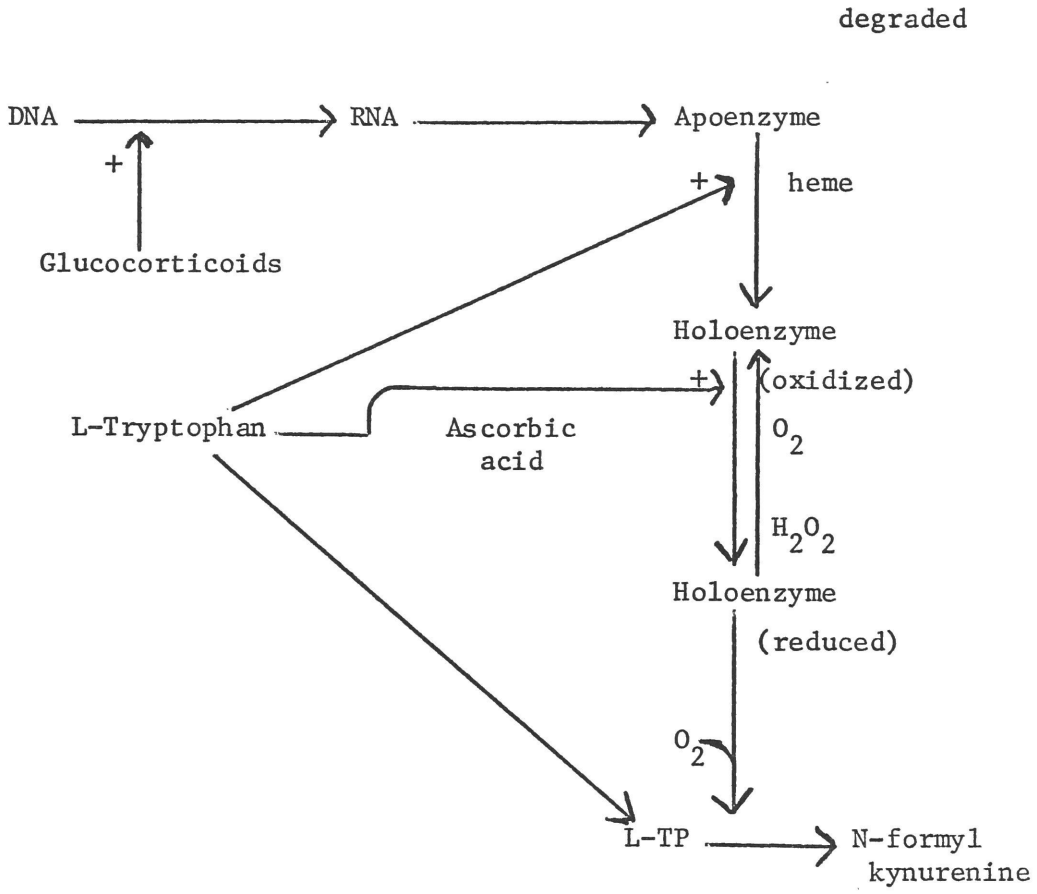

degraded
DNA
RNA
Apoenzyme
+
heme
Glucocorticoids
Holoenzyme
+
(oxidized)
L-Tryptophan
Ascorbic acid
O_2
H_2O_2
Holoenzyme
(reduced)
O_2
L-TP
N-formyl kynurenine

Figure 5

increase in activity, whereas if the chemicals were injected together they caused a 10-fold increase within 3 hours.

This enzyme does not exist in CNS tissue (Guroff and Lovenberg, 1970). However, the detection of kynurenine formylase in brain tissue from chick embryo raises the possibility that some of the metabolites of hepatic tryptophan pyrrolase may be converted to $NAD^+ + NADP^+$ in the brain (Peterkofsky, 1970).

The fate of tryptophan in the brain appears to be limited to the pathways drawn in Figure 6. The levels of serotonin are normally 1/10th of those of brain-tryptophan, making serotonin a major byproduct of brain-tryptophan. The existence of TP-oxaloacetate transaminase (question mark in Figure 6) has not been substantiated since the initial report (Tangen et al., 1965). The conversion of TP to tryptamine has only recently been reported and is only 1/10th of the amount converted to serotonin. Finally, the incorporation of tryptophan into protein is least of all major amino acids. Tryptophan has a frequency of occurrence in proteins of 0.012 as compared to 0.096 for phenylalanine (Dayhoff et al., 1969). Table I summarizes the brain levels of the main metabolites of tryptophan found in the brain.

C. Identification of Serotonin and Biosynthetic Enzymes in the Brain

The isolation and chemical identification of 5-HT was reported by Rapport and coworkers (1948). The 5-HT was recovered from serum and was shown to be a potent vasoconstrictor. This compound was found to be identical to enteramine (Erspamer and Asero, 1952), an intestinal chemical capable of contracting uterine tissue (Vialli and Erspamer, 1937). Since that time 5-HT has been detected in all nervous and many non-nervous tissue of vertebrates and invertebrates, as well as in most plants and fruits (Erspamer, 1966). The substance auxin, a potent growth factor in plants, is a derivative of 5-HT. The initial report of the presence of 5-HT in the mammalian brain was made 20 years ago (Twarog and Page, 1953; Amin et al., 1954). It was initially assumed

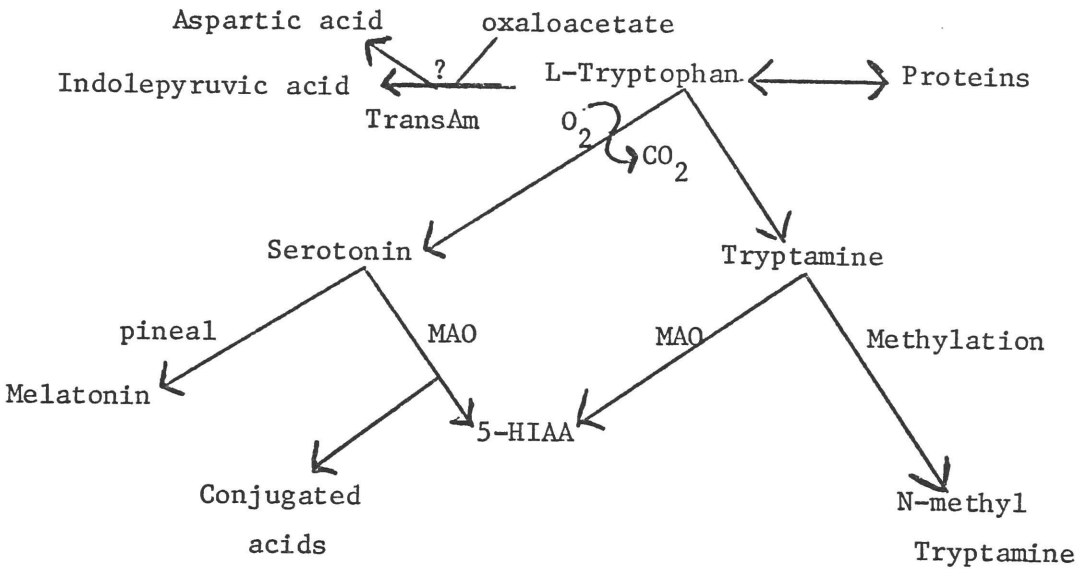

Aspartic acid
oxaloacetate
Indolepyruvic acid
?
TransAm
L-Tryptophan
Proteins
O_2
CO_2
Serotonin
Tryptamine
pineal
MAO
MAO
Methylation
Melatonin
5-HIAA
Conjugated
acids
N-methyl
Tryptamine

Figure 6

Table I

Compound*	Concentration (µmole/gm)
L-Tryptophan(1)	20
Serotonin (2)	3-2
5-Hydroxyindolacetic acid(3)	2-3
Tryptamine(4)	.125
NAD(5)	320
NADP(5)	10

*References for compounds:

(1) Azmitia et al., 1970

(2) Pohorecky, unpublished

(3) Neff et al., 1968

(4) Saavedra and Axelrod, 1972

(5) Mahler and Cordes, 1966

that the 5-HT in brain came from the intestine which has a much higher concentration of 5-HT (Erspamer, 1966). However, early indirect evidence suggested that the brain has to possess its own enzymes for 5-HT synthesis. For example, the demonstration that 5-HT did not cross the blood brain barrier ruled out direct transport of this amine (Udenfriend et al., 1957), although its hydroxylated precursor 5-hydroxytryptophan, can enter the CNS. Furthermore, total gastro-enterectomy, which removes the largest source of 5-HT, produced large decreases in 5-HT content in the spleen, plasma, and lung, but had little effect on brain 5-HT content (Bertaccini, 1960). Most importantly, intracranial but not intraperitoneal injection of tracer amounts of radioactive TP led to detectable levels of cerebral radioactive 5-HT (Gal et al., 1964). Since the L-aromatic amino acid decarboxylase responsible for conversion of 5-HTP to 5-HT was known to be present in CNS tissue (Lovenberg et al., 1962), a tryptophan hydroxylating enzyme was the only required enzyme for biosynthesis of 5-HT from L-TP which was not yet detected in the brain.

Grahame-Smith (1964) developed the first unequivocal in vitro assay for demonstrating the hydroxylation of TP to 5-HTP using brain stem homogenate from either dog or rabbit. DL-TP-C^{14} and NSD 1024 (a decarboxylase inhibitor) were mixed in an O_2 flushed flask containing the homogenate in Ringer's phosphate solution (pH 7.4). The hydroxylating reaction was terminated by heat (100° C for 3 min). Carrier DL-5-HTP was then introduced into the flask and this mixture was run by 2-dimensional ascending paper chromatography to separate DL-5-HTP from TP and 5-HT. The DL-5-HTP was enzymatically decarboxylated by addition of purified guinea pig kidney aromatic L-amino acid decarboxylase. Aliquots were used for radioactive and colorimetric determinations of 5-HT. Using this procedure, Grahame-Smith showed the enzyme had a stereospecificity for the hydroxylation of the naturally occurring L-isomer of TP.

This assay, although it directly verified the ability of the brain to hydroxylate tryptophan, was too laborious and not sensitive enough for detailed studies of the enzyme. Several other laboratories

began to develop new methods for measuring tryptophan hydroxylase (Green and Sawyer, 1966; Lovenberg et al., 1967; Renson et al., 1966; Ichiyama et al., 1968). The assay developed by Green and Sawyer (1966) was similar to that of Grahame-Smith (1964) but used instead a CG-50 resin column to separate and recover 5-HT. An important modification was introduced by Lovenberg's group. They utilized the fact that L-TP (1.4×10^{-2} M) and 5-HTP (5.4×10^{-6} M) have different affinities for decarboxylation by aromatic amino acid decarboxylase enzyme. However, their procedure also relied on separation of the product and therefore resulted in a loss of sensitivity.

The assay used by Renson's group tried to bypass the separation step by measuring the release of tritium into the medium caused by hydroxylation of TP labeled in the 5th position.

$$\text{TP-5-}{}^{3}\text{H} + 1/2\ O_2 + H_2O \longrightarrow \text{5-OH-TP} + {}^{3}\text{H}$$

Similar exchange reactions had been successful for other hydroxylase enzymes (Nagatsu et al., 1964; Pomerantz, 1964; Hutton et al., 1966). However, the assay revealed that like phenylalanine hydroxylase (Guroff et al., 1966) a migration of the tritium from position 5 to position 4 ("NIH" shift) occurred during the hydroxylation (Renson et al., 1966). More recent modifications of this technique (Lovenberg et al., 1971) now makes use of the fact that tritium in the 4th position of a 5-hydroxy-indole molecule exchanges quite readily in an acidic solution (Renson, 1971). However, some additional drawbacks of these assay methods have been discussed (Friedman et al., 1972).

The method of Ichiyama et al. (1968) determined the rate of hydroxylation by measuring the release of $^{14}CO_2$ after decarboxylation of 5-HTP obtained from L-tryptophan labeled at its terminal carboxyl group. This assay also makes use of the preferential decarboxylation of 5-HTP but was able to bypass the purification procedure by trapping and counting the $^{14}CO_2$ released. This was shown to be proportional to the amount of serotonin synthesized. The drawback inherent in this technique is that at high concentrations of TP direct decarboxylation occurs. This prevents the enzyme from being measured at its V_{max}.

A new assay procedure which directly determines the build up of 5-HTP by fluorometeric reading was developed by Friedman et al. (1972). Since this procedure employs the decarboxylase inhibitor NSD-1034, substrate level as high as 10^{-3} M may be used and the enzyme can be measured at its V_{max}.

D. Properties of Tryptophan Hydroxylase

Once these in vitro systems for tryptophan hydroxylase became available the properties of the enzyme began to be elucidated. In addition some major discrepencies arose due to the different assays employed. The presumed subcellular distribution and the cofactor requirements of tryptophan hydroxylase were dependent on the assay system used. These two properties were shown to be interrelated and the form of the enzyme used dictated its requirement for exogenous cofactors. It was shown that assays done on whole homogenate or particulate bound enzymes were not stimulated by the addition of cofactors if a reducing agent was used in the reaction medium. However, under similar conditions, the supernatant form of TP-hydroxylase was completely dependent on exogenous cofactors for enzymatic activity.

In subcellular distribution studies, most of the enzyme is recovered in a soluble form in the midbrain and brainstem regions (Ichiyama et al., 1970; Knapp and Mandel, 1972; Friedman et al., 1972; Lovenberg et al., 1968). In forebrain structures the enzyme is trapped in synaptosomes (pinched off nerve endings) and appears to be particulate. TP-hydroxylase in synaptosomes parallels the subcellular distribution of 5-HT (Michaelson and Whittaker, 1963) and 5-HTP decarboxylase activity (Arnaiz and De Roberts, 1964). The particulate bound tryptophan hydroxylase can be quite easily solubilized (Grahame-Smith, 1967; Ichiyama et al., 1968). Table II summarizes some of the properties of this enzyme.

The results obtained with whole homogenate or with the particulate form of the enzyme indicate a Km for TP of 2×10^{-5} M and, as stated above, no requirements for any cofactors.

Table II

Comparison of the Properties of Tryptophan Hydroxylase Activity

Tissue*	Form	KM of TP	KM of $DMPH_4$	KM of BH_4	O_2	pH
Guinea Pig[1]	HOMOG.	$2x10^{-5}M$				8.1
Brain	PART	$2x10^{-5}M$				8.1
	SOL.	$3x10^{-4}M$	$6x10^{-5}M$			8-8.5
Rat[2]	HOMOG.	$2x10^{-5}M$		5xSTIM		
Brainstem				100%		7.5
Rat[3]	SOL.	$3x10^{-4}M$	$3x10^{-5}M$	$5x10^{-6}M$		7.5
Brainstem						
Cat[4]						
Brain	HOMO.	$2x10^{-5}M$				7.6-7.9
Rabbit[5]						
Hindbrain	SOL.	$2.9x10^{-4}M$	$1.3x10^{-4}M$		20%	7.5
Rabbit[5]						
Hindbrain	SOL.	$5x10^{-5}M$		$3.1x10^{-5}$	2.5%	7.5

*References

1. Ichiyama et al., 1968, 1970
2. Green and Sawyer, 1966
3. Jéquier et al., 1967
4. Peters et al., 1968
5. Friedman et al., 1972

The soluble enzyme in order to be active must be fortified with a cofactor and a reducing agent to recharge it. The cofactor used in every earlier study of tryptophan hydroxylase was $DMPH_4$. This pteridine compound does not occur naturally. In an in vitro enzyme assay it causes the enzyme to have a lower affinity for L-TP and O_2 (Friedman et al., 1972). The naturally occurring pteridine is tetrahydrobiopterin (BH_4) which has a higher affinity for the enzyme (3×10^{-5} M) than does $DMPH_4$ (1.3×10^{-4} M) (Friedman et al., 1972). The structure of these compounds are shown in Figure 7. The enzyme in the presence of H_4-Biopterin has a much higher affinity for L-TP and O_2 as seen in Table II. These workers concluded that the majority of the experiments done with this enzyme system does not reflect measurements of the properties of soluble and particulate enzymes but rather reflects properties of the pteridine cofactor used in their assay mixtures. Furthermore, the reducing agents used by most workers are inhibitory. 0.05 M β-mercaptoethanol and DTT (dithiothreitol) inhibit the enzyme by 46 and 18 percent, respectively. On the other hand, the use of an enzymatic tetrahydro-pterin-regenerating system (Kaufman and Lovenberg, 1959) did not inhibit the hydroxylation reaction. It has been reported that Fe^{++} is necessary for optimal TP-hydroxylase activity (Lovenberg et al., 1968). However, Friedman's group showed that catalase could replace Fe^{++} and probably served the same purpose as in other pterin-dependent hydroxylases (Shiman et al., 1971; Kaufman, 1962). This function is the decomposition of H_2O_2 (Shiman et al., 1971), thus preventing the oxidation of the cofactor and possibly of the enzyme itself. The last possibility is depicted in Figure 5, Chapter I, B which pictures regulatory factors acting on TP-oxygenase. That enzyme is unable to bind O_2 if it is oxidized and must be in a reduced state before it can be functional. It is not known if the same is true for TP-hydroxylase.

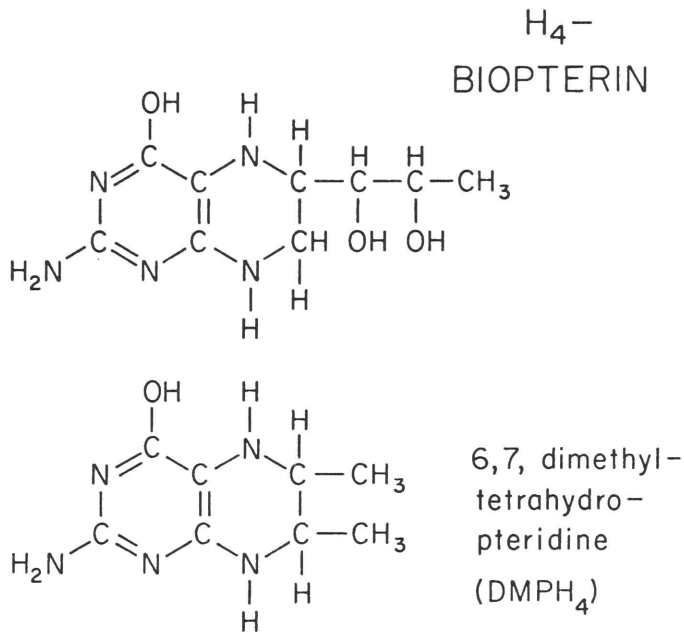


Figure 7. Comparison of the naturally occurring and synthetic pteridine cofactors.

E. Distribution of Tryptophan Hydroxylase

Since its first detection in dog and rabbit brain (Grahame and Smith, 1964) tryptophan hydroxylase has been shown to be present in every mammalian brain studied. The absolute values are similar for guinea pig, rat, mouse, hamster, rabbit, cow (Ichiyama et al., 1968) and cat (Peters et al., 1968).

The discovery by Falck (1964) that serotonin in histological sections of brain could be condensed by formaldehyde to form 3,4-dihydronorharman which has an intense yellow fluorescence has greatly facilitated regional studies of this amine. This technique showed the perikarya of the ascending serotonin-containing neurons in the mammalian brain were concentrated in the brainstem raphé (Dahlström and Fuxe, 1965). Axons from midbrain raphé cells extend forward by way of the medial forebrain bundle to various forebrain regions, being especially dense in limbic associated structures (Anden et al., 1966). The destruction of the raphé region has been shown to reduce forebrain 5-HT levels (Heller and Moore, 1965; Jouvet, 1967; Kostowski et al., 1968; Rosecrans and Sheard, 1969). Likewise, tryptophan hydroxylase decreases after such a lesion with an approximate half life of 48 hours (Kuhar et al., 1971).

This correlation between tryptophan hydroxylase and brain 5-HT levels is also reflected in their respective neuroanatomical distributions. Table III lists 5-HT, TP, and TP hydroxylase activity distribution for the cat brain (Peters et al., 1968). The distribution for the other enzymes involved in serotonin biosynthesis and degradation does not follow this pattern since these enzymes are not specific for the serotonin system but shared with the catecholamine-containing neurons (Garattini and Valzelli, 1965). Therefore detection of tryptophan hydroxylase activity is a reliable and specific indicator of the presence of serotonin in the tissue.

Table III

Neuroanatomical Distribution of TP, 5HT, and Tryptophan Hydroxylase

Tissue	V_{max}† m moles/g wet tissue/hr	5-HT mμmoles/g wet tissue*	Tryptophan† mμmoles/g wet tissue
Caudate	22.8 ± 2.5 (13)	14.2	30 ± 4
Septal	16.0 ± 1.5 (13)	17.3	20 ± 4
Anterior perforating substance	15.7 ± 1.8 (13)		
Hypothalamus	14.7 ± 1.0 (5)	14.2	
Amygdala	12.1 ± 0.9 (13)		32 ± 4
Mammillary bodies	11.8 (1)		
Rostral midbrain			
Midline tegmentum	11.1 ± 2.5 (5)		
Remainder	11.6 ± 3.0 (5)		
Caudal midbrain			
Midline tegmentum	17.3 ± 1.6 (5)	13.6	
Remainder	10.8 ± 2.5 (5)		
Thalamus	8.5 ± 0.9 (5)	6.2	36 ± 6
Hippocampus	4.3 ± 0.5 (13)		32 ± 4
Pons	4.0 ± 0.1 (5)	7.0	29 ± 5
Medulla	4.0 ± 0.2 (5)	6.6	28 ± 4
Cingulate gyrus	1.5 ± 0.2 (3)	5.3	31 ± 4
Association cortex	1.0 ± 0.3 (3)	0.62	
Visual cortex	1.0 ± 0.2 (3)	1.2	
Auditory cortex	1.9 ± 0.1 (3)		
Cerebellum	0.7 ± 0.3 (6)	1.1	72 ± 8
Pituitary	0.4 ± 0.2 (3)		

Figures in brackets denote numbers of samples

*Data taken from McGeer, McGeer and Wada (1963).

†Data taken from Peters et al. (1968).

F. Role of Tryptophan Hydroxylase in 5HT Biosynthesis

The functional significance of tryptophan hydroxylase activity for serotonin biosynthesis is well established. This enzyme is the first and rate-limiting step for serotonin biosynthesis in the brain from L-tryptophan (Ashcroft et al., 1965; Green and Sawyer, 1966; Jequier,,et al., 1967; Weber and Horita, 1965). Four lines of evidence support this: (1) The Km of this enzyme for L-TP (2×10^{-5} M, Friedman et al., 1972) is higher (lower affinity) than the Km of the L-aromatic amino acid decarboxylase for 5-HTP (5×10^{-6} M, Ichiyama et al., 1970); (2) The V_{max} of L-aromatic amino acid decarboxylase is 75 times greater than that of tryptophan hydroxylase (Peters et al., 1968); (3) The brain levels of 5HTP are negligible indicating rapid decarboxylation (Ashcroft et al., 1965); (4) The addition of exogenous amounts of L-aromatic amino acid decarboxylase to brain homogenates has no effect on the amount of 5-HT synthesized (Ichiyama et al., 1968; Peters et al., 1968). Changes in the activity of tryptophan hydroxylase would therefore most likely reflect an increased transmitter synthesizing capacity for the 5HT neurons.

An important requirement for the elucidation of function of any system is that regulatory controls be understood. The usual means by which synthesis rates are controlled is product feedback inhibition at the rate limiting step as seen in the norepinephrine synthesizing pathway (Udenfriend et al., 1965). However, tryptophan hydroxylase activity is not inhibited by 5HT (2×10^{-4} M) or 5HTP (2×10^{-4} M) (Grahame-Smith, 1964), although at a higher concentration 5HTP (10^{-3} M, Jequier et al., 1968) does inhibit the enzyme. It is surprising to note that in vitro TP levels above 2×10^{-4} M can inhibit the soluble tryptophan hydroxylase (Friedman et al., 1972).

The control by substrate availability (discussed below in Section I,1) is possible, but the recent clarification of the Km of this enzyme for its substrate (Friedman et al., 1972) make this type of control less dramatic than was first thought (Peters et al., 1968; Lovenberg et al., 1968). The enzyme is now believed to exist in a state similar to that in which most enzymes function with tissue concentrations of their

substrates in the range of the Km value of the enzyme for the substrate (Cleland, 1967). Tryptophan hydroxylase requires both molecular oxygen and reduced tetrahydropteridine cofactor to hydroxylate tryptophan. The specific sequence of this reaction is not clear but it most likely functions like tyrosine hydroxylase. This scheme is shown in Figure 8.

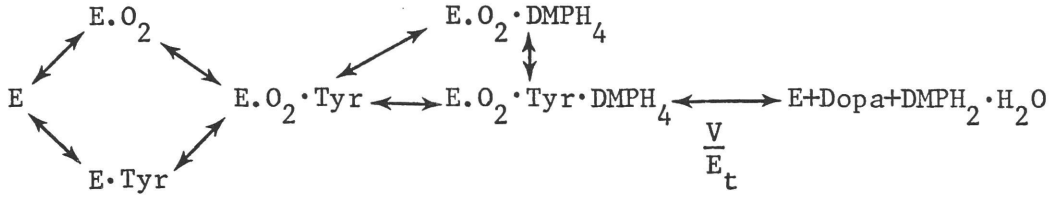
Molecular oxygen is necessary for enzyme activity. The availability of O_2 in the brain may be limiting since 100% O_2 has been shown to increase tryptophan hydroxylase activity both *in vivo* (Diaz *et al.*, 1968) and *in vitro* (Green and Sawyer, 1966). The amount of reduced cofactor may also be limiting. However, the cofactor (NADH) necessary to maintain biopterin in a reduced state is highly concentrated in CNS tissue. (Mahler and Cordes, 1966) and the pteridine reductase is believed to be present in excess in neural tissue (Cairnes *et al.*, 1972). Whether these factors serve as regulating factors in the *in vivo* system has not been studied in detail.

A final hypothetical regulatory mechanism for cerebral 5HT formation involves metabolites of liver tryptophan oxygenase. As mentioned above in Section I, B, the TP derivative with the highest concentration in the brain is NAD which presumably is partially synthesized in the liver. This cofactor participates in the degradation of serotonin in the brain in the aldehyde dehydrogenase reaction (Feldstein and Williamson, 1968).

G. Role of L-TP in Regulation 5-HT Synthesis

The Km of tryptophan hydroxylase for L-TP (5×10^{-5} M, Friedman, *et al.*, 1972) is close to the normal level of brain L-TP ($4\text{-}5 \times 10^{-5}$ mmoles/gm, equivalent to $4\text{-}5 \times 10^{-5}$ M; Peters *et al.*, 1968; McKean *et al.*, 1968). Therefore the cerebral enzyme might function at 1/2 V_{max} and be sensitive to changes in TP levels. The study of TP brain levels was handicapped until a sensitive and reliable tryptophan assay was developed (Denckla and Dewey, 1967). Until that time gross changes in TP plasma levels were achieved in order to assure increased levels in the brain.

Early studies attempted to regulate TP availability by restricting or supplementing the amount in the diet. A group of these studies are summarized in Table IV taken from Wurtman and Fernstrom (1972).



Assumptions:

1. $\frac{V}{E_t}$ is very small and rate limiting.

2. All steps are in rapid equilibrium.

Figure 8. Reaction mechanism of tyrosine hydroxylase (Tong H. Joh, 1973 personal communication).

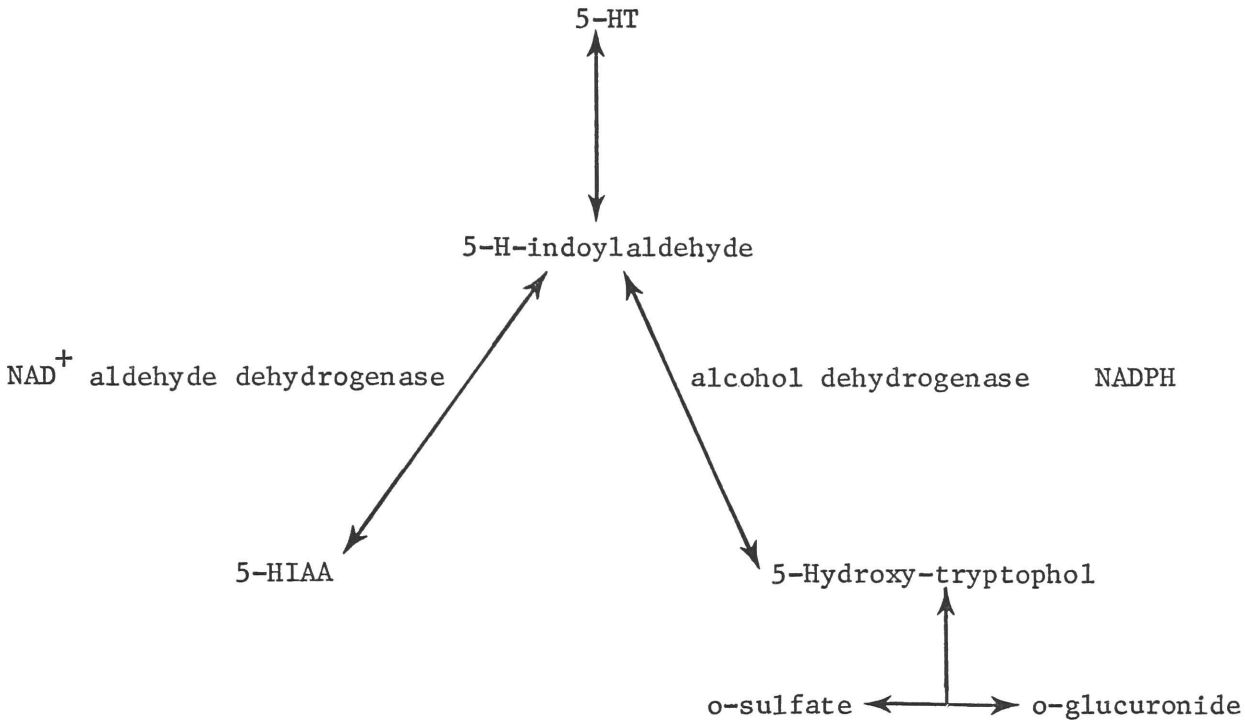


Figure 9. The systemic injection of 5 mg/Kg of L-Kynurenine sulfate, kynurenic acid, L-3 hydroxykynurenine, 3-hydroxyanthranilic acid and nicotinic acid all produce a similar effect on 5-HT degradation via this pathway, with a decrease in cerebral 5-HT levels and an increase in cerebral 5-HIAA levels (Green and Curzon, 1970).

Table IV

Effect of Dietary or Injected L-Tryptophan on Brain Serotonin Levels

Reference	Animal	Treatment
Zbinden et al., 1958	rabbit, guinea pig, rat, mouse	Tryptophan-deficient synthetic diet, 15-31 days
Gal et al., 1961	rat	Tryptophan-deficient diet, 6 weeks
Gal and Drewes, 1962	adult rat	Tryptophan-deficient diet (casein acid hydrolysate with added niacin), 5 weeks
Wang et al., 1962	18-day-old rat	Diet containing 1-5% L-tryptophan, 1-3 weeks
Green et al., 1962	rats weighing 35-46 g	Diet containing .09% tryptophan, .22% tryptophan (control), or 1.1% tryptophan, 15-17 days
Culley et al., 1963	adult rat	Tryptophan-deficient diet (casein acid hydrolysate); .5% L-tryptophan added for controls; 2-32 days
Boullin, 1963	weanling rat	Tryptophan-free synthetic diet; controls received 0.4% L-tryptophan; 14-35 days; pair-fed
Ashcroft et al., 1965	adult rat	L-Tryptophan, 800 mg/kg i.p.; killed after .5-8 hr
Eccleston et al., 1965	adult rat	L-Tryptophan, 400-1600 mg/kg i.p.; killed after 1-4 hr
Weber and Horita, 1965	adult rat	L-Tryptophan, 50-300 mg/kg i.p.; to rats pretreated with MAO inhibitor; killed after 2 hr
Thomas and Wysor, 1967	weanling rat	Tryptophan-poor diet (.056%) or control diet (.23%); 3-4 weeks
Weber, 1966	adult rat	L-Tryptophan, 204 mg/kg i.p.; killed after 2 hr
Moir and Eccleston, 1968	adult rat	L-Tryptophan, 50-1600 mg/kg i.p.; killed after .5-4 hr
	adult dog	L-Tryptophan, 50 mg/kg i.v.; killed after 1-4 hr (D,L-5-HTP, 10 mg/kg i.v.; killed after 1-4 hr)

Table IV (cont.)

Eccleston et al., 1968	adult dog	L-Tryptophan, 50 mg/kg i.v.; killed after 1-4 hr
Horita and Carino, 1970	adult rat	L-Tryptophan, 2500 mg/kg i.p.; survivors killed after 24 hr
Meek et al., 1970	adult spinal rat	L-Tryptophan, 100-750 mg/kg i.p.; killed after 2 hr.

Table IV (cont.)

Change in Brain Serotonin	Remarks
.48 vs .76 µg/g (rat) .47 vs .99 µg/g (mouse	----
Decreased by 70%	No change in brain norepinephrine
.16 vs .47 µg/g	Similar findings if sulfasuxidine added to diet.
.78 (1%, 2 weeks) vs .58 mg/g (controls)	High phenylalanine diet (7%, 1 week) depressed serotonin
Increased 32% with high tryptophan, unaffected by low tryptophan	High phenylalanine (9%) depressed serotonin, raised dopamine
.35 vs .54 µg/g after 4 days; .18 vs .51 µg/g after 32 days	Plasma tryptophan .6 vs .7 mg/100 ml after 4 days; .35 vs 2.1 mg/100 ml after 32 days
.12 vs .60 µg/g	No serotonin detectable in 11 of 19 animals; convulsions in 16, ataxis in 13 of 19
.62 vs .32 µg/g after 1 hr; .32 after 8 hr	Brain tryptophan increased 40-fold after 2 hr, 5-HIAA increased 4-fold after 4 hr
.62 vs .32 µg/g 1 hr after 400 mg/kg; bigger dose prolongs effect, but doesn't enhance peak	No 5-HTP detectable in brain, indicating that hydroxylation is rate-limiting; MAO inhibition causes brain serotonin to reach 1.27 µg/g with 400 mg/kg
Significant rise with 75 mg/kg	Viscera not needed for rise in brain serotonin; hence hydroxylation occurs in brain
.24 vs .39 µg/g (if niacin added to tryptophan-poor diet)	If no niacin added to tryptophan-poor diet, animals develop signs of pellagra, and brain serotonin is not depressed (.36 µg/g)
.73 vs .56 µg/g	----
.53 (.5 hr) or .63 (1 hr) vs .38 µg/g, after 50 mg/kg dose	Peak brain serotinin level and rate of rise of brain serotonin plus 5-HIAA as great after 60 mg/kg tryptophan as after 1600 mg/kg; hence, hydroxylation is rate-limiting

Table IV (cont.)

Change in Brain Serotonin	Remarks
Increment in each brain region related to its concentration in control dogs	Increment in regional serotonin contents after 5-HTP not related to concentrations in control animals; hence, plasma 5-HTP is not normal precursor for brain serotonin
Approximate doubling after 1 hr in midbrain, hypothalamus, thalamus, caudate, hippocampus, hindbrain	Approximate 4-fold increase in tryptophan concentrations of all regions, including those without detectable serotonin (cortex, cerebellum)
.78 vs .48 μg/g	Enormous dose no more effective than 50 mg/kg in elevating brain serotonin
Fluorescence intensity of serotonin-containing neurons increased with 500-750 mg/kg doses	Increase in extensor reflex with 500-750 mg/kg doses

The elimination of L-TP from the diet results in dramatic decreases in brain 5-HT. Diets which were only partially deficient in L-TP (0.09 compared with 0.22%, Green et al., 1962) had no effect on brain 5-HT, although brain catecholamines were elevated by 18%. Obviously the brain has the capacity to maintain normal levels of 5-HT despite substantial decreases in L-TP dietary intake. The additional L-TP could be obtained by increased catabolism of proteins in peripheral tissues. On the other hand the levels could also have remained constant because the degradation of 5-HT may have been decreased to match the decreased synthesis.

Increase in L-TP content in the diet leads to increased brain 5-HT levels as seen in Table IV. For example, elevating the L-TP in the diet 4-fold led to a 33% increase in brain 5-HT levels (Wang et al., 1962; Green et al., 1962). However, all of these studies examined whole brain changes after a given time period on the diet. The dietary study by Quay (1963) revealed that, at least in the hypothalamus, the change in 5-HT after an enriched diet may be transitory and affected indirectly by the stress imposed by the high L-TP diet.

A more direct approach to investigate the role of substrate on 5-HT synthesis and levels is the injection of large quantities of L-TP directly into the animal. This causes a substantial increase in brain 5-HT as seen in the table (Wurtman and Fernstrom, 1972). Although doses as high as 2,500 mg/kg have been given (Horita and Carino, 1970) the maximum increase achieved was similar to that obtained with a 50 mg/kg injection (Eccleston et al., 1968). The duration of the increase however, is longer with higher doses (Moir and Eccleston, 1968). The increase is usually of the order of 50% in 5-HT levels and is not greater in the presence of a monamine oxidase inhibitor. The magnitude of these increases appears to reach a plateau when brain levels of TP reach 90 nmoles/gm (Fernstrom and Wurtman, 1971).

These observed changes in brain 5-HT after L-TP injection appear to be changes in the functional pools of the transmitter. This is supported by five lines of evidence. First, neuroanatomical evidence shows the regional distribution of 5-HT follows a normal pattern after L-TP

administration, in contrast with changes in 5-HT levels after L-5HTP. Paradoxically after L-5HTP, increased 5-HT is detected in nonserotonergic neurons but not in serotonergic neurons (Moir and Eccleston, 1968; Airaksinen and McIsaac, 1968; Dahlstrom and Fuxe, 1965; Aghajanian and Asher, 1971; Meek et al., 1970). Secondly, a significant increase in the extensor reflex occurs two hours after a 500-750 mg/kg dose of L-TP (Meek et al., 1970). This is thought to be a direct disinhibiting action of 5-HT neurons, stimulated by high 5-HT levels in the spinal cord. Third, the firing rate of neurons in the midbrain raphé, which is believed to be depressed by increased 5-HT levels, is inhibited after a 100 mg/kg dose of L-TP (Aghajanian and Asher, 1971). The failure of this depression to develop after 100 mg/kg of L-5-HTP rules out the possibility that the inhibition was due to a general increase in brain 5-HT since injection of 5-HTP produces a more dramatic increase in 5-HT brain levels (Green and Sawyer, 1964; Costa and Rinaldi, 1958; Udenfriend et al., 1957). Fourth, in humans an oral dose of L-TP (7.5-12 gms) produces sedation, extreme drowsiness and an increase in slow wave sleep time (Griffith et al., 1972). Fifth, clinical studies have demonstrated that L-TP can potentiate the antidepressant effects of monamine oxidase inhibitors (Coppen et al., 1963). Although the studies with humans appear contradictory--L-TP producing sedation in the Griffith study and potentiating the antidepressant effects of MAO in the Coppen study--both support mood altering effects of this amino acid. The resolution must await better understanding and classification of human affective states.

A ciradian rhythm of brain 5-HT has been found in mouse, hamster, rat and cat (Albrecht et al., 1956, Matussek and Patschke, 1963; Dixit and Buckley, 1967; Scheving et al., 1968; Quay, 1968; Reis et al., 1969; Héry et al., 1972; Okada, 1971; Asano, 1971). Studies in the rat showed that levels peak in the afternoon and are correlated with the reduced activity of the animal, high levels, occurring when the animal is asleep (Asano, 1971; Okada, 1971). Measurements of circadian rhythm of brain TP in the rat showed a rhythm with peaks in the early morning (2 a.m.) and a peak of brain 5-HT four hours later (6 a.m.)

(Wurtman and Furnstrom, 1972). Although the result of this study, showing a peak of 5-HT at 6 a.m., disagrees with the above mentioned reports from other laboratories, it does reveal that the 5-HT and TP brain levels are somewhat out of phase with each other. Furthermore, it has been suggested that the 5-HT rhythm may be dependent on food intake since feeding rats every 4 hours abolish the variations (Young et al., 1969). But the reliance of 5-HT levels on food intake is not a direct relationship. It appears that fasting animals have higher L-TP plasma levels and brain 5-HT levels than recently fed rats (Perez-Cruet et al., 1972.

This paradox can be understood if the mechanism of L-TP uptake into brain and release from peripheral tissues is considered. L-TP is concentrated in brain tissues from plasma by an active energy requiring process (Snyder et al., 1970). This process appears to be shared by all neutral amino acids and the amino acids compete with each other for uptake (Blasberg and Lajtha, 1965; Guroff and Udenfriend, 1962). The amount of L-TP which will be taken up by the brain is proportional to the ratio of the concentration L-TP over the concentration of L-tyrosine, L-phenyalanine, L-leucine, L-isoleucine, and L-valine (Fernstrom and Wurtman, 1972). Therefore after a meal the L-TP ratio would be low but after starvation it appears, due to selective release of these amino acids, this L-TP ratio is high. Once in the plasma most of the L-TP is bound to albumin and only a small fraction exists in the free form (McNenamy and Oncley, 1958). It is assumed that the free form is the functional pool for exchange with tissue pools. Measurements of both forms of L-TP in plasma clearly showed that the free form increases during starvation while the total plasma L-TP levels remain unchanged (Knott and Curzon, 1972). Immobilization stress also causes an increase in the free form (Knott and Curzon, 1973). After both of these conditions brain 5-HT level increases. A work of caution should be added at this point. Although it appears that changes in L-TP and 5-HT levels are correlated, exceptions have been reported, for example, injection of probenecid, which prevents L-TP binding to albumin (McArthur and Dawkins, 1969; McNenamy and Oncley, 1958), produces a

large increase in free plasma and brain L-TP levels (Korf et al., 1972). However, despite these large changes no increase in 5-HT levels or build-up after pargyline (monoamine oxidase inhibitor) was found.

H. Behavior and Serotonin

The last section of the introduction will deal with the behavioral effects of 5-HT in the brain. The properties of the 5-HT system discussed previously suggests that 5-HT may act as a neurotransmitter. Its subcellular localization, synthesis, and distribution strongly suggest a neurotransmitter function for this amine. The data to be discussed in this section on behavior and neurophysiology will reinforce such a role for 5-HT. However, the critical evidence, release of 5-HT upon stimulation from identified nerve endings is lacking. Recent studies indirectly suggest that this occurs in the C.N.S. (Holman and Vogl, 1972; Andén et al., 1964). 5-HT perikarya are concentrated in the raphé region of the brainstem with the anteriorly projecting neurons located in the caudal midbrain at the level of the dorsal medial tegmentum (Dahlstrom and Fuxe, 1964). The axons from these neurons are fine (1-2 μm) and unmyelinated so that conduction velocities would be expected to be relatively slow (0.6-0.8 meters/sec, Couch, 1970). The projections ascend by way of the medial forebrain bundle and are highly branched near their terminations (Figure 10) in most forebrain areas (Andén et al., 1966; Fuxe et al., 1968; Heller and Moore, 1965; Sheard and Aghjanian, 1968). The firing rate of these cells is quite slow (1-2/sec, Sheard and Aghajarian, 1968) compared to the firing rate of the nearby reticular neurons (100-300/sec, Szerb, 1967). Together these facts imply that 5-HT neuronal system serves a general modulatory role rather than a precise, and rapid means of relaying information. Neurophysiological and biochemical studies support this view and further suggest that the main effect of 5-HT on emotional behavior is one of suppression.

Lesion and stimulation of the 5-HT system has been quite productive because of the precise localization of the perikarya in the dorsal medial tegmentum and its ascending fiber tract in the medial forebrain bundle. Lesions of either the dorsal medial tegmentum (Rosecrans and Sheard, 1967;

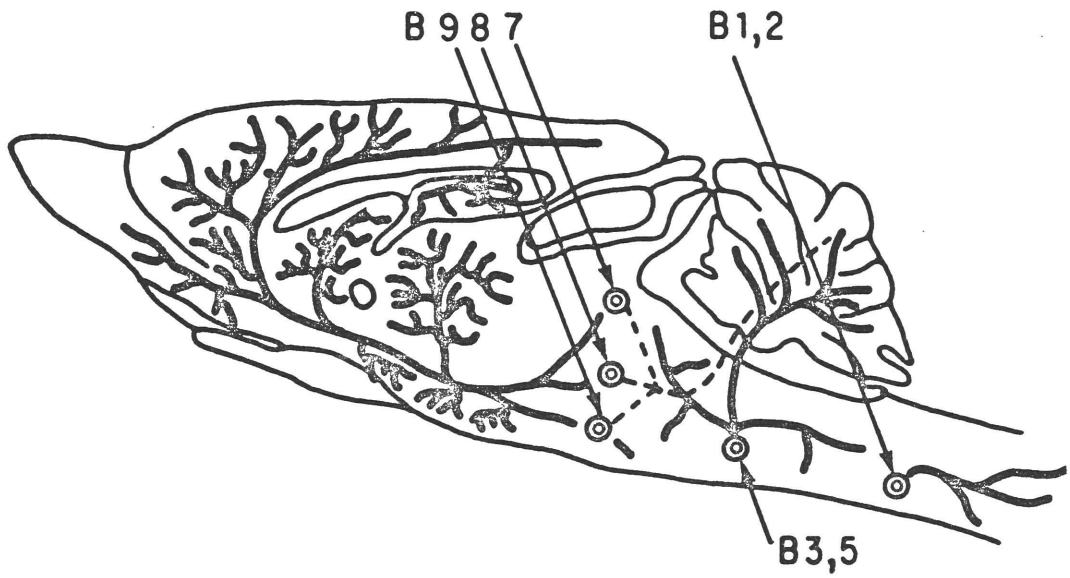


Figure 10. Rat brain showing the highly branched projections of the 5-HT ascending projections. The nuclear groups are numbered according to Dahlstrom and Fuxe (1964).

Jouvet et al., 1966; Kostowski et al., 1968; Renault, 1967; Lints and Harvey, 1969) or medial forebrain bundle (Heller and Moore, 1965; Heller et al., 1962; Lints and Harvey, 1969) lead to substantial decreases in forebrain 5-HT levels. Animals after lesions of the 5-HT system become hyperactive, display tremors, and perform stereotyped movements such as sniffing and licking (Kostowski et al., 1972, 1968). They become hypersensitive to external stimuli and show increased sensitivity to pain (Lints and Harvey, 1969). In addition animals become more aggressive towards conspecies (Heller and Moore, 1962). Most of these behavioral alterations return to normal despite the fact that 5-HT levels remain depressed after the destruction of the raphé area. Therefore it appears other systems--either the 5-HT neurons which survive or other transmitter systems--are able to readjust to the absence of normal amounts of 5-HT.

Stimulation of the dorsal medial tegmentum produces opposite results to the effects of lesions. The stimulation rate must be slow (1-3c/sec) since at higher rates (20-60c/sec) signs of arousal are obtained (Kostowski, 1968). The opposite results obtained by fast stimulation may be attributed either to direct stimulation of nearby reticular neurons which can be driven at a fast rate or to a conduction block of the 5-HT neurons. At low frequency stimulation, the first observed behavioral change is a decrease in orienting responses to external stimuli (Kostowski et al., 1968). This is followed by decreased motor activity and behavioral and EEG signs of sleep. These results, showing the slow driving rate required for 5-HT neurons, is in agreement with the general pattern of inhibitory systems in various brain areas which require low frequency stimulation to be maximally effective (Diecmann and Hassler, 1967; Buckwald et al., 1961; Akert et al., 1952, Hess,et al., 1950).

Direct biochemical studies on the effects of 5-HT on behavior are hampered by the fact that 5-HT does not cross the blood brain barrier (Garattini and Valzelli, 1965). For example, studies of cancer patients who have very high peripheral levels of 5-HT have revealed no apparent behavioral alterations in these patients (Sandler, 1968).

Direct loading with L-TP can be used to specifically increase functional 5-HT pools, as was discussed in Section 7. The administration of high doses of L-TP has been used to treat certain types of depression (Coppen et al., 1967) and produces light-headedness (Olsen et al., 1960), dizziness, euphoria, and severe drowsiness (Smith and Prockop, 1962). These effects are generally quite temporary and can be potentiated by monoamine oxidase inhibitors which prevent the breakdown of unprotected 5-HT in the brain (Berlet et al., 1965; Coppen et al., 1963; Pollin et al., 1961).

The synthesis of a halogenated phenylalanine compound p-chlorophenylalanine, provided a specific and protracted inhibitor of tryptophan hydroxylase (Koe and Weissman, 1966). Para-chlorophenylalanine (PCPA) markedly reduces levels of 5-HT with little effect of brain catecholamines in the rat (Volicier, 1969; Koe and Weissman, 1966). PCPA produces effects on behavior similar to those of raphé lesions. Animals display increased irritability, mutual agression (Koe and Weissman, 1966) sensitivity to external stimuli (Tenen, 1967; Swonger et al., 1970) and hyper-sexuality (Ferguson et al., 1970; Sheard, 1969). PCPA can disrupt conditioned suppression of negatively reinforced operant behaviors (Robichaud and Sledge, 1969; Brody, 1970). However, acquisition of active avoidance responses (Tenen, 1967; Schlesinger et al., 1968) and light discrimination tasks are facilitated (Stevens et al., 1967). These results rule out the explanation that a learning process is reduced. Furthermore, PCPA treated animals can learn to habituate to certain stimuli if enough trials are run even if the 5-HT levels are depressed (Conner et al., 1970).

The function of 5-HT as an inhibitory neurotransmitter has been proposed by several groups (Marrazzi and Hart, 1956; Gyermek, 1966). Direct application of 5-HT to neurons is generally inhibitory (Roberts and Strougham, 1967; Weight and Salmoiraghi, 1968; Legge et al., 1966). Therefore some of the behavioral and motor effects may be due to direct inhibition of motor sensory systems (Andén et al., 1966; Galambos, 1956; Hernandez-Peon et al., 1956). However, the transitory effects after PCPA and lesions argue for a more indirect role, although it may be possible that functional pools recover despite the general depression of amine levels.

It has been proposed that the 5-HT system might be activated by a catecholaminergic system and function as a gain control for cholinergic-limbic inhibitory system (Swonger and Rech, 1972). Therefore the forebrain acetylcholine system would have a central role in controlling emotionality. This acetylcholine system has been shown to be a powerful inhibitory circuit important for emotional behavior (Carlton, 1963, 1969). Neurophysiological evidence for 5-HT input to forebrain areas has been obtained. Direct application of 5-HT to single units of the hippocampus are generally inhibitory (Briscoe and Straugham, 1966). This circuit should be considered as a regulatory loop involving reciprocal innervations from forebrain areas on raphé neurons. Nauta (1958) described a monosynaptic pathway from the hippocampus to the midbrain raphé and Brodal (1969) describes a pathway from the mammillary area to this raphé region. Whatever the circuitry is, it is certainly quite diffuse, slow acting, and generally inhibitory.

Section II

MATERIALS AND METHODS

A. Materials

1. Chemicals - Drugs - Isotopes

Compound	Source
Corticotrophin (ACTH)	Organon, West Orange, New Jersey
Cycloheximide	Sigma, St. Louis, Mo.
$DMPH_4$	Calbiochem (Cal) Monsey, New York
Kidney Powder, hog, acetone dried	Sigma, St. Louis, Mo.
PCPA, D, L	Regis Chemical Chicago, Ill.
Morphine	Dr. Reis, D. Cornell University Medical School
Pargyline (Eutonyl TM)	Abbott Labs. Chicago, Ill.
Puromycin-dihydrochloride	Nutrition Biochem. Cleveland, Ohio
Aminonucleoside of Puromycin	Nutrition Biochem.
Reserpine	Sigma, St. Louis, Mo.
Unlabeled steroids	Steraloid, Inc. Pawling, N.Y.

Scintillation Compounds

Bio-solv	Beckman Instrument Co. Mountainside, N.J.
Liquifluor	New England Nuclear (NEN) Boston, Mass.
PPO (2,5-diphenyloxazole)	New England Nuclear (NEN) Boston, Mass.
POPOP (dimethyl-1,4-bis	

Triton X-100	Mallinckrodt, Linden, N.J.
Ethylene glycol	Mallinckrodt, Linden, N.J.
Para-dioxane	Mallinckrodt, Linden, N.J.
Methyl alcohol	Mallinckrodt, Linden, N.J.
Napthalene	Mallinckrodt, Linden, N.J.
Toluene, analytical	Mallinckrodt, Linden, N.J.

Isotopes

Compounds	Specific Activity	Source
Corticosterone-1,2,6,7-^{3}H	100 c/mmole	New England Nuclear (NEN), Boston, Mass.
Toluene-^{14}C	56 μc/mole	Packard, Downers Grove, Ill.
L-Tryptophan-1-^{14}C	8.9 mc/mmole	NEN, Boston, Mass.
	9.6 mc/mmole	Calbiochem, Monsey, New York
^{3}H-Valine	6 c/mmole	Schwartz-Mann, Orangeberg, N.Y.

2. Experimental Animals

Sprague Dawley rats were used for all studies to be reported here. Normal male rats (250-300 grams) obtained from Charles River breeding labs (Wilmington, Mass.) were used for studies conducted at The Rockefeller University. Adrenalectomies and sham operations were performed in the laboratory. For one series of experiments hypophysectomized rats were obtained from Holtzman Breeding Labs (Madison, Wisconsin). Studies done at the National Institute for Mental Health with Dr. Ermino Costa were done on female rates (180-220 grams), sham-operated or adrenalectomized by the supplier, Zivic Miller (Pittsburgh, Pa.). At Cornell University Medical College studies with Dr. Donald Reis used female rats (230-260 grams) obtained from Blue Spruce (Altamont, N.Y.). Animals were housed in group cages (4-6 per cage) unless otherwise stated. The animals were maintained on a 12 hr light-dark cycle with food and tap water (.8% saline to adrenalectomized rats) provided ad lib. The animals were maintained in rooms separated from the main part of the lab.

B. Methods

1. Brain Dissection

The rats were decapitated in a small animal guillotine, the brain removed within 30 seconds, and immediately placed on ice. Selected brain regions, identified using a rat atlas (Zeman and Innes, 1963; König and Kippel, 1963), were dissected, weighed, and placed on ice for subsequent procedures. Most of the dissected regions appear in Figure 11 which gives a general orientation and shows some of the major boundaries between brain areas. The cerebellum including peduncles was teased away from the brainstem. The midbrain was dissected with the rostral border extending from the anterior end of the superior colliculus ventrally to the posterior end of the mammillary body and the caudal border extending from the posterior end of the inferior colliculus ventrally to the anterior ridge of the pons (see Figure 11). The pons which was next to the midbrain was dissected using the ponto-cerebellar fibers as a landmark. The medullary region extended caudally from the pons to the obex. The hypothalamus extended from the posterior boundary of the mammillary body to a level just behind the optic chiasma and did not include the suprachiasmatic region. The separation of the hypothalamus from overlying thalamus was achieved by cutting around the hypothalamic region with a pointed scalpel and then lowering a pair of curved forceps on either side of the lateral borders of the hypothalamus and teasing this region from the thalamus. The thalamus was then separated from its attachment to the lateral internal capsule fibers. The preoptic area pictured in Figure 11 was then removed. Its bilateral oval appearance was quite distinct on the ventral surface of the rat brain. The anterior communicating artery and genu of corpus callosum were the rostral borders, the septum was the dorsal boundary, and the hypothalamus the caudal border of the preoptic region. The septum, lying on top of the preoptic region, was teased away from the overlying corpus callosum fibers. The brain was now placed on its ventral side and cut along the midline to expose the caudate area and the hippocampus. The caudate area, lying along side of the cortex, was teased off. The hippocampus was then visible

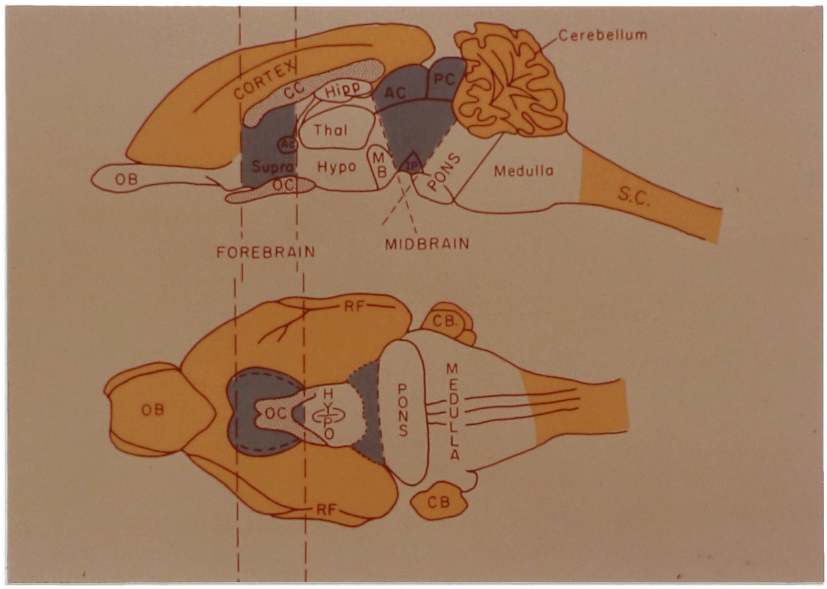


Figure 11. The rat brain, Ac, anterior colliculus, Ac = anterior commissure, CB = cerebellum, CC = corpus callosum, hipp = hippocampus, hypo = hypothalamus, ip = interpeduncular, MB = mammillary body, OB = olfactory bulb, OC = optic chiasma, PC = posterior colliculus, PO - preoptic, RF = rhinal fissure, Supra = supraoptic, SC = spinal cord, Thal = thalamus.

and has a characteristic hook shape about 1 1/2 cm in length. This structure was peeled away from the cortex with a spatula. The cortex was then placed flat and the amydala was separated by cutting along the rhinal fissure. A cortex sample was taken from the remaining tissue. While there certainly were slight errors at the boundaries, the landmarks were quite distinct and readily located. The amount of variation in the weight of the respective blocks was small (±10%).

2. Injection and Stress Procedures

a. Stress procedure: Animals were kept in group cages except when exposed to the various stress procedures.

Electric foot shock (EFS) or ether anesthesia was given 3 times a day (9:00 a.m., 5:00 p.m. and 11:00 p.m.) for two days and at 9:00 a.m. on the third day, 2 hrs before sacrifice. The shock consisted of 0.5 mA current delivered through the grid floor from a Lehigh Valley constant current source for 3 sec. Ether stress was produced by lightly anesthetizing animals in a tightly covered glass jar. Cold stress was produced by placing singly-caged rats with food and water present in a walk-in cold room (4-5° C) for 1, 3, 5, 8, or 48 hrs; sacrifice followed immediately upon removal from cold.

b. Injection of steroids: The steroids were given at doses ranging from 1.3-9 mg/kg body weight and injected into the peritoneal cavity in concentrations of 15 mg/cc ethanol. Control animals received a comparable injection of ethanol. In later experiments sodium salt-corticosterone-hemisuccinate* was used. This was dissolved in saline, 10 mg steroid/cc saline.

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*432 mg Corticosterone hemisuccinate (0.965 mMoles) placed in 2.9 ml acetone in a 20 ml beaker. With stirring 1.50 ml of 0.50 N NaOH was added dropwise (the crystals dissolved as they were converted). An additional 0.27 ml of 0.50 NaOH was required to bring the pH to 7.0 (a 0.10 ml aliquot was added to 3 ml of water for pH determination). An additional 2 ml of acetone was added to replace volume lost by evaporation. The contents of the beaker were transferred to a 30 ml glass stoppered tube, 3.5 ml water added and the aqueous phase was extracted 3 times with 8 ml volumes of ethyl acetate. The aqueous phase was transferred to a 50 ml round bottom flask and all traces of ethyl acetate were removed by rotary

c. Injection of protein synthesis inhibitors: Two injection procedures were used depending on what response was to be measured. (1) To study the effects of protein synthesis inhibition on the adrenal response the injection of cycloheximide (18 mg/Kg) was made into peritoneal cavity. The animals were exposed to ether anesthesia at various intervals after the injection and the corticosterone plasma levels measured 15 min after the exposure. (2) To study the effects of protein synthesis inhibitors on brain protein synthesis and on brain TP-hydroxylase activity the inhibitors were injected into the cerebellomedullary cisternum. To make this injection the animals were lightly anesthetized in ether and the fur from the back of the neck shaved. The head was then tilted forward and the injection (26 gauge needle) was made in the midline just below the dorsal caudal end of the skull. Cycloheximide (400 µg/40 µl), neutralized Puromycin (600 µg/40 µl), or 40 µl of 0.8% saline was injected in this manner. The animals were returned to their cages and sacrificed at various intervals afterwards.

d. Injection of morphine: Tolerant and withdrawal groups received morphine sulphate injected subcutaneously over a 16-day period, beginning with a 5 mg/kg dose administered twice a day (8:00 a.m. and 4:00 p.m.), and increasing up to a dose of 95 mg/kg given three times a day (8:00 a.m., noon, 4:00 p.m.). The tolerant animals were killed on the morning of the sixteenth day, 2 hours after an injection of 130 mg/kg morphine. The withdrawal group received a similar dose of morphine 48 hours before they were killed. A saline control group received injections of equal volume of isotonic saline on the same schedule as the tolerant group.

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evaporation. Traces of remaining insoluble material were removed by filtration. The final clear solution was lyophilized to yield 340 mg of white amorphous solid (Dr. Robert Purdy, Southwest Foundation, San Antonio, Texas, personal communication).

3. Tryptophan Hydroxylase Assay

a. Whole homogenate: Tryptophan hydroxylase activity was measured using Warburg flasks. The assay used was adapted from Ichiyama et al. (1968, 1970) and the general principle of the assay can be seen in Figure 12. The assay depends on the specific decarboxylation of terminal ^{14}C-carboxy from 5-OH-TP. This can be obtained if high levels of L-TP are avoided so that the amino acid will not be directly decarboxylated to form tryptamine (dashed lines). At levels of L-TP below 5 x 10^{-5}M this does not occur. Since TP-hydroxylase is rate limiting (Section F) the amount of $^{14}CO_2$ liberated should be proportional to the amount of radioactive $^{14}CO_2$ liberated. This has shown to be true for brain tissue (Ichiyama et al., 1968). The protocol followed for measuring tryptophan hydroxylase is as follows:

1) Fresh brain tissue (50-200 mg) homogenized (15 passes) in 2 ml of cold .32 M sucrose containing .1 M Tris Acetate, pH 8.1.

2) 2 ml of homogenate was added to the main compartment of Warburg flask kept on ice.

3) 20 λ of L-Tryptophan 1-C-14 (10-20 nmoles dissolved in 0.01N HCl) was added and the flask tightly sealed.

4) Reaction was stopped after shaking for 20 min at 37° C by the introductionoof 0.6-1.0 ml of 16% PCA (containing 8 x 10^{-3} M tiron).

5) Tubes were then shaken for an additional 2 hours and the radioactive CO_2 released was collected in the center well which contained a strip of filter paper (2 x 1 1/2 cm) soaked in 0.5 ml of 1 N NaOH.

6) Filter paper, NaOH, and 2 distilled H_2O rinses were transferred to vials containing 10 ml of Brays scintillation solution.

b. Particulate form of enzyme: In certain cases the enzyme was measured in the particulate fraction. This crude fractionation method included the nuclei, mitochondria, and synaptosomes. This procedure eliminates much of the free L-TP and other soluble components

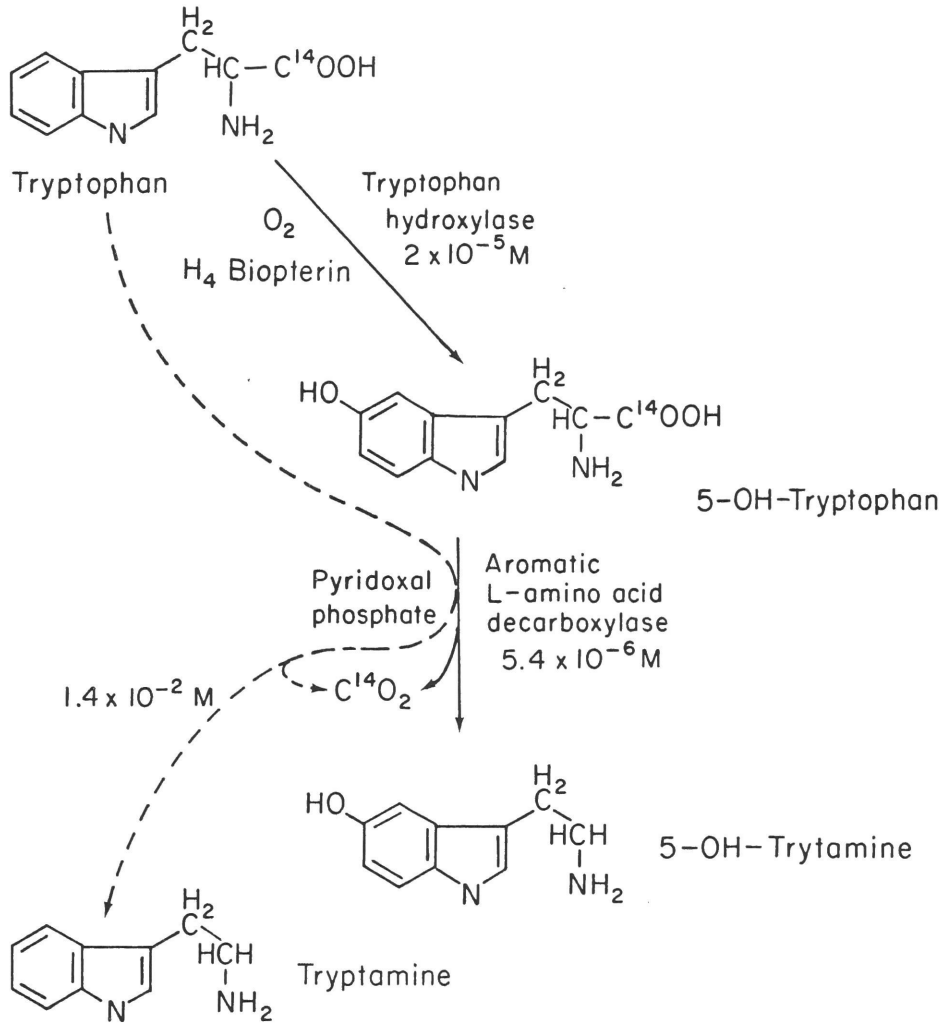

Figure 12. The scheme depicted above illustrates the principle of the assay developed by Ichiyama et al. (1968). The terminal carboxy group of TP-1-C^{14} is split by the decarboxylation of 5-OH-TP (straight line). However, at high TP levels this terminal carboxy group can be directly decarboxylated without being hydroxylated (dash line).

of the cell. No fortification of this fraction with cofactors or reducing agents is necessary in measuring TH activity. The basic procedure is shown in Figure 13. The pellet was resuspended after the high speed spin in 2 ml of the reaction mixture and TP-hydroxylase measured directly.

To study the effect of L-TP on TP-hydroxylase activity certain modifications were made to minimize the possibility of isotope dilution due to higher levels of L-TP in the sample from the TP injected animal. The procedure followed is outlined in Figure 14.

4. Corticosterone, TP, and 5-HT Measurements

a. Corticosterone: Blood for corticosterone determination was obtained in several ways. (1) A heart puncture was performed when the animals were under ether anesthesia. The animals were placed on their back and a 23 gauge syringe needle was inserted through the chest wall. Once the heart had been entered blood (approximately 2-3 cc) was slowly removed. (2) The tip of the tail of the rat was clipped and blood collected in a test tube (approximately 1 cc). (3) Finally, blood was collected in heparinized tubes when the animals were decapitated (approximately 5 cc). This was the technique used for most determinations.

The blood was spun in a table top centrifuge (Serval, NSE type 22637) at 7-8,000 rpm and the clear plasma transferred to 2 ml test tubes. If the corticosterone determination was not made within 24 hours the plasma was frozen at -5° C, otherwise it was kept in the refrigerator (5° C). The micromethod (50 µl of plasma) used for corticosterone was adapted from the method of Glick and coworkers (1964) with modifications by Gray (1972).

50 µl of plasma was transferred to a 2 ml test tube and 0.8 ml of dichloromethane (DCM) was added. The tube was gently shaken and the plasma carefully aspirated off. A .2 ml wash with 1 N NaOH and with distilled water was done and aspirated off. Next 0.5 ml of the DCM solution was transferred to another tube and 200 µl of acid mixture

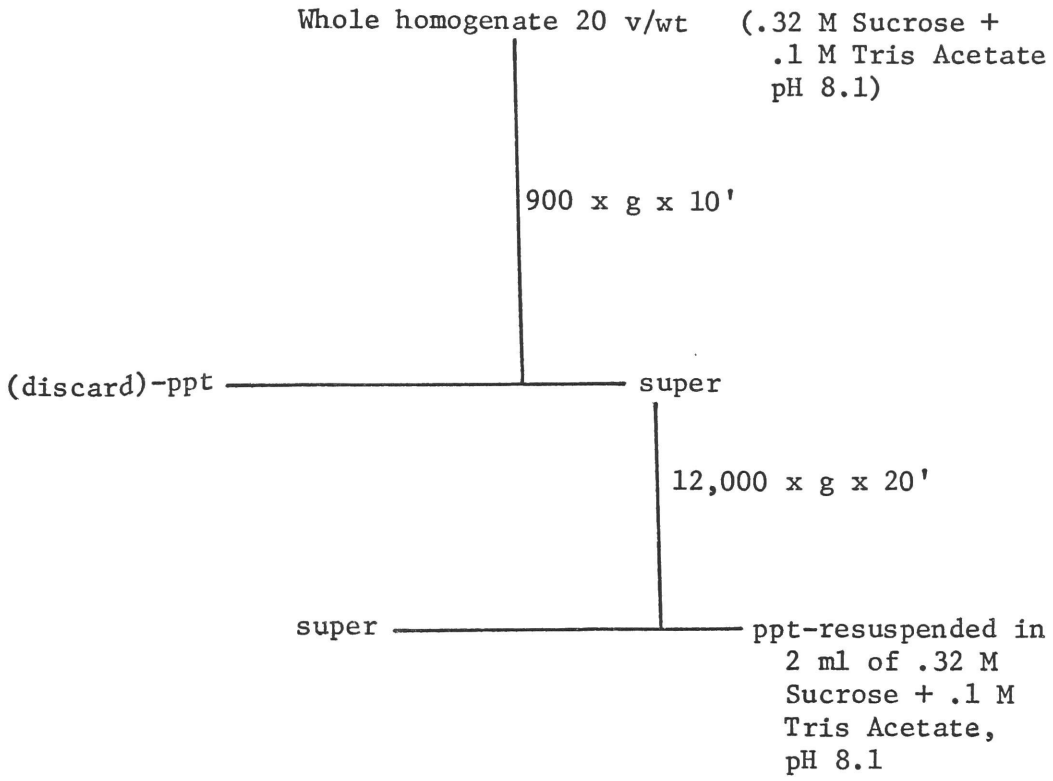

Figure 13. Procedue for particulate Fraction I

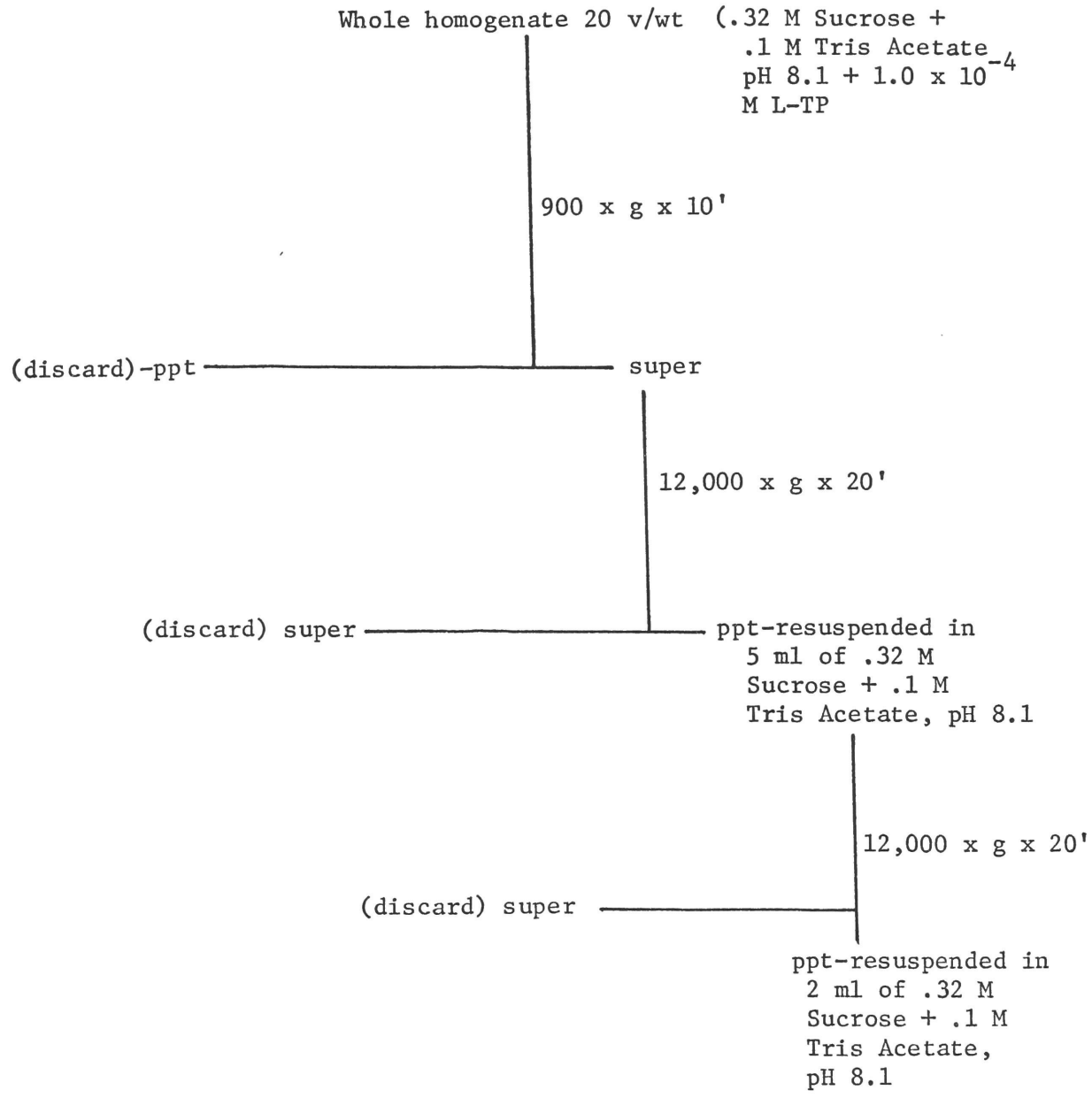

Figure 14. The tissues from both groups were homogenized in the presence of high levels of L-TP (1×10^{-4} M). Furthermore, once the first pellet was obtained it was resuspended in 5 ml of solution without the exogenous L-TP. After the second high speed spin the pellet was resuspended in 2 ml of the reaction mixture and the TP-hydroxylase assayed from this volume. Protein was determined at the termination of the assay procedure by the method of Lowry et al. (1951).

(7:sulfuric acid, 3:absolute alcohol) was added. The fluorescence was allowed 20 minutes to develop and read at 420 after activation at 370 in a spectrophotofluorometer (Aminco Bowman #4-8202).

b. Tryptophan and serotonin: Serotonin was measured by the method of Snyder et al. (1965). This method involves the extraction of serotonin from a salt-saturated suspension pH 10) (homogenate spun 900 g x 10 min) into an organic solution (1-butanol). The serotonin is returned to a pH 7 aqueous solution by the addition of heptane. Next the 5-HT reacted with ninhydrin (75° C x 30') to form a fluorescent product which is eight times more intense than the native fluorescence of 5-HT in acidic solution. The fluorescence is read in quartz cuvette in an Aminco-Bowman Spectrophotofluorometer. Serotonin normally fluoresces at 490 mμ after activation at 385 mμ; the peak of the standard 5-HT was checked before each series of measurements.

Tryptophan is assayed by the condensation method of Denckla and Dewey (1967). Figure 15 shows the main points of the assay. The extraction into organic phase with ethylacetate and subsequent re-extraction into the aqueous phase with 0.1 N HCl is included to remove excess $FeCl_2$ which can quench the fluorescence of Norharman. The samples are read spectrophotoflurometrically at 450 mμ after activation at 370 mμ. Peaks of fluorescence are checked on standards before readings of each series of samples.

c. Free plasma tryptophan: The separation of plasma TP into a free and bound form was obtained in a Centriflo membrane ultrafilter (Amicon). One ml of plasma was placed in the membrane cone which was supported by a polyethylene cone. The membrane cone was soaked in distilled water for 1 hour before use and stored in 10% ethanol-water solution between experiments. The cone was placed on top of a graduated centrifuge tube and spun at room temperature for 20 minutes at 800 G in swing out buckets in a table top centrifuge. Free amino acids (10%) passed through the filter while the bound form was retained.

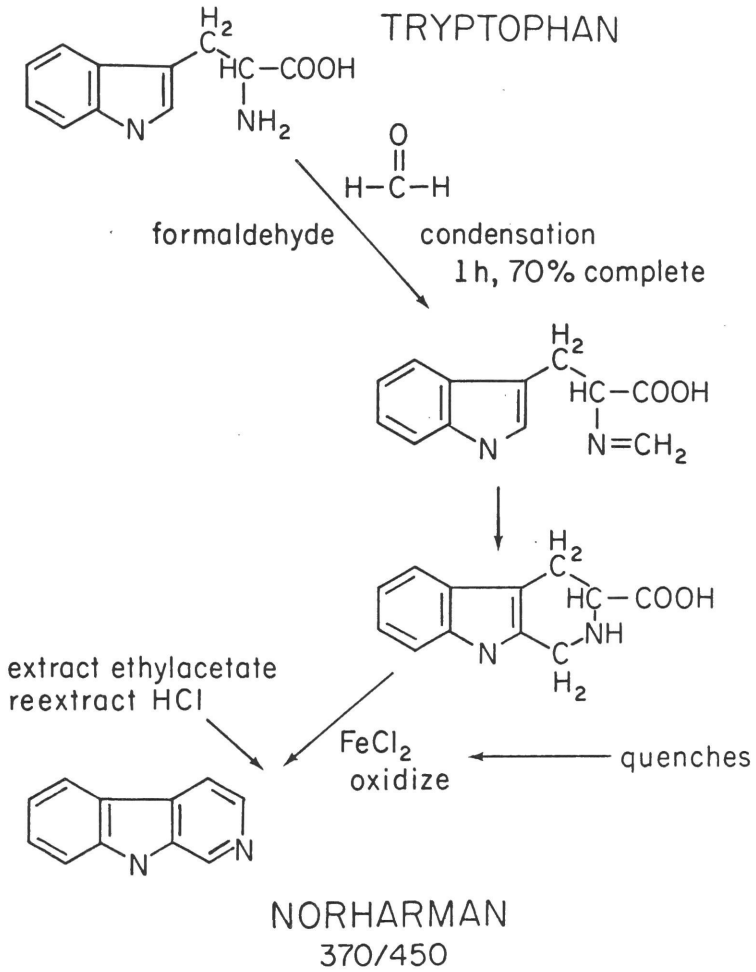

Figure 15. The condensation of L-TP to form norharman by treatment with formaldehyde (Denckla and Dewey, 1967).

d. Conversion index of 5-HT: A pulse intravenous injection of 500 c of ^{3}H-L-tryptophan per kilogram (5 c/mmole, New England Nuclear Co.) in 10 ml of phosphate buffer per kilogram (7.4 pH, 0.2 M) was made in the afternoon and the rats were decapitated either 20 or 40 minutes later. Sham-operated and adrenalectomized rats used as controls received only phosphate buffer. The brain was quickly removed and dissected into brainstem (pons, medulla, and midbrain) and telediencephalon. The latter included all structures anterior to a cut from the anterior part of the superior colliculus to the posterior edge of the mammillary bodies (Section 2-B, 1). These two brain areas were immediately frozen and they were kept frozen until assay of the specific activity of TP and serotonin (Section 2-B, 1). Blood was collected in heparinized tubes and immediately centrifuged to separate the plasma for assay of TP and corticosterone concentrations. The brain tissues were homogenized in the phosphate buffer, centrifuged, and the supernatant placed on dowex-50 columns where TP + 5-HT were separated as previously described (Costa et al., 1968). Radioactive TP and serotonin were estimated by liquid scintillation spectrometry in 2.0 ml aliquots from the aqueous phases used to estimate fluorimetrically amine and the amino acid concentrations.

The synthesis rate of 5-HT could be directly determined if the specific activity of brain TP ($\frac{\text{n curie TP}}{\text{m mole TP}}$) and newly, synthesized (NS) brain 5-HT ($\frac{\text{n curie 5-HT}}{\text{m mole 5-HT NS}}$) were known at a given time. This can't be measured after a pulse injection of ^{3}H-TP since the specific activity (SA) is constantly changing and the unlabeled 5-HT_{NS} cannot be distinguished from the existing 5-HT pools. However, an approximation of the synthesis was calculated, using similar calculations proposed for measurements of norepinephrine synthesis rate after a pulse injection of ^{14}C-tyrosine (Sedval et al., 1968). This value was called the conversion index (CI) (Azmitia et al., 1970) and was derived from the following equation: $R = \frac{SA(5\text{-HT})NS}{SA(TP)}$. Where R was the ratio of the specific activity of newly formed 5-HT over the precursor L-TP. This value should be constant after the initial increase following injection (Neff et al., 1971). The amount of 5-HT newly synthesized times R was

termed the conversion index. Thus by the following manipulations an approximation of the newly synthesized 5-HT could be determined.

$$\frac{R\ (n\ curie\ TP)}{(m\mu\ mole\ TP)} = \frac{(n\ curie\ 5\text{-}HT)}{m\mu\ mole\ 5\text{-}HT)NS}$$

The values not determined by experimental means were placed on the left side of the equation and termed CI.

$$CI = R\ (m\mu\ mole\ 5\text{-}HT)_{NS} = \frac{(n\ curie\ 5\text{-}HT)}{(\frac{n\ curie\ -\ TP}{m\mu\ mole\ TP})}$$

In order to express n curie as SA the left side of the equation was multiplied by $(\frac{m\mu\ mole\ 5\text{-}HT}{m\mu\ mole\ 5\text{-}HT})$

$$CI = \frac{(n\ curie\ 5\text{-}HT)}{(TP)} \quad X \quad \frac{m\mu\ mole\ 5\text{-}HT}{m\mu\ mole\ 5\text{-}HT}$$

$$CI = \frac{(5\text{-}HT)SA}{(TP)_{SA}} \quad X \quad (m\mu\ mole\ 5\text{-}HT)$$

The CI had the dimensions of n mole of 5-HT. There was no correction factor for time. This approximation is not to be confused with the synthesis rate of 5-HT which has the dimensions of n moles of 5-HT per unit time. Furthermore, CI neither corrected for continuous efflux rate of ^{3}H-5-HT from brain, nor the rapid change of SA of TP occurring at the initial times after labeling (Neff et al., 1971). To minimize for this last point the animals were sacrificed 20 or 40 min after injection since the SA of TP had already peaked and was slowly declining.

5. Other Procedures Used

a. Tyrosine hydroxylase assay: Tyrosine hydroxylase was assayed by the method of Nagatsu et al. (1964). This procedure measures the amount of radioactive L-dopa formed from radioactive tyrosine. The reaction is terminated by the addition of 2 ml of 5% TCA (200 μ gms of cold exogenous L-dopa is added as carrier and to determine

efficiency of recovery). This mixture is centrifuged and the supernatant mixed with alumina, EDTA, and Na Acetate at pH 8.5. After gentle stirring for 5 minutes the supernatant is decanted and the alumina transferred to a column. 30 ml of water is added to remove the radioactive tyrosine before the L-dopa is elated with 2 ml of 0.3 N Acetic Acid. This fraction is then counted in a scintillation counter. Recovery of L-dopa is around 65%.

b. Monoamine oxidase assay: L-Monoamine oxidase was determined by the method of Wurtman and Axelrod (1963). The tissue is homogenized in isotonic KCl and the ^{14}C-tryptamine (10,000 cpm) and 0.5 M phosphate buffer, pH 7.4 are added. The reaction is stopped after 20 minutes with 2 N HCl. The product, ^{14}C-indolacetic acid, is extracted with 6 ml of toluene and an aliquot is used for scintillation counting.

c. L-aromatic amino acid decarboxylase: The partial purification of L-aromatic amino acid decarboxylase was necessary to assay tryptophan hydroxylase activity in soluble preparation since the decarboxylase will mainly be concentrated in the particulate fraction. The decarboxylase was obtained from acetone dried hog kidney powder by the methods of Christenson et al. (1970) and Clark et al. (1954). The procedure followed is outlined in Figure 16. The enzyme was purified up to the 49% ammonium sulfate precipitation and dialyzed against .005 $NaPO_4$ buffer (pH 7.2) overnight. This preparation was devoid of oxidative enzymes. The decarboxylase activity was measured using 1-^{14}C-DOPA as substrate and collecting the radioactive CO_2 released during 20 minutes of incubation at 37° C (Lamprecht and Coyle, 1972).

d. Determination of in vivo protein synthesis: Animals were anesthetized with ether and injected intracisternally with either cycloheximide (400 µg/40 µl), puromycin (600 µg/40 µl) or 9.8% saline (40 µl) as described in Section II, B. After 1 hour ^{3}H-valine (100 µCi/0.1 ml) was injected intraperitoneally. The animals were decapitated 30 minutes later and the brains quickly removed and placed on ice. The midbrain, hippocampus and forebrain section (Preoptic-Septum) were dissected and placed on dry ice. The flow sheet (Figure 17) below describes the procedure followed to measure radioactivity in

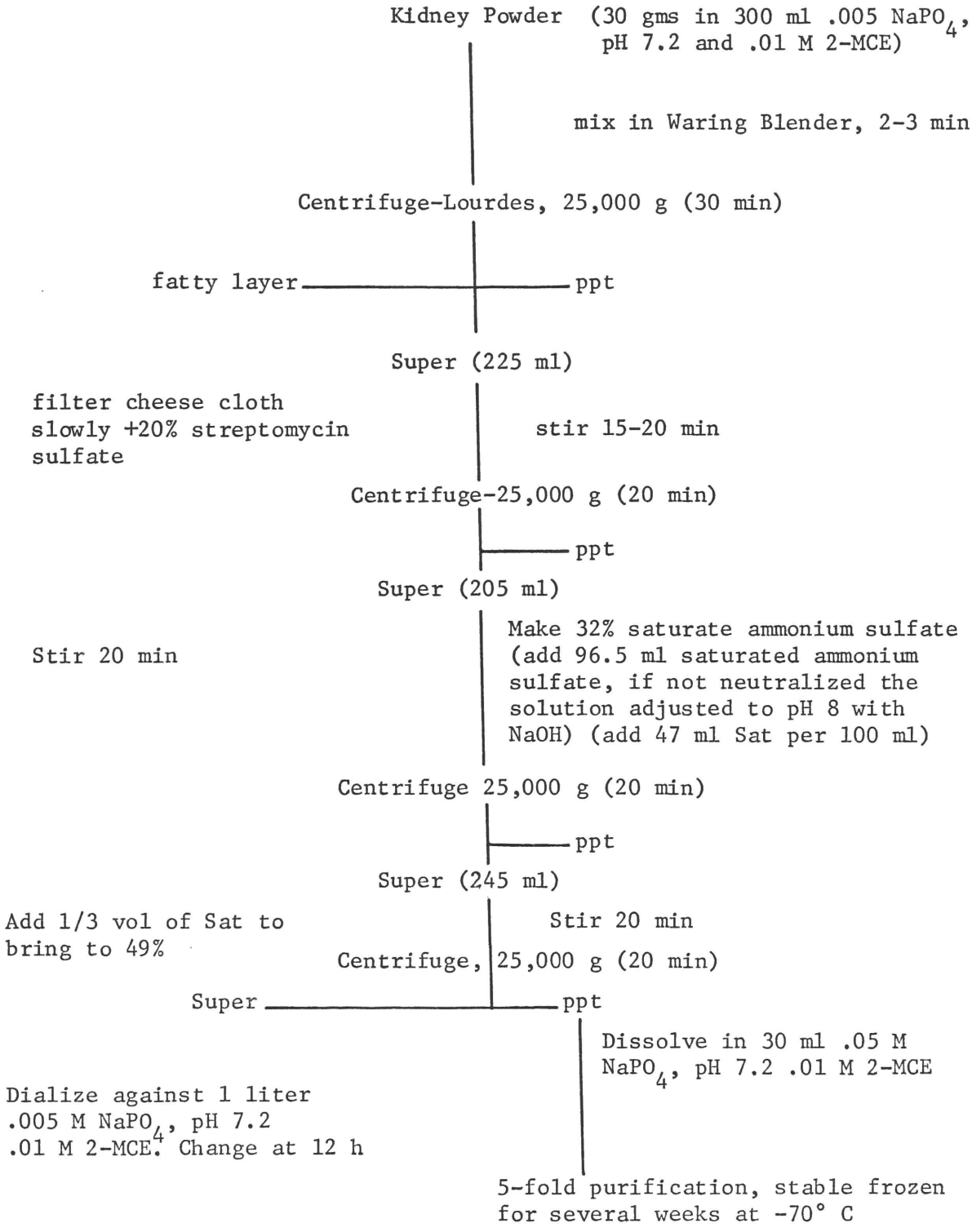

Figure 16. Decarboxylase for TP-OH assay. Extracts of acetone-dried hog or guinea pig kidney powder (devoid of oxidative enzymes).

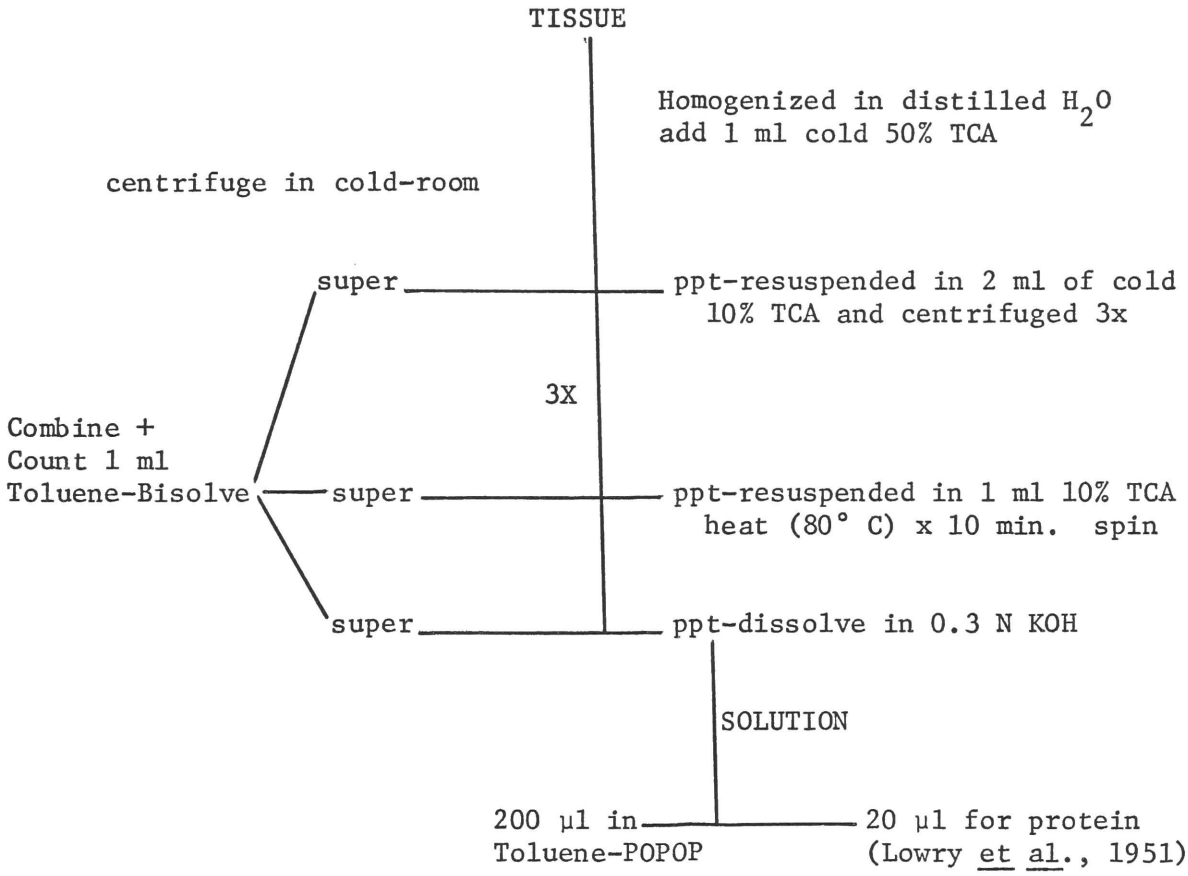

Figure 17. Flow diagram for the determination of radioactive counts in the soluble and protein fraction following an injection of radioactive amino acid.

supernatant and protein fraction.

e. Scintillation solutions and counting:

I. Bray's Solution (Bray, 1960)

180 gm Napthalene
12 gm PPO
0.6 gm POPOP
300 ml Methylalcohol
60 ml Ethyleneglycol
Para-dioxane - 3 liters

II. Toluene - POPOP - Biosolve (10% v/v)

1 gallon toluene
9
160 ml liquifluor
1 - Biosolve

III. NIMH mixture

23 gm PPO
11 - Triton x 100
21 - Toluene

These solutions vary in the amount of water which will be absorbed, Bray's solution has the highest capacity (2 ml). This mixture was used for most of the enzyme studies. The vials were placed in scintillation counter (Packard Tri Carb, model 3375) and permitted to equilibrate in the machine for at least 6 hours before counting was started. Samples were counted for 20 minutes. Counts per minute were corrected for efficiency by means of an automatic external standardization (AES). The efficiency of counting corresponding to each AEA reading was read from a standard curve prepared with a known amount of toluene-^{14}C quenched with varying quantities of 1 N NaOH. The number of pmoles of $^{14}CO_2$ collected could be calculated since the specific activity of the substrate and product were assumed to be equal. The enzyme activities expressed in most parts of this thesis will be expressed in terms of pmoles of $^{14}CO_2$ collected per hour per gram of brain tissue.

SECTION III

RESULTS

A. Properties of Enzyme Assay

Introduction

The assay developed by Ichiyama and coworkers (1968) for the measurement of TP hydroxylase from brain tissue was the most rapid and sensitive of the available methods. The enzyme assay did not require isolation of a product but depended on the collection of $^{14}CO_2$ from L-TP-1-^{14}C. The assay method was modified slightly so Warburg flasks could be used. Some of the properties of this assay system are discussed in the first part of the results section.

Results

The whole homogenate enzyme reaction rate was found to be linear with time for 20 minutes if assayed at 38° C and without reducing agent (Figure 18). The reaction at lower temperatures was linear for only 10 minutes. The reaction rate at 38° C was found to be proportional to the amount of whole homogenate brain tissue up to 200 mg (Table V). Tissue weights normally averaged 150 mg for most studies. Brain regions weighing over 200 mg (cerebellum, cortex) were trimmed until they weighed below this amount.

The concentration of the buffer was found to be important. The original assay method recommended a 1 Molar Tris-acetate buffer. However, comparison of enzyme activity in 1 Molar or 0.1 Molar revealed that at the higher concentration the buffer was inhibiting the reaction (Table VI).

The assay methods for soluble TP-hydroxylase required a reducing agent to maintain the cofactor in a reduced form. The compounds β-mercaptothanol (βME) or dithiothreitol (DTT) were commonly employed in various assay methods (Ichiyama et al., 1970; Jéquier et al., 1969; Knapp and Mandel, 1972). However, these compounds were found to be inhibiting in the enzyme reaction if the cofactor was maintained in a reduced form by a dihydropteridine reductase-reduced pyridine nucleotide system

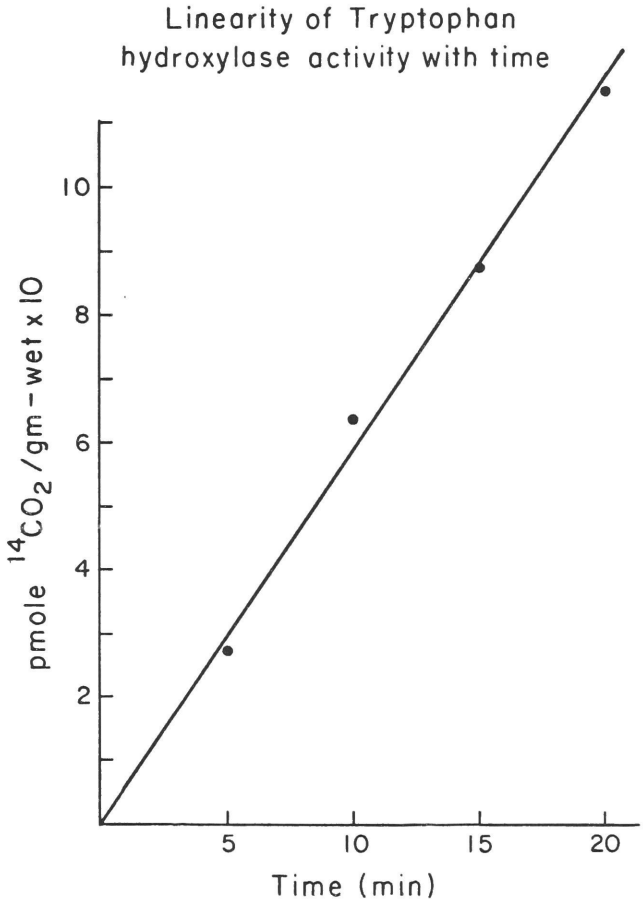

Figure 18. TP-hydroxylase activity per unit weight of whole brain homogenate at various time intervals. Reaction performed at 37° C at pH 8.1.

Table V

Effect of Tissue Concentration on THA in Whole Homogenate

Amount of tissue	No.	pmole $^{14}CO_2$/hr
50 mg	3	96 ± 24
100 mg	3	193 ± 21
200 mg	3	366 ± 25

Substrate = 5×10^{-6} M

Table VI

Effect of Concentration of Buffer on THA in Whole Homogenate

Buffer	No.	pmole $^{14}CO_2$/gm/hr
1M Tris Acetate pH 8.1	3	681 ± 54
.1M Tris Acetate pH 8.1	3	843 ± 57

Substrate = 5×10^{-6} M

(Friedman et al., 1972). The addition of βME to whole homogenate, which does not normally require a reducing agent, produced a significant inhibition of the enzyme activity (Table VII). Furthermore, the addition of pargyline, a monoamine oxidase inhibitor, had no significant effect on whole homogenate brain enzyme activity. The inclusion of $DMPH_4$, a synthetic cofactor not found in normal tissue but used in studies of the soluble enzyme, failed to have any effect on enzyme activity (Table VIII).

A crucial test for an enzyme assay is that the reaction be blocked by specific inhibitors of the enzyme. The discovery that D,L,-para-chlorophenylalanine (PCPA) selectively inhibits TP-hydroxylase allowed the specificity of the Ichiyama assay to be directly tested. The specificity of this compound was discussed in Section 1,H. The inhibition obtained is shown in Table IX and agrees with the in vitro results reported for effects of this compound on other assays for TP-hydroxylase activity (Jequier et al., 1967; Deguchi and Barchas, 1972; Koe and Weissman, 1966).

The effect of temperature on enzyme activity was measured. The activity was highest at 37° C (310° K) and decreased at lower temperatures as would be expected. Furthermore, an O_2 flush of the reaction vessel for only 10 seconds stimulated the enzyme reaction at all temperatures investigated (37° C, 25° C, and 15° C).

The amount of energy required for the reaction to occur is called the activation energy (E) of the enzyme. The E is normally independent of the temperature at which the enzyme rate is measured. To calculate this energy the logarithm of reaction rate is plotted against the reciprocal of the absolute temperature. This type of presentation of the data is called an Arrhenius plot. The E value of TP-hydroxylase appears above the lines in Figure 17 and was calculated from the following equation:

$$d(\log_{10} k) = \frac{E}{2.303R} \; d\left(\frac{1}{T}\right)$$

where k equals the reaction rate, T equals the absolute temperature, and R equals the ideal gas constant (1.987 cal/K°mole) (Figure 19).

Table VII

In vitro Effect of Reducing Agent (β-mercaptoethanol) on Whole Homogenate THA

Treatment	Conc.	pmole $^{14}CO_2$/gm/hr	% change
Control	-	2598 ± 217	
+ β-MCE	10^{-3}M	2106 ± 149*	-19
Control	-	1635 ± 65	
+ β-MCE	10^{-3}M	1305 ± 34*	-20

*P <.05; student's *t*-test

Substrate = 5 x 10^{-6} M

Table VIII

In vitro Effects of a Monoamine Oxidase (Pargyline) Inhibitor and a Cofactor ($DMPH_4$) on THA in Whole Homogenate

Treatment	Conc.	pmole $^{14}CO_2$/gm/hr
Control	-	795 ± 54
Pargyline	8×10^{-5} M	696 ± 144
Pargyline + $DMPH_4$	8×10^{-5} M + 2×10^{-3} M	675 ± 66

Substrate = 5×10^{-6} M

n = 3

Table IX

In vitro Effects of Para-Chlorophenyalanine (PCPA) on Whole Homogenate Tryptophan Hydroxylase Activity

Treatment	Conc.	No.	pmole $^{14}CO_2$/gm/hr	Relative Activity
Control	-	3	1608 ± 36	1.00
PCPA	10^{-2}M	3	237 ± 6*	.15
"	10^{-3}M	3	243 ± 4*	.15
"	10^{-4}M	3	177 ± 1.5*	.11

*P <.001, student's t-test
Substrate = 5 x 10^{-6} M

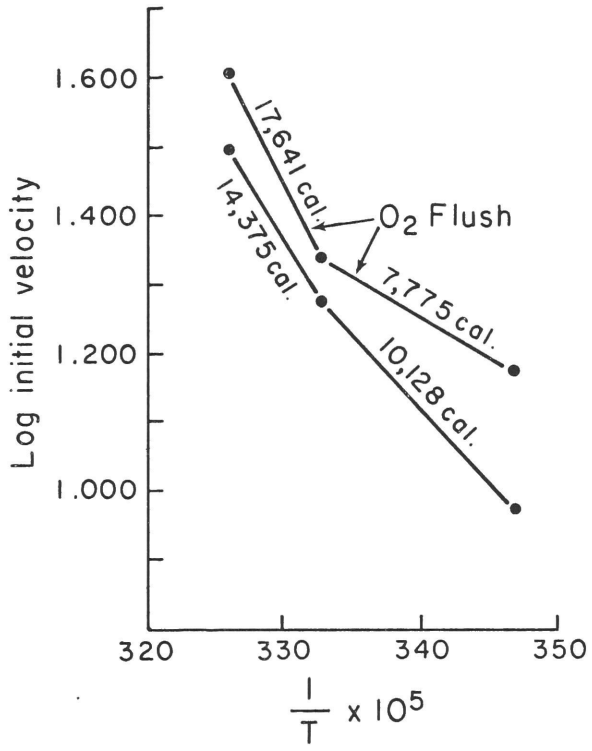

Figure 19. Arrhenius plot of TP-hydroxylase activity at 3 temperatures (37°, 25°, 15° C). The numbers above the line are the activation energy (E). The top line was obtained from tubes flushed with 100% O_2 for 10 seconds.

The discontinuity seen at 25° C was surprising since the Arrhenius plot of most enzymes gives a straight line. Four explanations for this break observed at 25° C were suggested by Dixon and Webb (1964). 1) The small change in pH due to the temperature difference might affect the enzyme molecule. 2) Since the enzyme assay used depends on TP-hydroxylase and L-AAA decarboxylase, the break may represent the activation energies of two enzymes, one being dominant at higher temperatures and the other being dominant at lower temperatures. However, the activation by O_2 observed at all temperatures appears to rule out that one of the E values represents L-AAA decarboxylase which is not stimulated by O_2. 3) The enzyme may exist in 2 forms of differing activities. The enzyme urease (3.5.1.5, Sizer, 1943) has an activation energy of 8,700 cal in the reduced form and 11,700 cal in the oxidized form. In a mildly reducing medium a discontinuity was observed to occur at 22° C. The E values for TP-hydroxylase changed in the opposite direction. However, this mechanism may nevertheless be relevant since evidence that TP-hydroxylase exists in a reduced and oxidized form has been presented (Friedman et al., 1972). 4) Finally, the enzyme may be dissociated into subunits at higher temperatures. Fumarate hydratase (4.2.1.2, Massey, 1953) was shown to have a break around 22-32° C with higher E at the higher temperatures in an alkaline solution. This enzyme is the only other known enzyme with an upward break in the Arrhenius plot. As seen in Figure 16, TP-hydroxylase also has an upward bend. Furthermore, fumarate hydratase had an E value of 9,300 cal below 32° C (305°k) and 14,800 cal above this temperature. TP-hydroxylase had corresponding E values of 10,128 cal and 14,375 cal. Massey (1953) suggested that in the case of fumarate hydratase this break was due to a dissociation of the fumarate enzyme into subunits of smaller molecular weights as the temperature was increased. Whether TP-hydroxylase exists as a composite of smaller subunits or whether this enzyme exists as part of enzyme complex, either of which dissociates at higher temperatures cannot be determined from the available data.

Discussion

The assay system was shown to be a reliable indicator of the properties of TP-hydroxylase described by other workers using more complicated procedures. The failure of $DMPH_4$ and βMCE to stimulate the enzyme substantiates the findings of other laboratories that no cofactor or reducing agents were necessary when the enzyme was measured in whole homogenate.

In whole homogenate the reaction rate reflects the *in vivo* milieu in which the enzyme normally functions. Assay of TP-hydroxylase with this procedure revealed that the enzymes activity had a faster rate but a puzzling higher activation energy at temperatures above 25° C.

B. TP-Hydroxylase Activity, Regional Distribution and Effects of Adrenal Steroids

Introduction

The following section will describe the neuroanatomical distribution of TP-hydroxylase in the rat brain and the effect of bilateral adrenalectomy on this distribution. The pattern of TP-hydroxylase follows that of 5-HT levels in the rat brain and the level of the enzyme decreases after adrenalectomy. This decrease in TP-hydroxylase activity found in most brain regions is paralleled by decreases in the *in vivo* conversion rate of ^{3}H-L-TP to ^{3}H-5-HT. The decrease of the enzyme, TP-hydroxylase, produced in the midbrain region by adrenalectomy is partially reversed by adrenal steroid injection. The prevention of this increase due to steroid injection by a protein synthesis inhibitor, cycloheximide, is demonstrated.

Results

A positive correlation between 5-HT and TP-hydroxylase was found in the rat brain. The enzyme activity found in various brain areas is shown in Table X with the activity expressed with respect to the activity found in the midbrain. This rank ordering of TP-hydroxylase was in agreement with the rank order of the distribution of 5-HT levels, determined

Table X

Rat Brain - Regional Distribution

Tryptophan Hydroxylase Activity		5-HT Levels*
Midbrain	1.00	1.00
Hypothalamus	1.16	1.02
Caudate	.84	1.00
Medulla	.97	.72
Pons	.56	.98
Hippocampus	.32	.50
Cerebral Cortex	.21	.15
Cerebellum	.06	.17

*Taylor et al., 1972.

by Taylor et al.,(1972). These results agree with data from the cat where the neuroanatomical distributions of 5-HT levels and TP-hydroxylase activity had a similar pattern (Table III, Section I). A more detailed distribution of the enzyme in the rat was undertaken using the brain dissection scheme described in Section II, B.2. The presence of 5-HT in most areas of the mammalian brain has been reported (Andén et al., 1966) and TP-hydroxylase activity should have a similar widespread distribution throughout the brain. The results obtained are shown in Figure 20. The open columns represent the enzyme activity per unit weight in normal rats. The regions with the highest activity were the hypothalamus, septum, and midbrain while the regions with the lowest activity were the hippocampus, cortex, and cerebellum. It has been shown that if the raphé nuclei are concentrated in a midline disection of the midbrain, this area has the highest amount of enzyme activity in the brain (Deguchi et al., 1973).

The investigation of the involvement of adrenal steroid regulation began by comparing normal rats with bilaterally adrenalectomized rats. The animals were operated in the lab and maintained on .8% saline for 1-2 weeks. The distribution of the enzyme activity of adrenalectomized rats seen in the hatched bars of Figure 20 shows a different distribution from normals. The absence of the adrenal glands produced a significant decrease in enzyme activity in most brain areas studied. The largest decrease occurred in the midbrain region (-75%) where the cell bodies of the ascending raphé 5-HT neurons are concentrated. However, the hypothalamus and the hippocampus showed no change in enzyme activity. Although the reason for this is unknown, it suggests that tryptophan hydroxylase activity may be regulated by surrounding tissue in each brain area or that certain region receive independent serotonin fibers which may be regulated by separate mechanisms.

The fact that TP-hydroxylase is the rate-limiting step in serotonin biosynthesis (Section I, F) suggested that the dramatic decrease seen in in vitro enzyme activity of certain brain areas might be reflected in a comparable decrease in 5-HT synthesis rate in vivo. This prediction was tested by determining the conversion index of 5-HT after a pulse injection of ^{3}H-L-TP.

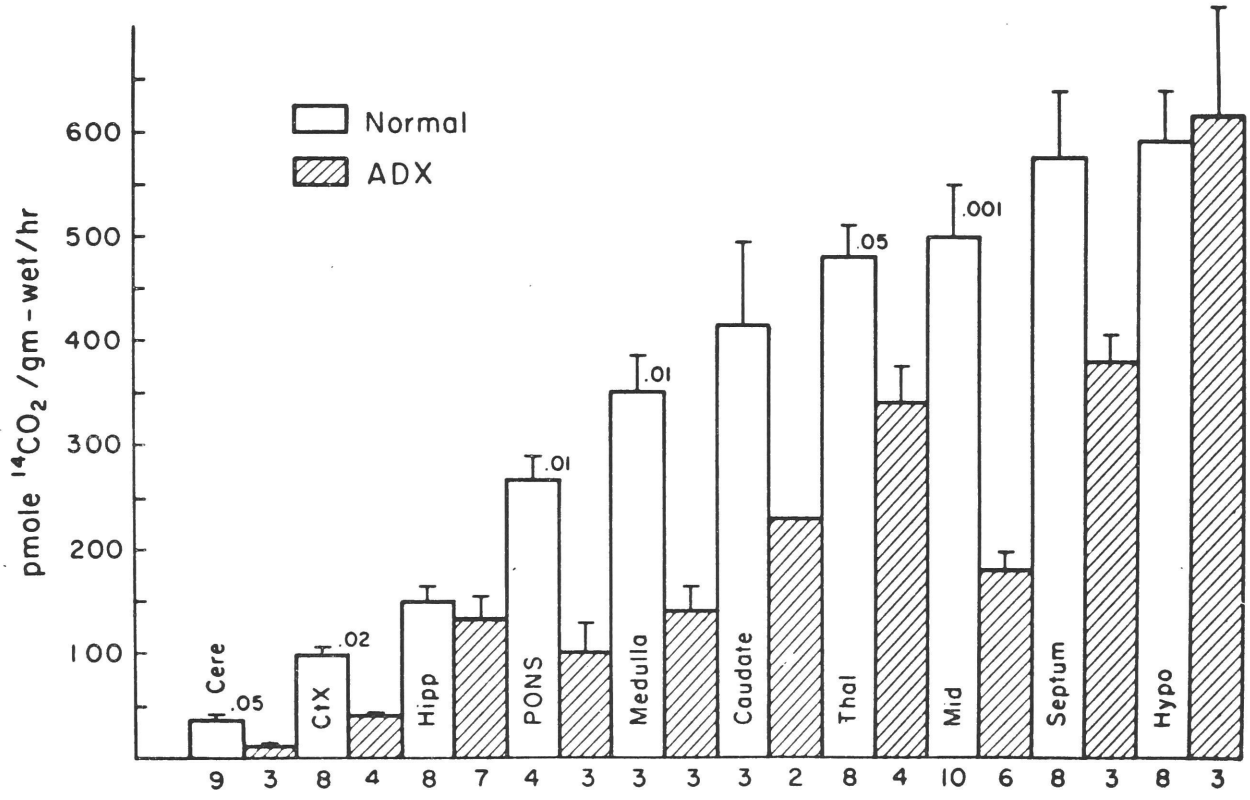

Figure 20. The regional distribution of TP-hydroxylase activity in normal and adrenalectomized rats. Cere = cerebellum, ctx = cortex, hipp = hippocampus, Thal = thalamus, Mid = midbrain, Hypo = hypothalamus. Significance determined by student's t-test with respect to adrenalectomized brain region. The number of animals is below each column. Horizontal bars represents S.E.M.

Table X lists the concentrations and specific activity of TP in plasma, brainstem, and telediencephalon of adrenalectomized and sham-operated rats 20 and 40 minutes after the injection of ^{3}H-TP. The concentration of TP of adrenalectomized rats was significantly greater than that of sham-operated rats only in the plasma of experiment 2 (Table XI) which might be due to adrenal steroid regulation of tryptophan oxygenase (Greengard, 1963).

The serotonin concentrations in brainstem and telediencephalon of sham-operated and adrenalectomized rats are comparable, which shows that 10 days after adrenalectomy the adrenal steroids are not essential for the maintenance of steady-state concentrations of brain serotonin. The serotonin specific activity was lower in adrenalectomized rats than in sham-operated rats in both forebrain and midbrain, however, this was significant only in the midbrain of animals in experiment 1 of Table XII. It has also been reported that in control rats the turnover rate of serotonin in brainstem is faster than in telediencephalon; accordingly the data reported in Table XII shows that the specific activity of serotonin in brainstem of sham-operated rats declines from 20 to 40 minutes after labeling; in contrast, that of telediencephalon is still increasing from 20 to 40 minutes. In addition, the data listed in Table XII shows that the specific activity of serotonin in brainstem of adrenalectomized rats but not that of sham-operated rats is similar at the two times studied; in contrast the specific activity of serotonin in teledicencephalon appears to increase both in sham-operated and adrenalectomized rats. This consideration indicates that in adrenalectomized rats the serotonin turnover rate is decreased in the brainstem and is unaffected in telediencephalon. These indexes are listed in Table XII and show that in the brainstem of adrenalectomized rats the conversion index is significantly lower than that of sham-operated rats. However, in telediencephalon, adrenalectomized and sham-operated rats there was a much smaller nonsignificant decrease. The failure of the telediencephalon to show a significant decrease in activity is consistent with the enzyme studies showing no change in hypothalamic or hippocampal TP-hydroxylase activity after bilateral adrenalectomy (Figure 20).

Table XI

Concentrations and Specific Activity of Tryptophan in Plasma, Brainstem, and Tele-Diencephalon of Sham-Operated and Adrenalectomized Rats Injected with ^{3}H L-Tryptophan

Condition	Plasma		Brainstem		Tele-Diencephalon	
	Plasma Level (mμmol/g) Mean ± S.E.	Specific Activity$_{4}$ (mμCi/mμmol 1x10^{-4} Mean ± S.E.	Tissue Level (mμmol/g) Mean ± S.E.	Specific Activity (mμCi/10^{-4}/mμmol) Mean ± S.E.	Tissue Level (mμmol/g) Mean ± S.E.	Specific Activity (mμCi/10^{-4}/mμmol) Mean ± S.E.
Adx (20 min)	116 ± 7.2 (9)	26.18 ± 1.21 (4)	23.60 ± 1.63 (9)**	20.20 ± 0.46 (5)	25.9 ± 1.96 (8)	20.29 ± 0.99 (4)
Sham (20 min)	101 ± 4 (10)	23.60 ± 2.18 (5)	18.15 ± 0.89 (10)	22.60 ± 2.21 (5)	21.6 ± 0.98 (10)	20.52 ± 1.78 (5)
Adx (40 min)	130 ± 10 (10)***	9.84 ± 0.41 (5)*	18.90 ± 0.68 (10)	17.36 ± 2.63 (3)	16.19 ± 0.78	13.43 ± 1.19 (10)
Sham (40 min)	89 ± 4 (10)	8.00 ± 0.38 (5)	21.00 ± 1.18 (10)	17.90 ± 2.80 (4)	17.3 ± 0.92	11.15 ± 1.23 (9)

*P <.02 when compared to sham operated rats.

**P <.01 when compared to sham-operated rats.

***P <.001 when compared to sham operated rats.

Table XII

Conversion of ^{3}H Tryptophan into 5-HT in Brainstem and Tele-Diencephalon of Sham Operated and Adrenalectomized Rats

Condition	Tissue Level ($m\mu mol/g$) Mean ± S.E.	Specific Activity ($m\mu Ci/m\mu mol\ 1x10^{-4}$) Mean ± S.E.	Conversion Index ($m\mu mole$) Mean ± S.E.	Tissue Level ($m\mu mol/g$) Mean ± S.E.	Specific Activity ($m\mu Ci/m\mu mol\ 1x10^{-4}$)	Conversion Index ($m\mu mole$) Mean ± S.E.
Adx (20 min)	3.59 ± 0.16 (10)	11.2 ± 1.01 (3)**	1.62 ± 0.28 (3)***	1.96 ± 0.16 (10)	7.04 ± 1.7 (4)	1.44 ± .22 (4)
Sham (20 min)	3.23 ± 0.089 (10)	41.3 ± 7.89 (3)	4.68 ± 0.34 (3)	1.96 ± 0.33 (9)	10.2 ± 2.2 (4)	1.92 ± 0.61 (4)
Adx (40 min)	3.01 ± 0.13 (10)	13.5 ± 1.25 (4)	1.90 ± 0.18 (3)**	1.31 ± 0.06 (15)	15.3 ± 1.78 (10)	1.01 ± 0.14 (9)
Sham (40 min)	3.38 ± 0.15 (10)	22.3 ± 5.53 (6)	3.65 ± 0.62 (6)	1.38 ± 0.14 (12)	20.0 ± 2.88 (6)	1.44 ± 0.56 (6)

*P <0.2 when compared to sham operated rats.

**P <.01 when compared to sham operated rats.

***P <.001 when compared to sham operated rats.

The data reported in Tables XI and XII show that the decrease of the conversion index in brainstem of adrenalectomized rats is neither related to a change in substrate concentration nor to a change in the serotonin concentration.

In the next series of experiments the midbrain region was chosen for more detailed studies since this region showed the largest decrease after adrenalectomy in in vitro enzyme activity and in the in vivo conversion index. To determine whether the absence of corticosteroids was responsible for this decrease, corticosterone, the principle glucocorticosteroid in the rat, was administered to adrenalectomized rats. A partial but never complete restoration of the enzyme activity in the midbrain was observed (Figure 21). The increase was dependent on the dosage and length of time after hormone injection. Seventeen hours before the rat was killed 0.4 mg of corticosterone produced a 43 per cent increase in enzyme activity compared to that in untreated adrenalectomized rats (E-bar); and a dose of 2 mg produced an increase of 71 per cent (D-bar). Repeated daily doses of 1 mg for 5 days resulted in a 107 per cent increase in activity over that in adrenalectomized (C-bar) rats.

A significant increase was detected in TP-hydroxylase in midbrain even 4 hours after a 3 mg dose of corticosterone (F-bar). To determine the specificity of this induction the action of corticosterone was compared with comparable doses of dexamethasone (synthetic steroid), desoxycorticosterone (DOC, synthetic mineralcorticoid) and combination of ACTH and corticosterone. This last group was included to ascertain if the effect of corticosterone was due to feedback suppression of ACTH levels known to be elevated after adrenalectomy. The results are shown in the left side of Figure 22. It can be observed that all 3 steroids were equally effective at the doses used and that the effect was neither antagonized nor potentiated by simultaneous injection of ACTH. The right half of the figure presents the effects of corticosterone, and ACTH on hypophysectomized rats obtained from Holtzman labs. These animals have no plasma ACTH or corticosterone although their adrenals are intact. Furthermore, the secretion of mineralocorticoids is not totally suppressed after hypophysectomy since substantial aldosterone secretion can be

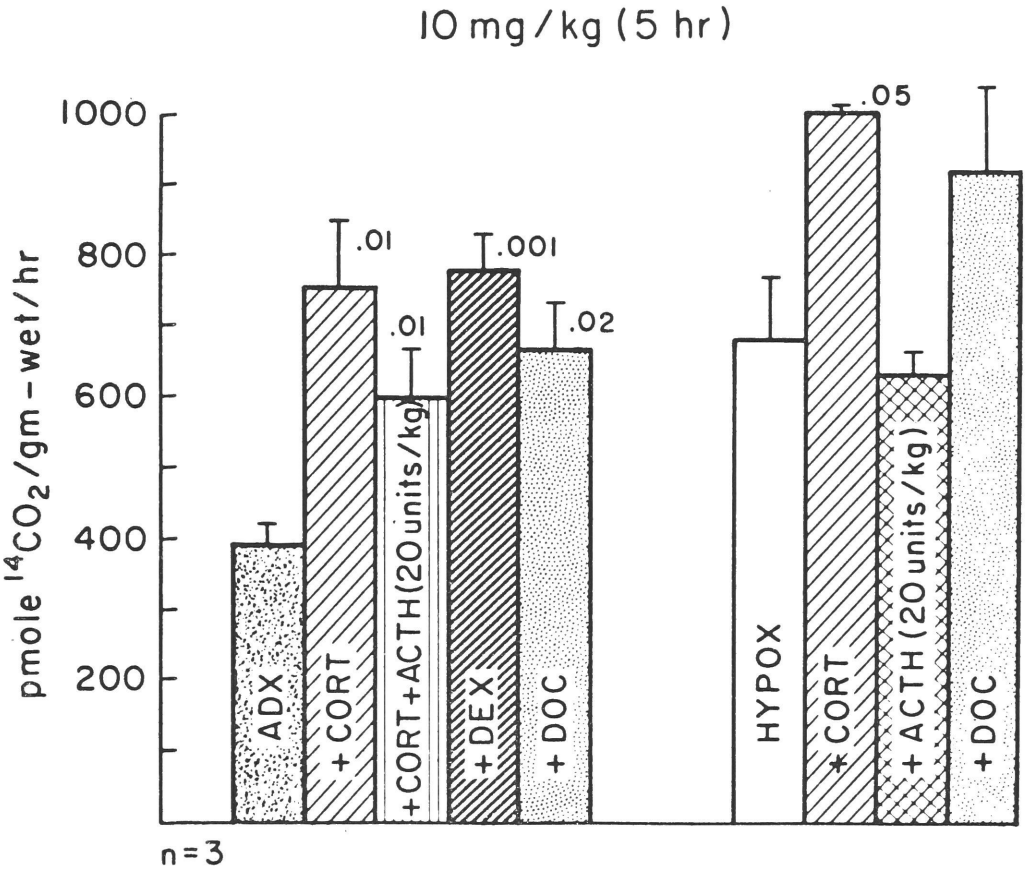

Figure 22. The effects of steroids or ACTH injection in adrenalectomized (part A) or hypophysectomized (part B) animals. Steroids were dissolved in .2 cc ethanol and given intraperitoneally. Animals sacrificed 5 hours after injection and the midbrain region assayed for TP-hydroxylase activity. Significance test was determined by student's t-test with respect to control injected group. Vertical bar represents S.E.M.

stimulated by K^+ and angiotensin II (Forsham, 1968). The results in part B show that corticosterone was more effective than DOC in Hypox rats. The failure of ACTH to increase TP-hydroxylase may appear inconsistent since the animals had their adrenals intact. However, a single injection of ACTH is not sufficient to stimulate corticosterone secretion in hypophysectomized rats (Ganong, 1971, Liddle et al., 1962).

The significant increase detected within 4 hours in midbrain enzyme activity in adrenalectomized rats after a 3 mg dose of corticosterone allowed the opportunity to determine whether de novo protein synthesis was responsible for the increase in the enzyme activity induced by corticosterone. Cycloheximide (350 µg), an inhibitor of protein synthesis in higher organisms (Korner, 1966; Wettstein et al., 1964), was administered into the cisterna magna to suppress brain protein synthesis. Control experiments, with radioactive leucine injected intraperitoneally 30 minutes after the cycloheximide injection, showed that protein synthesis in the midbrain and brainstem was inhibited by about 80 per cent. Injection of cycloheximide alone into adrenalectomized rats resulted in a decrease of 38 per cent in tryptophan hydroxylase activity in the midbrain after 4 hours (Table XIII). A similar decrease was detected in normal rats 4 hours after cycloheximide injection.

The ability of corticosterone to cause an increase in tryptophan hydroxylase activity in the midbrain of an adrenalectomized rat within 4 hours after injection was completely inhibited by the presence of 350 µg of cycloheximide. Two groups of adrenalectomized rats were injected with 3 mg of corticosterone (Table XIV). One group received at the same time an intracisternal injection of cycloheximide in saline; the other group received saline alone. Four hours later, the midbrains of the rats injected with corticosterone and saline had 175 per cent higher activities of tryptophan hydroxylase than the midbrains of the rats injected with corticosterone and cycloheximide. These results show cycloheximide prevented the induction by corticosterone of tryptophan hydroxylase, and led instead to the decrease of enzyme activity observed in adrenalectomized rats treated with cycloheximide alone.

Table XIII

Tryptophan hydroxylase activity in the midbrain of rats subjected to corticosterone treatment following 10 days of adrenalectomy. *P <.01, †P <.05 students t-test, compared to uninjected adrenalectomized animals.

No.	Condition	Dose	Time	TP-Hydroxylase Activity (pmole/gm/hr)
8	Normal		-	1500 ± 220*
8	Adrenalectomy	-	-	575 ± 20
4	" "	1 mg daily	5 days	1160 ± 50*
4	" "	2 mg	17 hrs	930 ± 35*
4	" "	.4 mg	17 hrs	820 ± 110†
8	" "	3 mg	4 hrs	.845 ± 25*

Table XIV

Prevention of corticosterone effect on tryptophan hydroxylase by cycloheximide. The tryptophan hydroxylase activity in midbrain is expressed as counts per minute of labeled CO_2 evolved per gram of tissue per 1-hr incubation period (mean ± S.E.M.).[2] The statistical significance was determined by means of student's t-test. Differences between enzyme activities in A, B, and C are due to the fact that these experiments were run at different times with different batches of isotope. Normalization of the activities to those in adrenalectomized animals permits a comparison of the three experiments. Abbreviations are: ADX, adrenalectomy; cort., corticosterone; and cyclo., cycloheximide.

Treatment of ADX	Animals (No.)	Activity	Normalized to ADX
		Part A	
None	4	958 ± 12 P >.005	1.00
3 mg cort.	4	1,408 ± 6	1.47
		Part B	
None	8	314 ± 19 P >.05	1.00
Cyclo.	10	337 ± 22	.74
		Part C	
3 mg cort.	4	1,063 ± 10 P >.005	1.47
3 mg cort. + cyclo.	4	386 ± 8	.54

Discussion

The neuroanatomical distribution of TP-hydroxylase agrees with the neuroanatomical distribution of 5-HT in the rat (Taylor et al., 1972) and compares well with the results presented in Section I.E for the cat brain (Peters et al., 1968). Data for the hamster was similar with the exception of the hypochalamus (Ichiyama et al., 1968) which showed a relatively low enzyme activity level.

The decrease in enzyme activity following bilateral adrenalectomy on the tryptophan hydroxylase is supported by a similar change in in vivo conversion index of ^{3}H-L-TP into 5-HT in brainstem region. Earlier reports on the effects of adrenalectomy on 5-HT brain levels are contradictory. Various reports showed that adrenalectomy caused a decrease (DeMaio, 1959) increase (Put and Meduski, 1962; Pleifer et al., 1963), or no change (Garattini et al., 1961) in whole brain 5-HT content. The in vivo study of 5-HT formation presented in this section demonstrated that despite unchanged levels of brainstem 5-HT the CI was reduced by 75% in adrenalectomized compared to normal animals. This finding of altered turnover rate of serotonin and unchanged steady-state concentration of brain serotonin is consistent with other reports. Injection of morphine (Way et al., 1968), inhalation of 100% oxygen, ether anesthesia (Diaz et al., 1968) and LSD injection (Lin et al., 1969) produced no change in 5-HT levels, but large changes in turnover. These data support the concept that in the 5-HT system of the brain the synthesis rate and degradative rate of the amine are balanced with each other. Therefore an increase in synthesis would result in an increase in utilization or an increase in utilization would result in an increase in synthesis with brain levels remaining constant. The possibility that utilization might regulate synthesis exists despite the lack of feedback inhibition of 5-HT on tryptophan hydroxylase. One mechanism for such a control could be the generation of reduced cofactors from NAD^{+} necessary for the hydroxylation reaction, and provided by the aldehyde dehydrogenase reaction shown in Figure 2.

The studies done with hormone replacement demonstrated that several adrenal and synthetic steroids partially reversed the effects of adrenalectomy on midbrain TP-hydroxylase. The results showed little

specificity among the different steroids at the high dose used in these experiments. The effect obtained with corticosterone on TP-hydroxylase activity within 4 hours was blocked by the simultaneous injection of the protein synthesis inhibitor, cycloheximide. This suggested that the increase in enzyme activity in the midbrain might be dependent on de novo protein synthesis.

C. Effects of Cold Stress, Morphine and Corticosterone on Enzyme Activity

Introduction

In the previous section it was shown that adrenal corticosteroids are important for maintaining normal levels of TP-hydroxylase activity. The activity of this enzyme in the midbrain of the rat decreased after bilateral adrenalectomy and could be partially restored by administration of corticosterone to adrenalectomized rats. In the present section the increase in midbrain TP-hydroxylase activity in normal rats produced by the elevation of endogenous, circulating corticosterone will be presented. The elevation in plasma corticosterone levels was accomplished by stressing the animals and by taking advantage of the natural diurnal rhythm in adrenal corticosterone secretion. The effect of morphine was also tested since this drug has been shown to increase 5-HT turnover (Way et al., 1968; Shen et al., 1970; Hitzemann et al., 1972) and is a potent stimulator of the pituitary-adrenal axis in unanesthetized animals (George and Way, 1955; Lotti et al., 1969).

Results

To determine the effect of stress-induced secretion of adrenal steroids on TP-hydroxylase activity, normal rats were given electric foot shock (EFS) or ether stress. The graphic summary of the effects of chronic stressors on TP-hydroxylase activity in the midbrain and on corticosterone plasma levels, compared to unhandled controls, is shown in Figure 23. A significant increase in TP-hydroxylase activity occurred after EFS (20.5%) and after ether stress (20.0%) (Figure 23A). Plasma

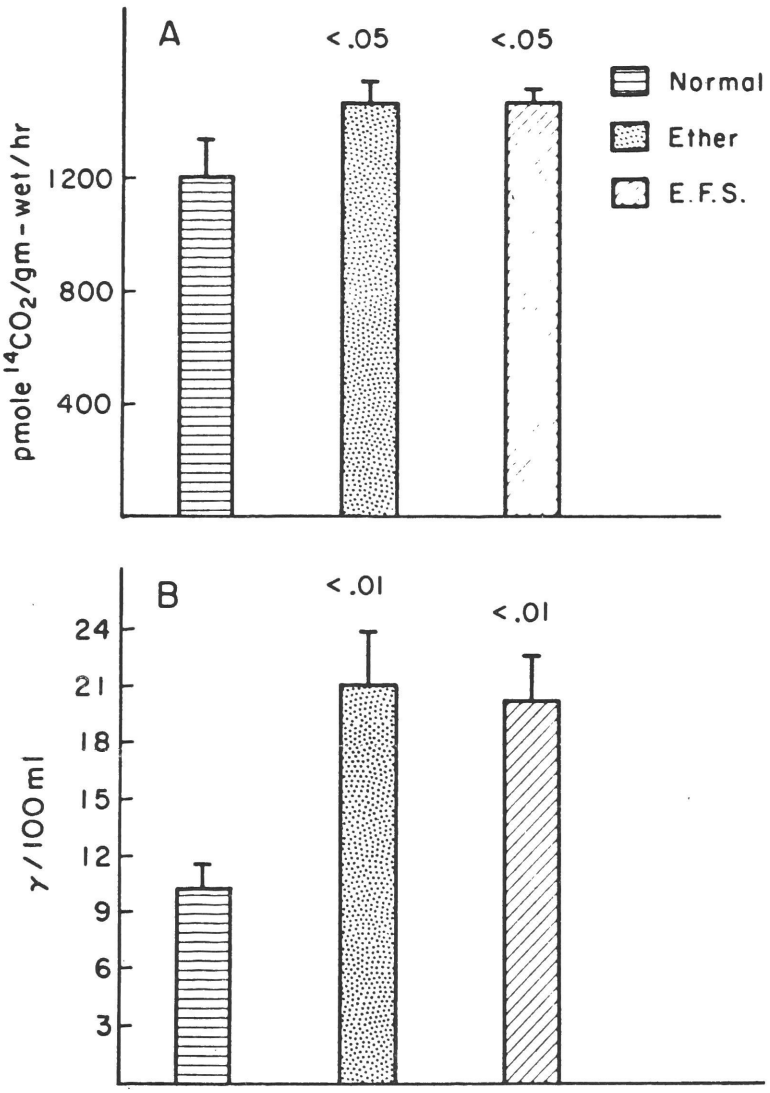

Figure 23. Tryptophan hydroxylase activity in the midbrain of repeatedly stressed rats. The enzyme activity is expressed as counts/min of labeled $^{14}CO_2$ evolved per gram of tissue per 60 min incubation period. Ether or electric foot shock (EFS) was given 3 times a day for 2 days and once on the third day 2 hr before sacrifice. Number of animals in each group was 4. Horizontal bars represents S.E.M. B, Plasma corticosterone levels for animals in part A expressed as μg/100 ml of blood plasma. Significance determined by student's t-test with respect to normal animals. Number of animals in each group was 4. Horizontal bars represents S.E.M.

corticosterone levels were also significantly increased, by approximately 100% in both experimental groups at the time of sacrifice, i.e. 2 hr after the application of the final stress (Figure 23B).

The hormonal response to cold stress is both intense and prolonged. The effects of cold stress on midbrain TP-hydroxylase activity and plasma corticosterone are shown in Table XV and Figure 24. Table XV summarizes data from 6 experiments and Figure 24 presents the average per cent change at each interval of cold stress. The corticosterone level was highest after 5 hr of exposure. There was a smaller, but still significant increase over normal levels after 8 hr of cold exposure, but no significant elevation after 48 hr of cold stress. This pattern of corticosterone response to cold is similar to that reported by Maickel et al. (1961). Increases in the midbrain TP-hydroxylase activity reflected the increases in corticosterone levels (Table XV and Figure 24). There was a statistically significant 70% increase after 5 hr and a significant 25% increase after 8 hr over control levels, but a 12% decrease after 48 hr which was statistically significant in one of two experiments. The TP-hydroxylase activity changes at 5 and 8 hours in rat midbrain are consistent with a report by Gal et al. (1968), who, using a different assay procedure, showed that 8 hr of cold stress increases whole brain TP-hydroxylase activity by 40%.

As a further test of a correlation between adrenal steroid levels and TP-hydroxylase activity in normal rats, the daily fluctuation of plasma corticosterone and midbrain TP-hydroxylase activity was measured. The level of corticosterone in the rat is low in the morning and high in the evening (Critchlow, 1963). The rats were sacrificed either at 8:00 a.m. or 6:00 p.m. The results in Figure 25 show a 400% increase in resting corticosterone level from morning to evening and a smaller 38% increase in TP-hydroxylase activity in the same time interval.

The enzyme assay employed to measure these changes involves the decarboxylation of 5-hydroxytryptophan (5-HTP) after the initial hydroxylation of 1-^{14}C-tryptophan. It is conceivable that a large increase in the decarboxylase after stress might cause direct release of $^{14}CO_2$ from

Table XV

Changes in Midbrain Tryptophan Hydroxylase (TH) Activity and Plasma Corticosteroid Levels during Prolonged Cold Stress

Exp.	No. of Animals	Treatment	TH activity pmole $^{-14}CO_2$/gm/20"	Plasma corticosterone (µg/100 ml)
1	4	Control	451 ± 20	12.2 ± 3.0
	4	5 hr cold	761 ± 48†	27.5 ± 5.1†
2	3	Control	487 ± 101	--
	3	5 hr cold	893 ± 107*	--
3	4	Control	558 ± 9	14.2 ± 3.0
	3	8 hr cold	706 ± 26‡	21.3 ± 4.2*
4	4	Control	466 ± 57	--
	4	8 hr cold	583 ± 72*	--
5	5	Control	434 ± 55	10.2 ± 1.0
	5	48 hr cold	396 ± 30	12.1 ± 3.4
6	4	Control	564 ± 26	--
	4	48 hr cold	476 ± 19*	

*P <0.05, student's t-test.

†P <0.01, student's t-test.

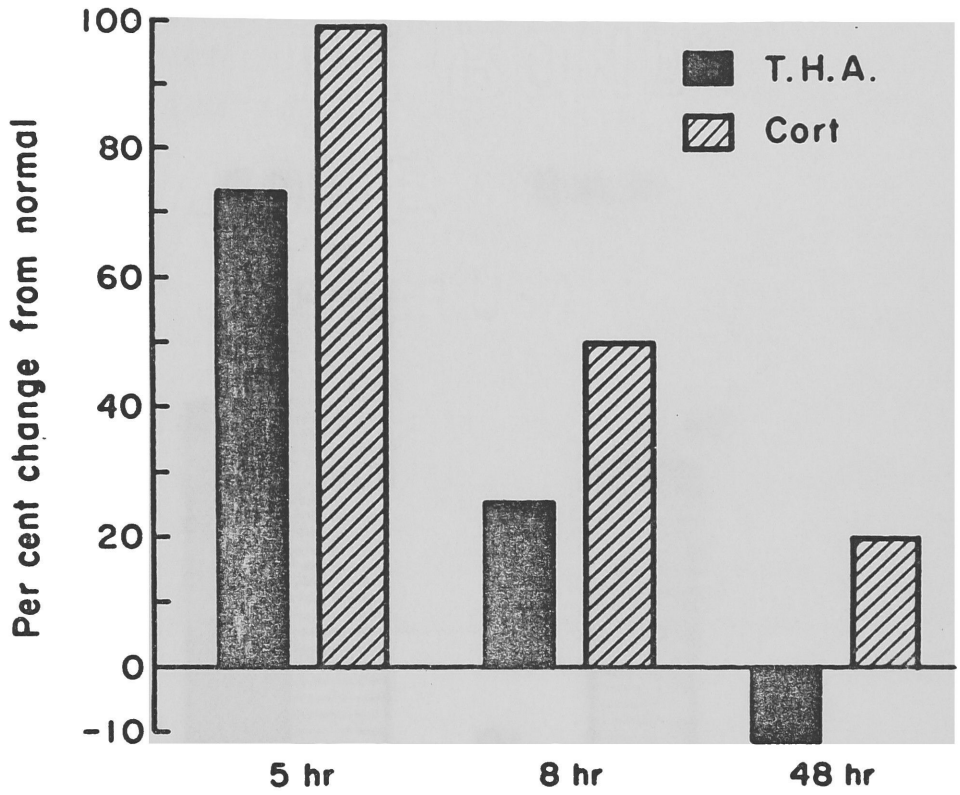

Figure 24. Tryptophan hydroxylase activity and corticosterone plasma level changes during cold stress. Levels expressed as the average per cent change from normal, based on data presented in Table XIII.

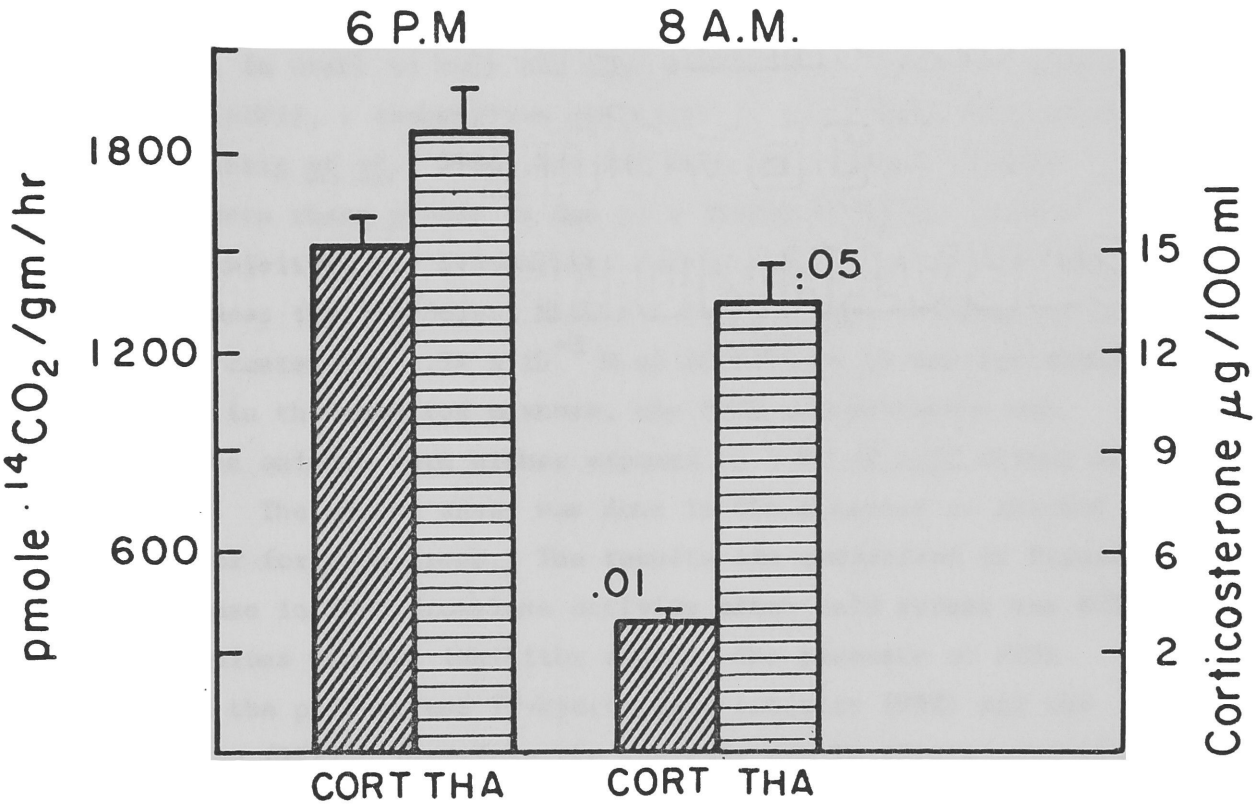

Figure 25. Diurnal changes in tryptophan hydroxylase activity and plasma corticosterone levels. Significance test was determined by student's t-test with respect to PM group. Horizontal bar represents S.E.M.

tryptophan. This possibility is remote since the tryptophan concentration in the reaction mixture (approximately 5×10^{-6} M) is several orders of magnitude lower than the K_m of the decarboxylase enzyme for tryptophan (2×10^{-3} M) (Lovenberg et al., 1962; Ichiyama et al., 1970). The K_m is an approximation of the substrate concentration necessary to saturate one-half of the active sites of the enzyme. In contrast, the K_m of the decarboxylase for 5-HTP (2×10^{-5} M) is much lower than that for tryptophan and therefore 5-HTP would be preferentially decarboxylated in this system.

However, in order to rule out this possibility directly, parachlorophenylalanine (PCPA), a competitive inhibitor of tryptophan hydroxylase was used (Lovenberg et al., 1968; Koe and Weissman, 1966). If the difference between these groups is due to a change in decarboxylase enzyme, then inhibiting the hydroxylase enzyme should not abolish the difference between the 2 groups. Midbrain samples were homogenized in 0.32 M sucrose containing 1.54×10^{-3} M of DL-PCPA or in sucrose alone. After dilution in the reaction mixture, the PCPA concentration was 1×10^{-3} M. The animals were either exposed to 5 hr of cold stress or left unhandled. The enzyme assay was done in the presence or absence of the inhibitor for each group. The results are summarized in Figure 26. The increase in TP-hydroxylase activity after cold stress was 83% over control values without inhibitor added. The presence of PCPA abolished both the post-stress TP-hydroxylase activity (99%) and the control activity (95%). Therefore the increase due to stress most likely reflects tryptophan hydroxylase activity.

If the changes in the midbrain TP-hydroxylase after stress and during the diurnal rhythm are attributable to adrenal secretion, then bilateral adrenalectomy would be expected to prevent them from occurring. Rats, adrenalectomized 5-14 days before the experiment, were given repeated EFS or 5 hr cold stress. All animals appeared in good health after the stress sessions. Plasma corticosterone determination at sacrifice showed all animals to have been completely adrenalectomized. The midbrain TP-hydroxylase activity of these animals is summarized in Figure 27. The

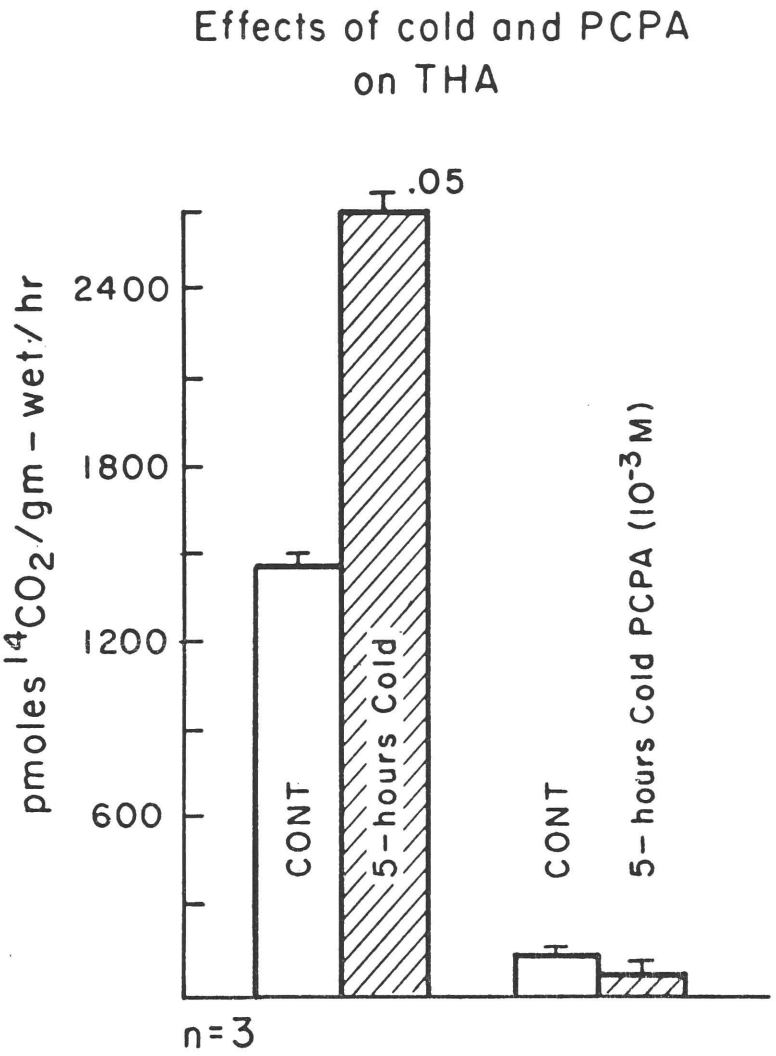

Figure 26. Effects of P-chlorophenylalanine (PCPA) on the increase of tryptophan hydroxylase (TH) activity induced by cold stress. Significance test was determined by student's t-test with respect to control group. Horizontal bar represents S.E.M.

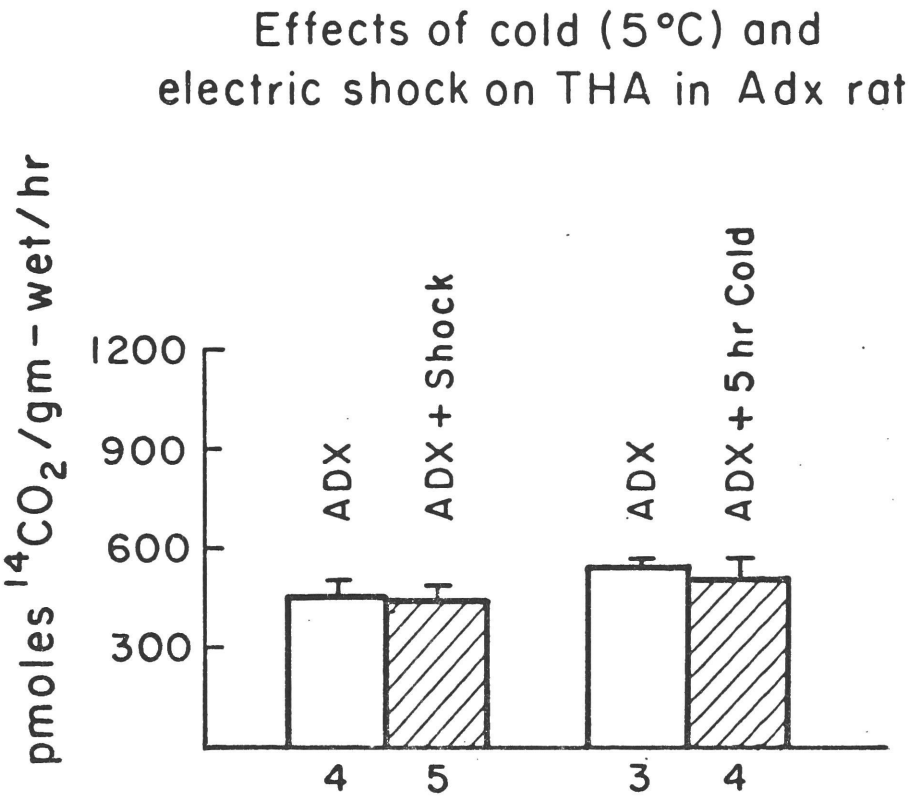

Figure 27. Effects of cold (5° C) and electric shock on THA in Adx rats.

enzyme activity of the adrenalectomized rats failed to show any change after stress, although, as noted previously, the increase in normal rats, 20% for shock and 70% for cold stress, was significant (Figure 23 and Table XIV).

It was clear that the effects of stress on TP-hydroxylase activity were somehow mediated by adrenal secretion, either casually or permissively. In order to demonstrate directly the stimulation of TP-hydroxylase activity by corticosterone, normal rats received exogenous corticosterone in ethanol (5 ml/0.1 ml) or were given ethanol (0.1 ml) alone intraperitoneally 4 hours before sacrifice. The increase of 20% due to corticosterone administration (Table XVI) was consistent with the stimulatory effect of this steroid observed in adrenalectomized rats.

A possible complicating factor in these experiments was the tissue concentration of free L-TP. Large fluctuations in tissue levels could influence the specific activity (SA) of L-TP in the reaction mixture and produce differences in $^{14}CO_2$ released even though the enzyme activity was not significantly changed. The concentration of radioactive exogenous substrate in these studies was 5×10^{-6} M and the amount of endogenous substrate in the reaction mixture was approximately 2×10^{-6} M. Therefore, a decrease of endogenous L-TP to zero levels in stressed animals would increase the $^{14}CO_2$ liberated by 29%. To study this problem the following experiments were done.

First, direct measurements of plasma, and brain levels of L-TP and brain levels of 5-HT were performed in control and cold stressed animals. The results are shown in Figure 28 and revealed small changes in free plasma L-TP, and in midbrain and forebrain L-TP and 5-HT levels as a result of the stress procedure. However, these changes in brain L-TP were transitory and were not sufficient in magnitude to account for the observed change in $^{14}CO_2$ liberated.

Secondly, the effects of various substrate concentrations on TP-hydroxylase activity from 5 hours cold stressed normal and adrenalectomized rats were tested. Unstressed adrenalectomized rats were chosen as controls for two reasons. 1) There was no difference between TP-

Table XVI

Effects of Corticosterone and Ethanol Injected Intraperitoneally in Midbrain of Normal Rats

Treatment	No.	pmole $^{14}CO_2$/gm/hr
Control	4	1386 ± 84
+ 5 mg cort./.1 cc ethanol	4	1488 ± 66*
+ .1 cc ethanol	4	1242 ± 60

*P <.05, compared to ethanol injected control.

Substrate concentration = 5×10^{-5} M

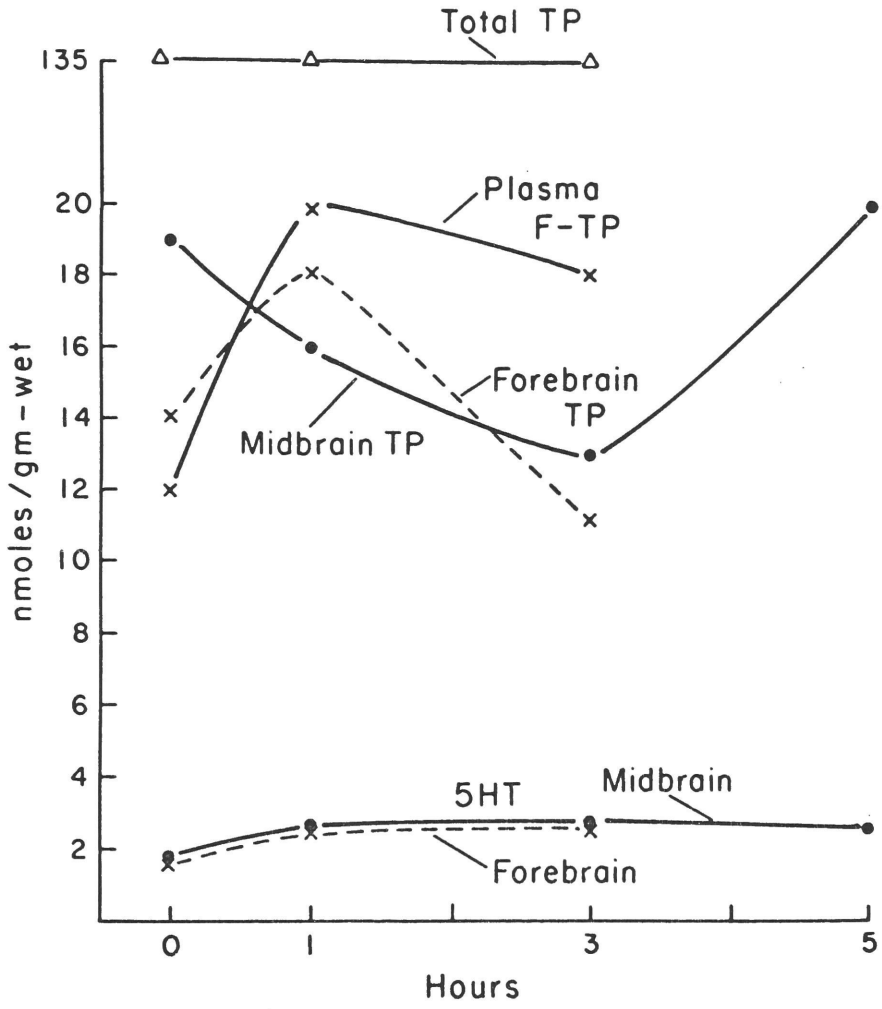

Figure 28. Changes in TP and 5-HT levels in rat after cold stress. Plasma levels of TP were determined for whole plasma (total TP) or for the free form of TP (F-TP).

hydroxylase activity of cold stressed adrenalectomized and control adrenalectomized rats (Figure 27); 2) the use of adrenalectomized rats eliminated the possibility that uncontrollable stressors might elevate adrenal steroids.

The enzyme activities of cold stressed and adrenalectomized animals were tested at 3 exogenous substrate concentrations (5×10^{-6} M, 1×10^{-5} M, and 5×10^{-5} M). The results are presented in Figure 29 in the form of a Lineweaver-Burke plot assuming an endogenous L-TP concentration of 2×10^{-6} M. It can be seen that enzyme activity from both groups had a K_m of 2×10^{-5} M which agrees with the values present in Table II (Section I, D). Furthermore, the activity for the stressed group was significantly increased at all substrate concentrations. The results showed that there was a 25% difference in the maximum reaction rate between the two groups. Finally, in the last experiment the brainstem (midbrain, pons and medulla) and forebrain (POA, septum, and hypothalamus) particulate form of the enzyme was measured in cold stressed and adrenalectomized rats. This procedure separates the bound enzyme from the cytosol fraction where much of the free L-TP would be located. The particulate form of the enzyme, like the whole homogenate, does not require exogenous cofactors or decarboxylase enzyme for optimal activity as seen in Table II (Section I, D). The results of this study are presented in Table XVII. The results again demonstrate that the enzyme activity in cold stressed animals is significantly higher (30%) than that of adrenalectomized controls. Taken together these three experiments rule out the possibility that the difference in TP-hydroxylase activity seen after stress can be attributed to large fluctuations in tissue L-TP levels.

The fact that the *in vitro* assay was not dependent on endogenous TP levels left the question of whether TP levels could regulate the enzyme activity *in vivo*. To determine if the increase in TP-hydroxylase activity was a result of the increase in plasma free L-TP level observed in Figure 28 animals were injected intraperitoneally with 100 mg/kg of L-TP. The results are shown in Table XVIII. The results clearly show that the enzyme was not stimulated by L-TP, but was in fact significantly

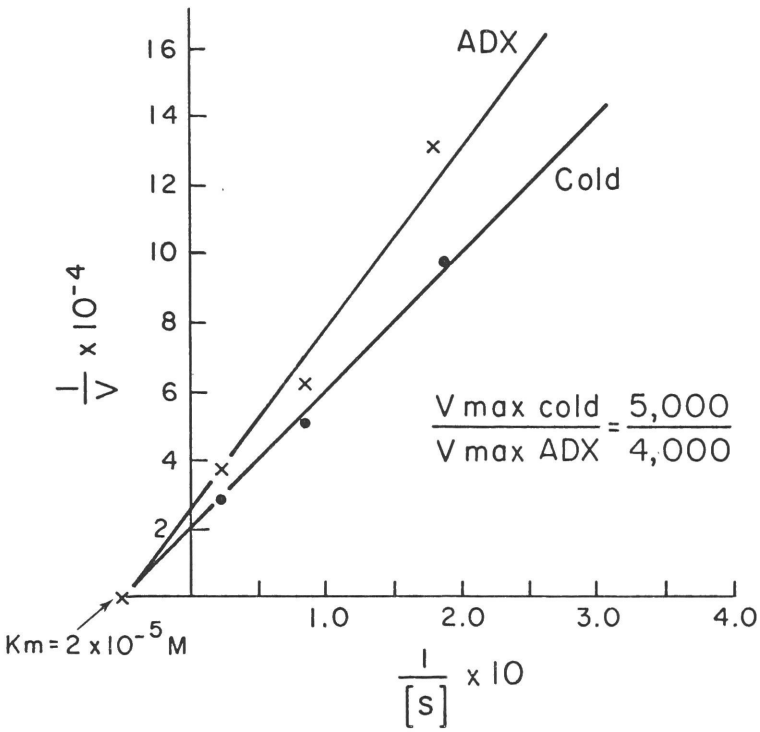

Figure 29. A Lineweaver-Burke plot of 5-h cold stressed and adrenalectomized rat brain tryptophan hydroxylase activity.

Table XVII

Comparison of Particulate Enzyme from Adrenalectomized or 5-hour Cold Stressed Normal Rats

Treatment	No.	Brainstem THA	% Change	No.	Forebrain THA	% Change
Normal + Cold Stress	(7)	1074 ± 99	31*	(4)	489 ± 15	27†
Adrenalec-tomized	(7)	819 ± 48		(4)	384 ± 15	

*P <.05, †P <.01; student's t-test

Substrate = 5×10^{-6} M

Table XVIII

Effects of Injection of L-TP (100 mg/kg) on Forebrain and Hypothalamus Particulate* TP-Hydroxylase Activity after 4 Hours

	No.	pmole $^{14}CO_2$/gm protein/h
Control	4	11,200 ± 900
Tryptophan	4	8,580 ± 500†

*Isolation procedure II

†P <.05, student's t-test

Substrate = 5×10^{-6} M

inhibited. The isolation procedure II (Figure 14, Section II, B.3) used for this study minimized any difference in cerebral L-TP tissue level. The inhibition shown was in marked contrast to the results found with hepatic TP-oxygenase which is known to be stimulated by circulating L-TP (Figure 4, Section I, B).

The last study in this section deals with the effects of morphine induced tolerance and withdrawal in rat TP-hydroxylase activity. It has been reported that changes in in vivo 5-HT turnover accompanied chronic administration of morphine (Way et al., 1968; Shen et al., 1970; Hitzemann et al., 1972) although not all workers can reproduce this effect (Cheney et al., 1971). To test if TP-hydroxylase also changed rats were treated as described in Section II. The results are presented in Figure 30. The enzyme level in tolerant animals showed a 40% increase when compared with unhandled controls and in a 17% increase when compared with the saline group. Thus tolerance to morphine is accompanied by an increase of TP-hydroxylase in the midbrain which is consistent with the reported increase of serotonin turnover. After 48 hours of withdrawal, the enzyme level has dropped from its tolerant level but still remains elevated when compared with unhandled controls ($p < .01$) though not with saline controls.

The increase in TP-hydroxylase activity found after injection of saline alone (Figure 30) suggested that some of the increase observed following morphine treatment might be related to activities of the pituitary-adrenal axis. The measurements of tyrosine hydroxylase and monoamine oxidase in the caudate nucleus of these same animals failed to show any increase after saline injection. In fact, tyrosine hydroxylase was significantly reduced by this treatment (Reis et al., 1970). These results argue for the change in TP-hydroxylase being selective and not a general effect influencing all brain enzymes.

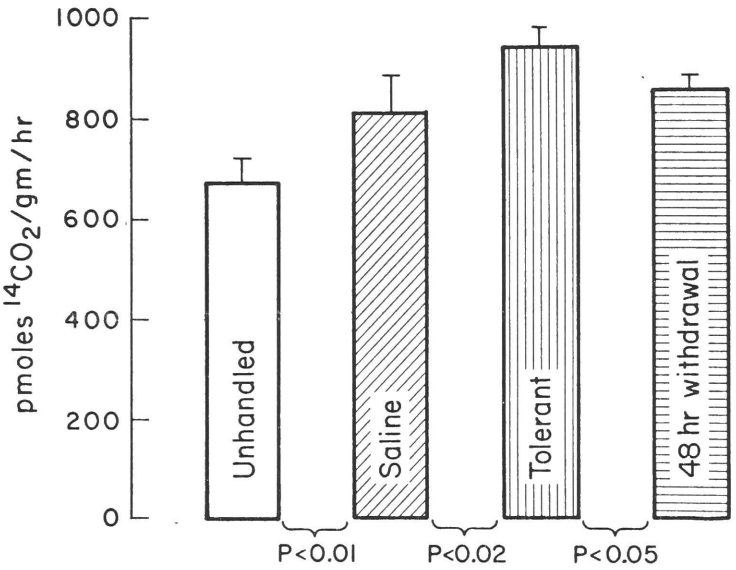

Figure 30. Effect of chronic morphine injections on TP-hydroxylase activity in female rats. The experimental procedure is described in Section II, B.2-d. Statistics determined using student's t-test on 8 determinations for each group.

Table XIX

Enzyme Activity in Caudate Nucleus

Enzyme activity in caudate nucleus during tolerance to and after 48 hr of abstinence from morphine in rat.

NS = not significant ($P < 0.05$) by student's t-test.

	Tyrosine hydroxylase (nmoles/g/20 min ± S.E.M.)	MAO (counts/min/g/20min ± S.E.M.)
Native control	0.077 ± 0.013	212 ± 15
Saline control	0.047 ± 0.013	175 ± 23
Change from naive control	($P < 0.01$)	(NS)
Tolerant	0.100 ± 0.030	205 ± 36
Change from saline control	($P < 0.05$)	(NS)
Withdrawal	0.100 ± 0.037	243 ± 18*
Change from saline control	(NS)	($P < 0.02$)

*Change from tolerant animals significant ($P < 0.01$)

Discussion

The evidence presented in this section extends earlier evidence in Section III, B showing stimulation by exogenous corticosterone of TP-hydroxylase activity in the midbrain of adrenalectomized rats. The levels of TP-hydroxylase showed a positive correlation with plasma corticosterone levels observed at sacrifice. Elevation of plasma corticosterone by EFS, exposure to cold, and ether led to increases in midbrain TP-hydroxylase activity. Plasma corticosterone levels and TP-hydroxylase activity both had a similar diurnal variation with low levels in the morning and elevated levels in the early evening. Furthermore, results showing increased TP-hydroxylase after repeated injections of saline or morphine, both of which elevate plasma corticosterone, are also consistent with a corticosterone-mediated increase of midbrain tryptophan hydroxylase levels. This is further supported by the observation that bilateral adrenalectomy prevents the increases in midbrain TP-hydroxylase activity due to EFS and cold stress, and the observation that corticosterone administration to normal rats increases TP-hydroxylase activity.

The increase, followed by a return to normal levels of TP-hydroxylase activity during prolonged cold stress, suggests that this enzyme may be turning over rapidly in the midbrain. These results indicated that the TP-hydroxylase activity decreased by nearly half from its peak at 5 hr to its value at 8 hr after the beginning of cold stress (see Figure 24). A decrease in TP-hydroxylase activity of over 25% was reported in Section III, B to occur within 4 hr after intracranial administration of cycloheximide, a protein syntehsis inhibitor. These decreases might be due to destruction of newly formed enzyme, to transport of the newly formed enzyme out of the midbrain by axonal transport to nerve endings, or to some other factor. These points will be considered further in the next section.

A correlation between enzyme activity and the *in vivo* conversion index of 5-HT after adrenalectomy was discussed in Section II, B. Preliminary comparisons can also be made with some of the results presented in this section from data available in the literature. Diethyl ether (Diaz *et al.*, 1968) and EFS (Thierry *et al.*, 1968) both led to an increase

in 5-HT turnover rate in the rat. The situation following morphine treatment appears to be unclear because of the following 2 reasons: 1) The majority of the reported results have shown an increase in turnover (Way et al., 1968; Shen et al., 1970; Hitzemann et al., 1972) but other workers failed to find any change in the turnover rate (Cheney et al., 1971). It is conceivable that this discrepancy might be due to the amount of stress the control animals were subjected to in the different experiments, the stress causing an increase of the 5-HT turnover in the control animals. 2) Measurements of soluble TP-hydroxylase activity were found not to change in morphine treated animals (Schechter et al., 1972). These workers used the synthetic cofactor $DMPH_4$ and the reducing agent β-mercaptoethanol soluble enzyme. However, when the enzyme activity was measured in the particulate fraction which does not require addition of cofactor or reducing agent, an increased activity was observed resulting from morphine treatment (Knapp and Mandel, 1972). These workers confirmed that with $DMPH_4$ and reducing agent, the soluble enzyme activity was not changed. This phenomenon suggest that the regulation may be associated with cofactor availability and will be discussed in detail in the general discussion.

It was discussed earlier that serotonin in the brain is affected by variations in the availability of TP (Section I, G). However, the availability of TP was not obviously involved in the corticosterone regulation of midbrain 5-HT levels or turnover. The studies with adrenalectomized rats showed a 75% decrease in midbrain-brainstem 5-HT conversion index in vivo without changes in tissue TP concentration or specific activity (Section III, B). Results reported in Figure 28 showed no change in midbrain L-TP after 5 hours of cold stress although enzyme activity and brain 5-HT levels were both increased. Tagliamonte et al. (1971) reported no change in whole brain and plasma TP levels after 2 hr of cold stress. Furthermore, Gal et al. (1968) found a 40% increase in whole brain TP-hydroxylase activity after cold stress in rats maintained on both normal and TP-deficient diets. In addition, there was a significant difference between TP-hydroxylase activity of cold stressed and adrenalectomized rats in the particulate fraction after removal of most of

the free tissue TP. Finally, studies on the whole homogenate showed an increase after cold stress in the V_{max} without a change in the apparent K_m.

D. Mechanism of Action--Rapid Changes

Introduction

This last section will present experiments done in an attempt to elucidate the mechanism underlying the steroid induced increase of TP-hydroxylase. The studies up to this point were consistent with the model shown in Figure 29. The fact that corticosterone was known to be taken up by brain cell nuclei (McEwen et al., 1972) and the ability of cycloheximide to prevent the steroid induced increase in TP-hydroxylase activity (Section III, B) suggested a possible involvement of the genome in the control of de novo TP-hydroxylase synthesis (Figure 29). According to the model the uptake of steroid by the midbrain cell bodies of serotonin neurons would result in an increase synthesis of new enzyme. This newly formed enzyme would subsequently be rapidly transported to the serotonin terminals in the forebrain area. This model will be referred to as the uptake-synthesis transport model (UST-model).

Some of the data to be presented in this section is not consistent with UST-model. Significant increases in TP-hydroxylase are observed in midbrain and forebrain region within 1 hour after steroid injection and significant decreases occur in the same areas within 1 hour after intracranial administration of a protein synthesis inhibitor cycloheximide. The changes were not due to direct interaction of the enzyme with corticosterone or protein synthesis inhibitor during the assay in vitro. The failure of a decrease after 1 hour of injection of puromycin suggest that the decrease after 1 hour of cycloheximide is independent of its disruption of protein synthesis since both drugs had similar inhibitory effects on in vivo amino acid incorporation into cerebral proteins.

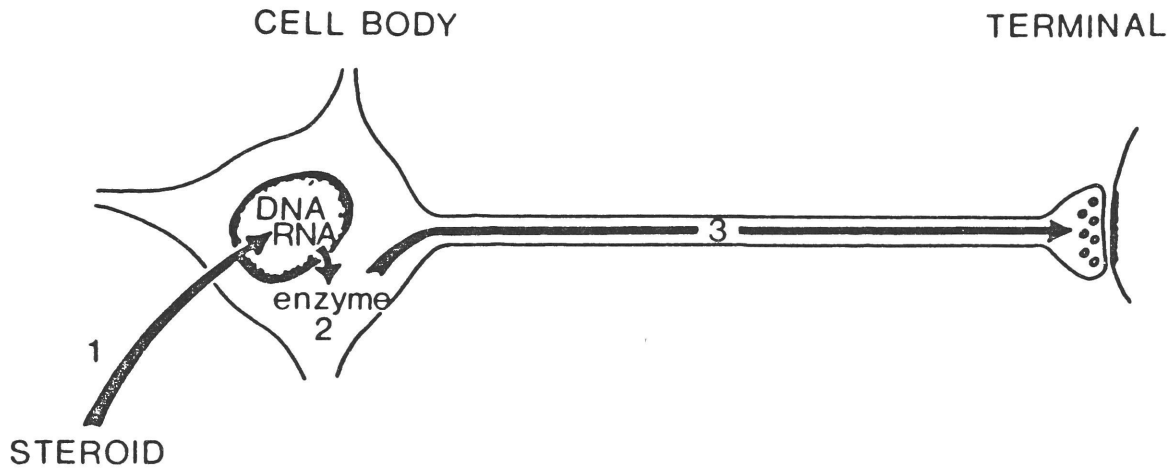

Figure 29. Uptake, Synthesis, Treensport model "UST". A 3-stage mechanism for the action of steroid on a neuron resulting in an increase synthesis of transmitter at terminal.

Results

To determine if the steroid induced increase in TP-hydroxylase occurred first in the cell body and was subsequently transported to the nerve terminals, the enzyme activity was measured after short intervals in both midbrain and forebrain regions. Figure 11 in Section II, B-1 shows schematically the regions dissected for this study. The forebrain region includes the preoptic area and the septum and contains 5-HT axons and terminals only (Section I, G). The distance between these 2 areas (2-10 mm) was thought to be sufficient to detect transport of the enzyme from midbrain to forebrain. The results obtained after 1 and 4 hours following corticosterone injection are shown in Figure 30. The results reveal a significant increase in forebrain as well as midbrain TP-hydroxylase within 1 hour. This increase is inconsistent with a model which requires transport of enzyme from midbrain region to the forebrain region (which is about 20 mm anterior) unless the transport has a rate greater than 10 mm/h (240 mm day). This rate is consistent with reported fast transport calculation (Lasek, 1970) but there is evidence that the transport rate of TP-hydroxylase in the spinal cord is 5-7 mm/day (Meek and Neff, 1972). To investigate the phenomenon of transport measurements of TP-hydroxylase activity were performed within short-time intervals of intracisternal injections of cycloheximide, a protein synthesis inhibitor. This compound was shown to decrease TP-hydroxylase activity in normal and Adx rats (Table XIV). If the rapid increase obtained after corticosterone injection in forebrain enzyme activity (Figure 30) was due to an increase in the catalytic efficiency of existing enzyme, cycloheximide would be expected to have no effect on the forebrain enzyme activity. On the other hand, if cycloheximide did decrease forebrain enzyme activity it would indicate rapid transport was possible. The results obtained after intracisternal injection of 450-750 µgms of cycloheximide are shown in Figure 31. The figure shows that both midbrain and forebrain enzyme activity are significantly decreased 1 hour following cycloheximide injection, but not at shorter intervals (20-30 minutes). This reduction found in whole homogenate was also evident in the soluble form of TP-hydroxylase in the brainstem of rats treated 1 hour before with

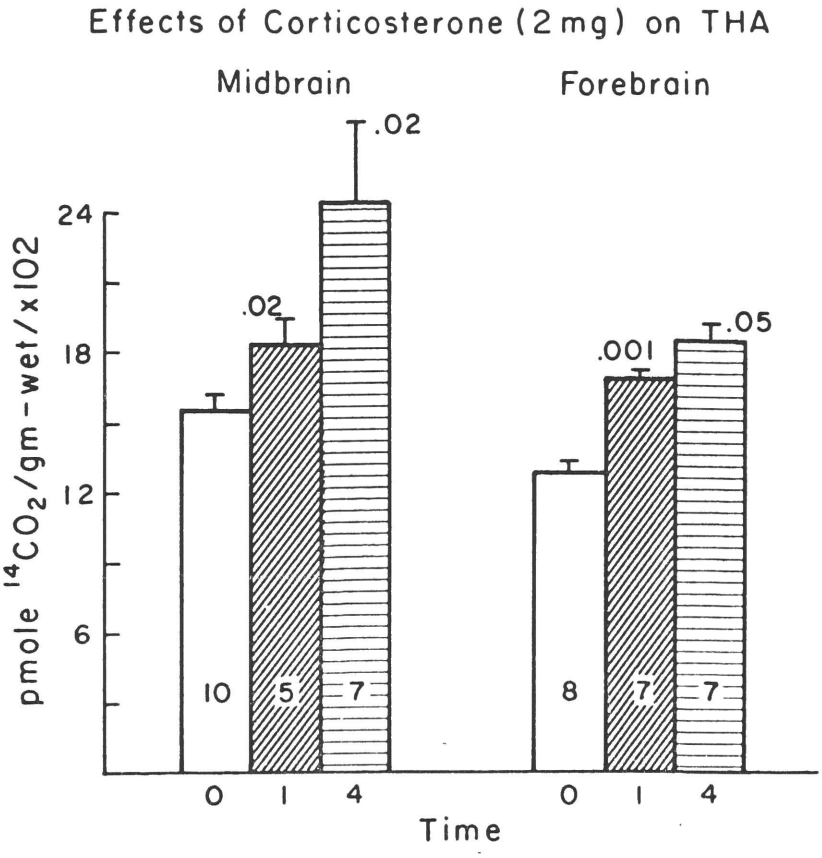

Figure 30. The effects of corticosterone hemisuccinate - Na - salt (2 mg/300 mg) injected intraperitoneally. The numbers in the columns are the animals in each group. The horizontal bars are S.E.M. Time is in hours. The statistics determined by Student's t-test with respect to control injected rats.

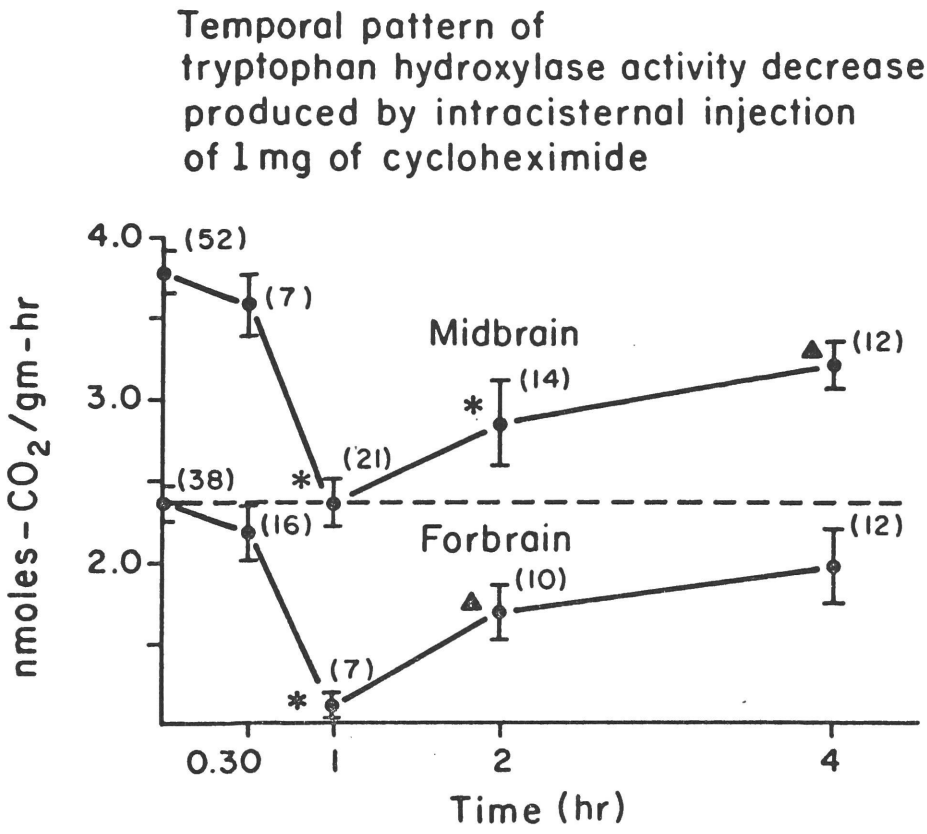

Figure 31. Effects of intracisternal injection of cycloheximide (400 µgm) in .8% saline (40 µl) on TP-hydroxylase activity in midbrain (mid) and forebrain (fore) regions. Statistics performed using Student's t-test with respect to control injected animals. At least 4 samples per point.

cycloheximide. The tissue was spun at 100,000 x g for 30 minutes and this preparation was fortified with an excess of exogenous cofactor and decarboxylase enzyme following the procedure of Ichiyama et al., 1970. The reduction was from 235 ± 14 pmole/mg-protein/hr to 183 ± 18 pmole/mg-protein/hr (N=22) and was significant at $p < .05$ using the student's t-test.

A reduction in the amount of inhibition of whole homogenate midbrain tissue TP-hydroxylase activity occurred after 4 hours post-injection compared to the inhibition after 1 hour post-injection (Figure 31). This was unexpected since, as will be shown in Table XX, the degree of inhibition of protein synthesis produced by intracisternal injection of cycloheximide was still maximal at 5 hours post-injection, and agrees with the time effects observed by other workers (Barondes and Cohen, 1967; Serota, 1971).

The failure of the enzyme activity to be reduced 20-30 minutes after cycloheximide injections argues against a direct effect of cycloheximide on TP-hydroxylase activity. Further evidence against a direct effect was obtained since the in vitro presence of corticosterone (1×10^{-6} M) and cycloheximide at 3 different concentrations failed to have any significant effect on TP-hydroxylase activity.

Table XXI shows that at levels of 10^{-3}, 10^{-4} and 10^{-5} M no decrease was observed in enzyme activity. To determine if an in vitro effect would develop over time, the brain homogenate was incubated in the presence of the steroid or inhibitor for 20 minutes at 37° C. The tubes were then oxygenated and the radioactivity added. The reaction was terminated after 10 minutes.

These studies showed that the in vivo inhibition by cycloheximide was not due to a direct interaction of this compound with the enzyme. However, the effect of cycloheximide may be independent from its action on protein synthesis. To test this possibility a second protein synthesis inhibitor was used. Puromycin inhibits protein synthesis in eukaryotic cells by a process different from that of cycloheximide (Fiale and Davis, 1965; Yarmolinsky and dela Haba, 1959), and the structure of these two compounds are quite different. These two drugs were shown to have

Table XX

In vitro Effects of Cycloheximide on Whole Homogenate Tryptophan Hydroxylase Activity

Treatment	Concentration	M	THA pmole $^{14}CO_2$/gm/hr
Control		(4)	1959 ± 120
+ Cycloheximide	10^{-5}	(8)	1923 ± 78
+ Cycloheximide	10^{-4}	(4)	2235 ± 63
+ Cycloheximide	10^{-3}	(4)	1962 ± 150

Substrate concentrations = 1×10^{-5} M

Table XXI

In vitro Effects of Corticosterone and Cycloheximide on Tryptophan Hydroxylase Activity in Whole Homogenate after 20 minutes Preincubation

	THA
Control	2091 ± 105
Corticosterone (1×10^{-6} M)	2184 ± 84
Cycloheximide (1×10^{-5} M)	2088 ± 120

Enzyme activity expressed pmole $^{14}CO_2$/gm-wet tissue/hr

Substrate concentration = 1×10^{-5} M

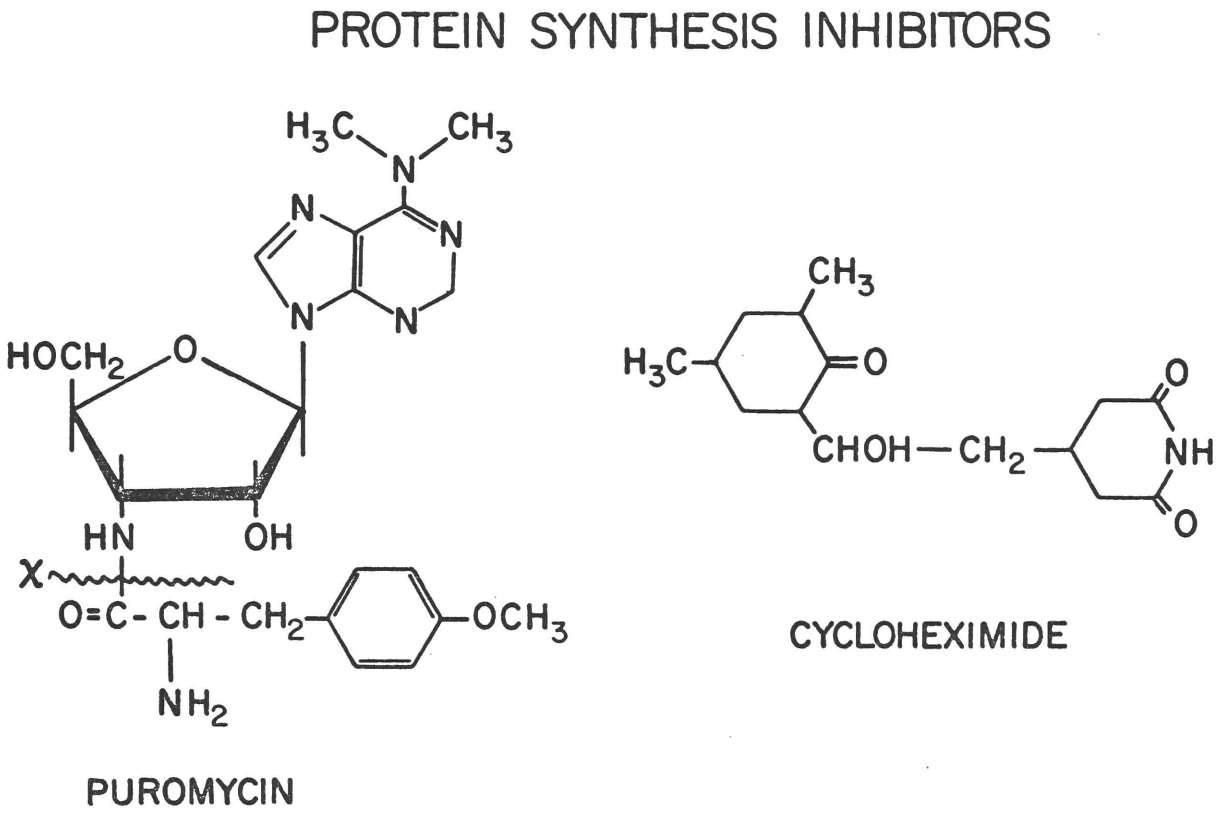

Figure 32. Structures of 2 protein synthesis inhibitors known to block mammalian *in vivo* protein synthesis.

comparable effects on brain protein synthesis. The results listed in Table XXII show that the intracisternal injection of cycloheximide (450-750 gms) or puromycin (600-1000 µgm) produced significant inhibition of *in vivo* ^{3}H-Valine incorporation into proteins in midbrain and forebrain at 1 and 4 hours. The inhibition of TP-hydroxylase activity produced by these drugs was then determined. The brain areas were assayed for TP-hydroxylase activity at 1 and 4 hours. The results are presented in Table XXIII. The table clearly shows that the 1 hour decrease of TP-hydroxylase activity produced by cycloheximide was not obtained if puromycin was injected, but at 4 hours the decrease with both compounds was similar. The decrease in the midbrain (16%) was significant for both compounds and is probably directly attributable to a decrease in enzyme amount. The injection of the aminonucleoside derivative of puromycin (Fig. 32, structure above wavy line labeled X) was without any inhibitory influence on enzyme activity.

A final experiment in this series was performed to verify that the decrease found with cycloheximide and adrenalectomy was not a consequence of an inhibition of the decarboxylase activity. The decarboxylase enzyme was purified as outlined in the methods section and 100 units (mmole/hr) added to the reaction mixture. The L-aromatic-amino acid decarboxylase cofactor, pyridoxal phosphate, was added so that its concentration was 1×10^{-4} M. The results obtained in normal, 1-hour cycloheximide, and 2-weeks of adrenalectomy are presented in Figure 33. It can be seen that the addition of decarboxylase enzyme and cofactor was without effect. This strengthens the belief that the changes detected by the TP-hydroxylase assay used was reliable and specific.

Discussion

The results presented in this section raised serious objections of the UST-model (Figure 29). The rapidity with which the enzyme activity was able to fluctuate in different brain areas argues against these changes being attributed to an increase in the amount of TP-hydroxylase present unless one assumes that the enzyme can be synthesized locally at the nerve terminals or that the enzyme is transported at a fast rate.

TABLE XXII

Effects of Intracerebral Puromycin and Cycloheximide After 1 or 5 hours on *in vivo* Incorporation of ^{3}H Valine in Brain

Brain Region	DPM/mg protein	Saline n = 6	Cycloheximide 1	Cycloheximide 2	Puromycin 1	Puromycin 2
Midbrain (1 h)	Incorporated[3]	541±35	147	132	223	158
	TCA-Soluble	1475±32	2573	2210	1395	1286
Forebrain (1 h)	Incorporated	532±49	132	110	291	205
	TCA-Soluble	1475±32	2719	2174	1399	1206
		(n = 2)				
Midbrain (5 h)	Incorporated	1222 931	560	794	514	644
	TCA-Soluble	3492 2951	4366	4885	3138	3778
Forebrain (5 h)	Incorporated	1144 874	569	794	766	773
	TCA-Soluble	3881 2893	3638	4860	2968	3666

^{3}H Valine (6 Ci/mmole)

30^{1} Incorporation Time

Table XXIII

Effect of Cycloheximide, Puromycin, and Aminonucleotide on Midbrain and Forebrain Tryptophan Hydroxylase Activity

		Midbrain		Forebrain	
		No.	pmole/gm-h + SEM	No.	pmole/gm-h + SEM
Control		38	3872 ± 117	38	2377 ± 93
Cyclo	1	21	2355 ± 160*	7	1110 ± 77*
	4	12	3268 ± 187*	12	1963 ± 228
Puro	1	7	4188 ± 180	7	2418 ± 168
	4	7	3241 ± 121*	6	2077 ± 165
Amino	1	7	4705 ± 347	7	2775 ± 262
	4	7	3837 ± 199	6	2223 ± 141

*P <.001, student's t-test

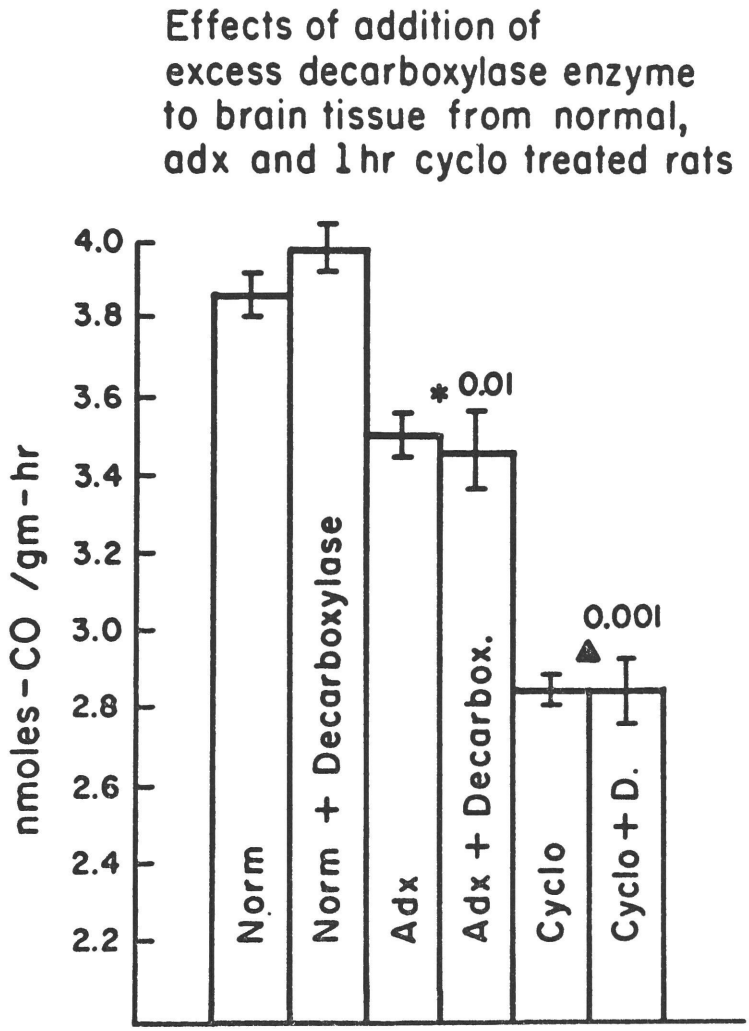

Figure 33. Effects of exogenous L-aromatic-amino-acid decarboxylase on in vitro midbrain TP-hydroxylase activity in normal, adrenalectomy and cycloheximide treated rats.

It has been shown that protein synthesis does occur in the synaptosomal fraction of nerve tissue (Austin et al., 1970; Droz and Koenig, 1970; Autilio et al., 1968; Cotman and Taylor, 1971; Gambetti et al., 1970). Studies with the electron microscope ruled out the possible contamination by bacteria or mitochondria in this fraction (Cotman and Taylor, 1971; Gambetti et al., 1970), unless the mitochondrial contents had broken open during preparation. The components necessary for protein synthesis were also shown to be present. Ribosomal type RNA associated with synaptosomal membrane fraction (Edström et al., 1962; Koenig et al., 1965) and amino acid activating system (Austin et al., 1970) were found in neuronal synaptosomal fractions. This synthesis was blocked by both puromycin and cycloheximide (Autilio et al., 1968). Nevertheless, the amount of protein synthesized here appears to be small and predominantly incorporated into membrane fractions (Autilio et al., 1968), and since TP-hydroxylase has been shown to be a soluble enzyme, this type of mechanism is not obviously relevant.

The possibility that TP-hydroxylase is rapidly transported after being synthesized in the cell body was discussed in the results section. The evidence against this mechanism was essentially that the changes occurred too quickly to be compatible with the reported transport rate in spinal cord and the half-life measurement of TP-hydroxylase in whole brain. Furthermore, if de novo synthesis was involved an induction lag would certainly increase the time required for the increased production of the enzyme in the cell body. Therefore, it can be assumed that a mechanism which requires induction and transport is not compatible with the results following cycloheximide and corticosterone injection presented in this section, but are compatible with the 4-hour results following puromycin and cycloheximide effects on midbrain and forebrain TP-hydroxylase activity.

A similar rapid decrease was recently reported for tyrosine hydroxylase in mouse and rat brain following cycloheximide and acetoxycycloheximide injection (Flexner et al., 1973). The authors concluded that the inhibition was independent of effect of these compounds on protein synthesis because of the rapid reversal of the inhibition, and was probably due to metabolism of some active-inhibitory product from cycloheximide and acetoxycycloheximide. The results presented in this section with the effects of puromycin are consistent with such a view, but do not provide any direct evidence to support this hypothesis.

The fast increase in TP-hydroxylase after steroid injection agree with the rapid large increase in forebrain 5-HT turnover (168%) found after corticosterone and ACTH injection (Millard et al., 1972). Increases in 5-HT levels have also been reported after corticosterone injection (DeMaio and Marbbrio, 1954; Kato and Valzelli, 1958) although very high doses were used and the results have not always been reproduced (McKannee et al., 1966). 5-HT turnover has been shown to be rapidly increased following stress (Thiery et al., 1968; Corrodi and Hockfelt, 1968; Goldberg and Salama, 1969; Bliss et al., 1968, 1972; Welch and Welch, 1968; Barchas and Freedman, 1963).

The rapidity of the changes referred to in this section and the results presented above have obvious physiological advantages over a system which might require many hours to respond to a stimulus. During a stress a large demand for transmitter exists and a system with axons as widespread as 5-HT neuronal system would not logically benefit directly from increased synthesis of its rate limiting enzyme in the cell body region. The regulation would be most productive if it occurred throughout the nerve and could rapidly increase or decrease the activity of the rate limiting enzyme. A possible mechanism for achieving such a type of rapid control will be presented in the general discussion.

SECTION IV

General Discussion

This thesis describes an interaction between the neurochemical 5-HT system and the pituitary-adrenal axis. Adrenal involvement in brain serotonin metabolism was first suggested by the decrease of normal levels of TP-hydroxylase in most brain areas after adrenalectomy. Three steroids--corticosterone, dexamethasone, and desoxycorticosterone--given in supra-physiological doses (10 mg/kg) partially restored the TP-hydroxylase in the midbrain area. The physiological relevance of this type of regulation by adrenal steroids was demonstrated in normal stressed animals. These rats were shown to have increased TP-hydroxylase activity following procedures which stimulated the release of corticosterone into the plasma (Figs. 20 and 21; Table XIII). This correlation between corticosterone and midbrain TP-hydroxylase was also evident in the diurnal rhythm of these two systems. The importance of the adrenal for this regulation was shown by subjecting adrenalectomized rats to the same stressful procedure which had led to changes in normal rats (Fig. 24). As expected, no increase in TP-hydroxylase activity was found in adrenalectomized rats.

The results found in *in vitro* enzyme activity should be directly relevant to *in vivo* 5-HT metabolism, because TP-hydroxylase functions as the rate-limiting enzyme in serotonin synthesis (Section I, F). In support of this the *in vivo* conversion index for metabolism of L-TP to 5-HT in brainstem was significantly reduced from normal in adrenalectomized rats (Table XI). Furthermore, numerous examples of increased 5-HT turnover following stress and steroid injection have been reported (Section III, B, C discussion). This correlation between enzyme activity and turnover, and an absence of large changes in 5-HT levels supports the concept that the serotonin system exists in a steady-state, with synthesis and utilization rates in balance with each other.

The increased enzyme activities found were initially assumed to agree with the UST model in Figure 28. The scheme depicted a mechanism whereby a neuron was activated by nuclear *uptake* of corticosterone to

produce de novo enzyme synthesis followed by axonal transport to nerve endings. The following lines of reasoning supported this mechanism: (1) midbrain nuclear uptake of radioactive corticosterone (McEwen et al., 1972), (2) increase of TP-hydroxylase activity in the cell body region after stress and steroid injection (Section II, B, C), (3) cycloheximide was able to block the increase of enzyme activity following corticosterone injection (Table XII), (4) rapid reduction of the increased enzyme activity between 5-8 hours of cold stress paralleling the decrease in plasma steroid levels which suggested transport of the enzyme away from the cell bodies (Table XIII, Figure 21). Furthermore, changes in turnover were observed to occur quite soon after steroid injection (Millard et al., 1972) or stress (Thierry et al., 1968; Corrodi and Hockfelt, 1968; Goldberg and Salama, 1969; Bliss et al., 1968, 1972; Welch and Welch, 1968; Barchas and Freedman, 1963). However, measurements using spinal transections demonstrated that the enzyme had a transport rate of 5-7 mm/day (Meek and Neff, 1972) and leision of raphé area demonstrated that the enzyme had a half-life of 48 hours (Kuhar et al., 1971). These results suggest the changes observed in turnover in forebrain areas were too fast to be attributed to an increased supply of enzyme from the cell body to the terminals.

To directly determine whether TP-hydroxylase was being rapidly synthesized in the cell bodies and transported at a rapid rate, midbrain (cell body) and forebrain (nerve endings) brain areas were assayed for TP-hydroxylase at short time intervals following steroid injection (Fig. 29). The results demonstrated a significant increase in enzyme activity in both regions within one (1) hour. A similar protocol was followed using the protein synthesis inhibitor, cycloheximide (Fig. 30). In this instance the activity was found to be strongly depressed after one (1) hour in midbrain and forebrain but a smaller depression occurring after 4 hours while the effect of the inhibitor on protein synthesis was still maximal (Table XXII). The results showed that TP-hydroxylase activity can fluctuate rapidly in midbrain and forebrain in agreement with the turnover studies mentioned above. Moreover, the changes seem to be independent of de novo enzyme synthesis since puromycin did not mimic the effects of cycloheximide.

As mentioned above, the half-life of TP-hydroxylase has been estimated to be forty-eight (48) hours (Meek and Neff, 1972; Kuhar et al., 1971). However, there is some evidence that the turnover in the midbrain may be faster. The action of PCPA is effective in depressing 5-HT brain levels for up to six (6) days (Koe and Weissman, 1960). Several groups have suggested that this may be due to the synthesis of a deformed TP-hydroxylase molecule by the incorporation of PCPA into the peptide sequence during de novo protein synthesis (Guroff, 1969; Gal and Millard, 1971). The onset of an irreversible reduction of soluble TP-hydroxylase has been reported to occur within four (4) hours after injection in the brainstem region (Lovenberg et al., 1970; Knapp and Mandel, 1972). This irreversible inhibition, if it occurs as suggested above, means the enzyme must have a rapid synthesis and transport rate in the brainstem, comparable to the changes after four (4) hours of steroid treatment, five (5) hours of cold stress, and 4 hours after cycloheximide and puromycin injection. Nevertheless, the changes observed after one (1) hour in forebrain after steroid and cycloheximide injection are not consistent with a hypothesis which requires de novo enzyme synthesis.

An alternative mechanism which more logically explains the rapid changes reported in this thesis has not been experimentally demonstrated. However, from several lines of investigations in other laboratories it appears that the reduced cofactor biopterine (BH_4) required for normal enzyme activity might be involved in this rapid regulation of TP-hydroxylase activity. Studies done with chick sympathetic ganglion in tissue cultures demonstrated a significant increase in catecholamine content, believed to be regulated by tyrosine hydroxylase, after the addition of oxidized biopterin to the medium (Côté et al., 1970). In the rat brain the concentration of BH_4 is 0.75 µg/gm (Guroff et al., 1972). In the conditions of the assay used in most studies the in vitro concentration was 2.3×10^{-7} M, approximately two hundred (200) fold below the K_m value reported for TP-hydroxylase (Friedman et al., 1972). It is surprising that under these conditions addition of the synthetic cofactor ($DMPH_4$) has no stimulating effect (Table VII). Furthermore, studies done on soluble TP-hydroxylase fortified with $DMPH_4$ failed to change after

morphine treatment (Schechter et al., 1972) or adrenalectomy (Renson, 1972; Deguchi and Barchas, 1972). The assay system used bypassed any regulatory influence which the cofactor would exert.

This reduced cofactor is required for maximum activity of the phenylalanine, tyrosine, and tryptophan hydroxylase enzymes (Hayaishi, 1968, 1969). The reaction scheme for hepatic phenylalanine hydroxylast has been well studied (Kaufman, 1969). This mechanism is shown in Figure 34. The reaction scheme shows that a second enzyme system is required to continually supply the hydroxylase enzyme with reduced BH_4. This enzyme, dihydropteridine reductase, requires NADPH to supply the hydrogen atoms to reduce BH_2. This reductase enzyme had been postulated as being rate-limiting for adrenal tyrosine hydroxylase activity (Musacchio et al., 1972), but now believed to be present in excess amounts (Craine et al., 1972).

The possible involvement of BH_4, NADPH, or pteridine reductase in the changes in TP-hydroxylase activity after adrenal steroid administration, stress, or cycloheximide injection needed to be directly tested. However, there was a precedent for a protein synthesis inhibitor sensitive NADPH-mediated response to a hormone of an hydroxylase enzyme system in the adrenal gland (Haynes et al., 1960, Haynes and Berthet, 1957; Simpson and Estabrook, 1969). A series of hydroxylation reactions change cholesterol to the various steroids, and each reaction requires molecular O_2 and NADPH. The proposed sequence of events initiated by ACTH in the adrenal cortex is shown below (Haynes et al., 1960; Krebs et al., 1966). Figure 35 shows that ACTH stimulates cyclic AMP levels which in turn increases glycolysis. The increase of glucose-6-phosphate would lead to an increase in the NADPH level, presumably by way of the pentose phosphate shunt. Interestingly, the stimulation produced by ACTH in the adrenal is blocked at the first step (desmolase enzyme) by either cycloheximide or puromycin (Ferguson, 1962, 1963; Garren et al., 1965). Figure 36 illustrates the in vivo effects of cycloheximide (20 mg/kg) on the ability of the adrenal cortex to respond to ACTH released after ether anesthesia (Azmitia, unpublished). The rapidity of the inhibition by cycloheximide and the rate of recovery from inhibition are similar to the

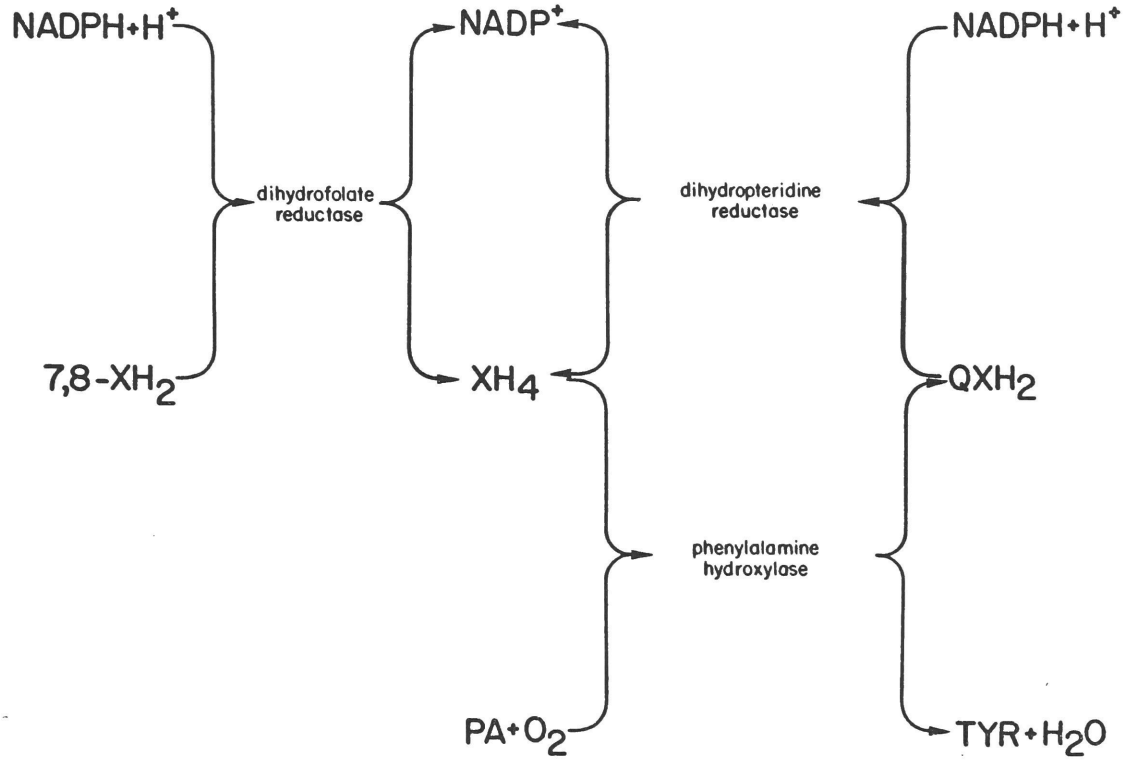

Figure 34. Reaction scheme for phenylalamine hydroxylase taken from Kaufman (1969). The reduction of Quinonid biopterine ($Q-H_2$) to reduced tetrahydro-biopterine involves a NADPH enzyme dihydro-pteridine reductase.

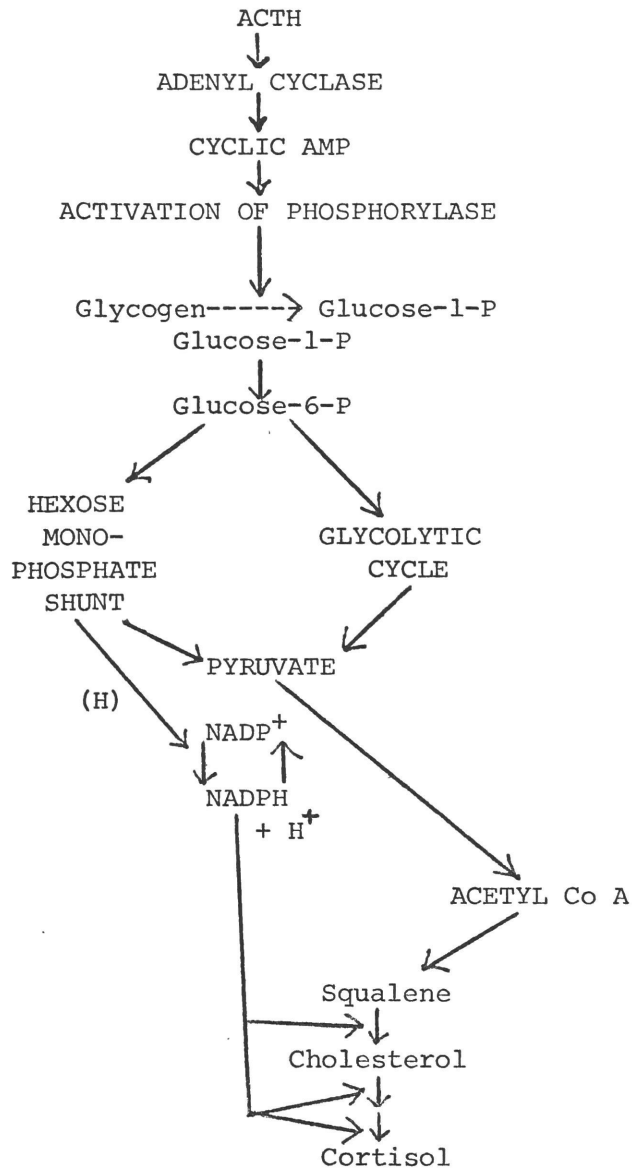

Figure 35. An outline of the main biochemical steps involved in the action of corticotropin on adrenal steroid biosynthesis (Haynes et al., 1960; Krebs et al., 1966).

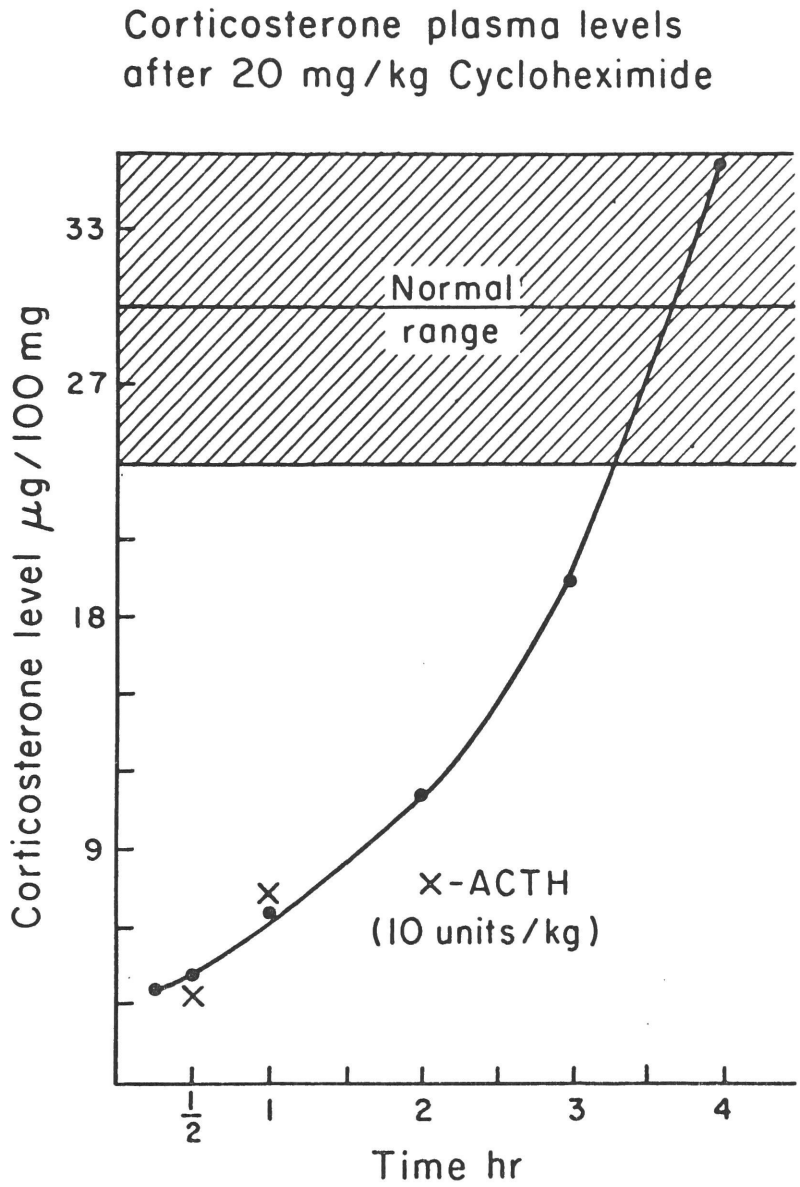

Figure 36. Plasma corticosterone levels in the rat 15 minutes after ether stress at different times (dots), following a intraperitoneal injection of cycloheximide (20 mg/kg). The X represents the corticosterone plasma level following ACTH injection (10 units/kg) to determine if the inhibition was due to an absence of circulating ACTH.

results shown in Figure 30 where the effects of cycloheximide on TP-hydroxylase in the brain are shown. The in vitro inhibition of ACTH stimulated corticosterone synthesis produced by puromycin in adrenal slices is abolished by the addition of NADPH to the medium (Ferguson, 1962). The explanation of this phenomenon remains obscure. Whether this would occur for the cerebral tryphophan and tyrosine hydroxylase, both of which are reduced after one (1) hour of cycloheximide injection needed to be tested.

The apparent similarities between the hydroxylase systems has allowed certain speculations to be made. A cofactor model for regulation of TP-hydroxylase based on the mechanisms presented in Figures 33 and 34 is shown in Figure 37. This scheme is consistent with the data presented in this thesis. The possible involvement of reduced pteridine cofactor is stressed. A final point seen in the cofactor model is that the productions of ATP and NADPH are linked, as stressed by other workers (Krebs and Veech, 1969). Therefore, the TP-hydroxylase activity may be a direct consequence of the energy state of the neuron or surrounding glia, which can rapidly fluctuate.

The hypothesis outlined in Figures 35 and 37 are directly testable. It has been shown that 6-aminonicotinamide is converted in vivo to an analog of $NADP^+$, which is a potent inhibitor of 6-phosphogluconate dehydrogenase in neural tissue (Herken et al., 1969). This compound results in the complete inhibition of the pentose phosphate shunt in rats resulting in increase 6-phosphogluconate levels by 2 hr, with a maximum increase by 6 hr (Harkonen and Kauffman, 1973). TP-hydroxylase activity in midbrain and forebrain regions and plasma corticosterone levels were measured in rats 6 hours after i.p. injection of 6-aminonicotinamide (70 mg/kg). The results are presented in Figure 38. The only significant decrease occurred in forebrain TP-hydroxylase activity. This finding is consistent with the report showing that the pentose-phosphate shunt enzymes are highly localized in brain synaptasome fraction (Sims et al., 1973). The results indicate that the hydroxylase enzyme might be controlled by different mechanism in the cell body and in the terminal of serotonin neurons.

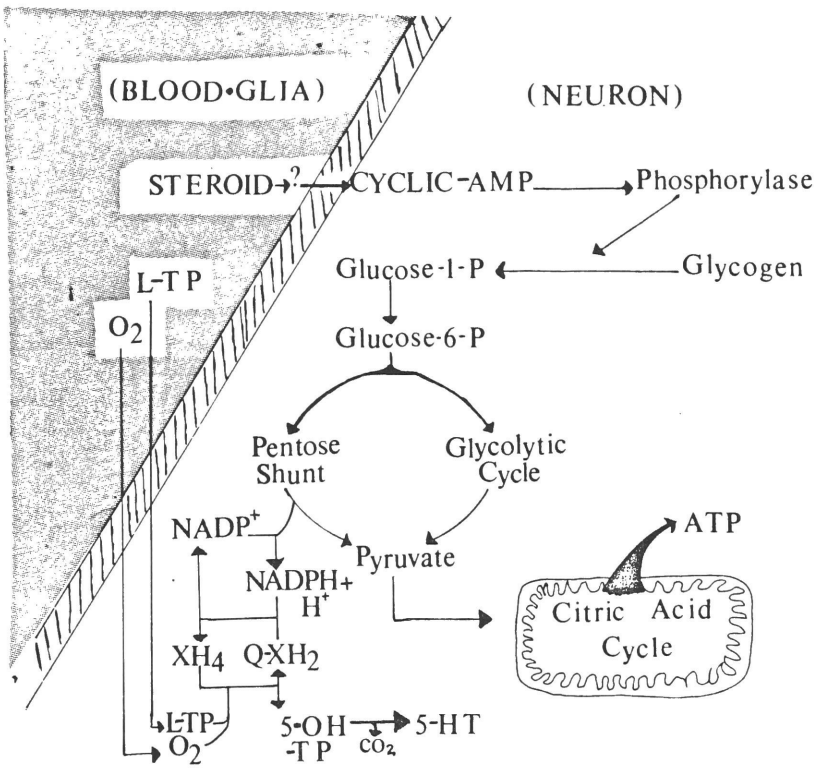

Figure 37. A scheme to explain the rapid changes in TP-hydroxylase activity through the availability of reduced NADPH generated by the pentose phosphate shunt. XH_4 = tetrahydro-biopterine, $Q\text{-}XH_2$ = quinonoid dihydro-biopterine.

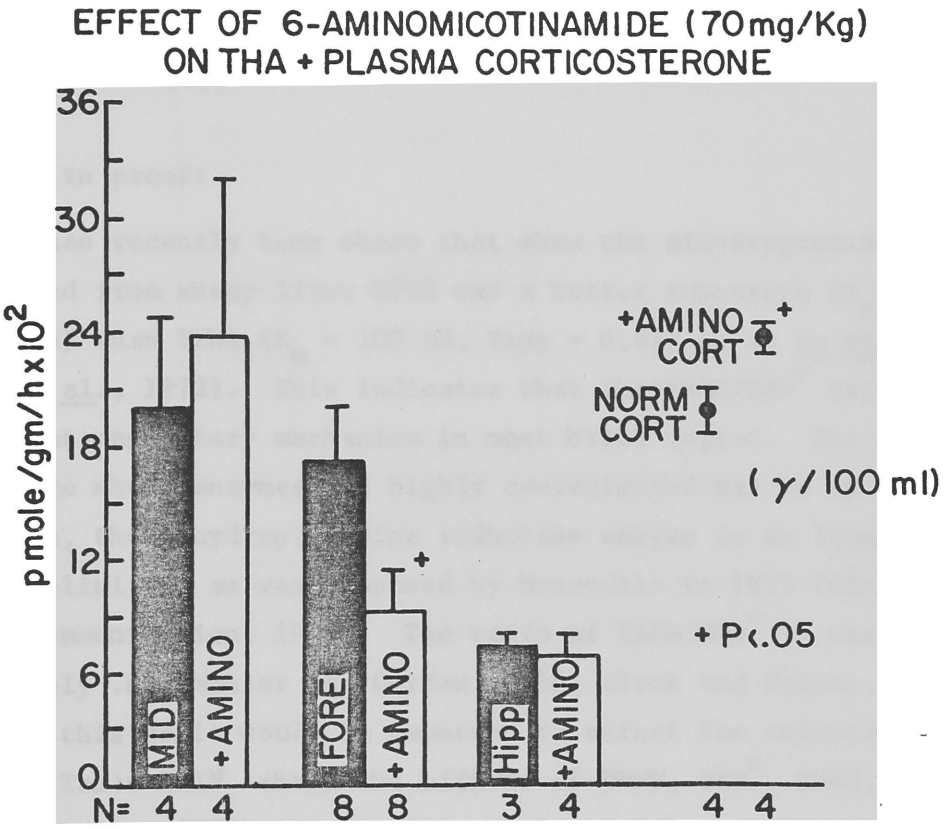

Figure 38. Effects of 6-aminonicotinamide (70 mg/kg) on brain tryptophan hydroxylase activity and plasma corticosterone level 6 hours after i.p. injection.

The significant increase in plasma corticosterone was unexpected since NADPH is known to be involved in the synthesis of this steroid in the adrenal gland (Haynes *et al.*, 1960; Estabrooks, 1969). The results do, however, confirm the stimulatory action of 2-deoxy-D-glucose (2-DG) on plasma corticosterone levels (Smith and Root, 1969). 2-DG produces *in vivo* a competitive inhibitor of intracellular glucose utilization which blocks both glycolytic and pentose pathways (Brown, 1962). The mechanism of action is unknown.

Note added in proof:

It has recently been shown that when the dihydropteridine reductase was isolated from sheep liver DPNH was a better substrate (K_m = 2.2 μM, Vapp = 2.45) than TPNH (K_m = 100 μM, Vapp = 0.88) in an *in vitro* assay (Craine *et al.*, 1972). This indicates that the NADH/NAD^+ ratio may be an important regulatory mechanism in most brain region. The synaptosomal region where shunt enzymes are highly concentrated may be an exception. Furthermore, the dihydropteridine reductase enzyme is no longer believed to be rate-limiting as was proposed by Musacchio in 1971 (Côté, L., personal communication, 1973). The ratio of NADH/NAD^+ in whole brain is approximately .66 (Mahler and Cordes, 1966; Glock and McLean, 1955) and changes in this ratio would be expected to affect the reductase enzyme activity. Table XXIV shows the effects of DPNH, DPN^+, TPNH, TPN^+, (Friedman *et al.*, 1972), a reducing complex and biopterin on TP-hydroxylase activity. TPNH did but DPNH and BH_4 did not significantly increase whole homogenate enzyme activity. When DPNH and BH_4 were added together a strong stimulation of TP-hydroxylase was measured. Comparison of exogenous DPNH (10^{-4} M) or DPN^+ (10^{-4} M) revealed a significant difference with DPNH associated activity higher in normal tissue, but no difference was found if cycloheximide (10^{-4} M) was present in the reaction mixture (Table XXV). TPNH (10^{-4} M) was effective in increasing TP-hydroxylase activity in 1 hr cycloheximide injected rats but the increase did not restore activity to normal levels, possibly due to the interference of cycloheximide in the tissue. These results are consistent with the results of several laboratories (Gal *et al.*, 1966; Nakamura *et al.*, 1965) which

Table XXIV

Effects of Various Cofactors on Tryptophan Hydroxylase Activity in Normal Brain Tissue

		Midbrain		Forebrain
Control	32	3191 ± 146	29	2836 ± 165
TPNH	9	4101 ± 502§	9	2909 ± 511
DPNH	26	3556 ± 132	13	3168 ± 306
		o = 2.78		
DPN^+	9	2864 ± 179	--	--
Biopterin	3	2870 ± 329	3	2940 ± 390
BH_4 and DPNH	9	4877 ± 342†	6	4148 ± 1312*
Complex	4	3178 ± 564		

Student t-test; †P <.001; * P <.005; ††P <.01; §P <.05

Table XXV

Effect of Cofactors on THA in Cyclo-treated Brain Tissue

Animal	No.	Midbrain	No.	Forebrain
Control	5	3181 ± 447	4	2875 ± 548
+ TPNH	5	4444 ± 879	5	3221 ± 905
+ Cyclo	3	3114 ± 104		
Cyclo + DPNH	4	2957 ± 71		
Cyclo + DPN^+	5	2834 ± 153		
Cyclo	5	1410 ± 314	5	1241 ± 272
+ TPNH	5	2299 ± 174*	5	2011 ± 358

Student's t-test; *P <.05.

demonstrated that a TPNH stimulates TP-hydroxylase activity in brain tissue in the absence of strong reducing agents such as DTT or β-MCE.

This leaves two choices which involve cofactors NAD^+ and $NADP^+$ for explaining the changes in TP-hydroxylase activity. 1) The activity of this enzyme is regulated by ratio or availability of NADPH (Figure 37). The cycloheximide reduction would be attributed to a disruption of either NADP linked dehydropteridine reductase or NADPH-linked dehydrogenase enzymes of the shunt pathway, neither of which has direct experimental verification. The indirect evidence favoring this consist of the reduction produced by 6-aminonicotinamide (Figure 38), stimulation produced by NADPH in in vitro TP-hydroxylase activity with normal and cycloheximide treated brain tissue (Table XXIV and Table XXV), and the recent report that both serotonin and catecholamine neurons have very high levels of NADPH-linked dehydrogenase enzymes (Kauffman et al., 1973, Sims, K.L., personal communication, 1973). 2) The activity of TP-hydroxylase is regulated by ratio or availability of NADH. Cycloheximide would produce its inhibition in a manner analogous to its effect at site 1 in the energy production cycle of mitochondria isolated from rat and guinea pig liver (Garber et al., 1973). This inhibition of NAD^+ linked enzymes occurred only in intact mitochondria and no direct effect was observed in purified NAD^+ linked dehydrogenases. The report that dihydropteridine reductase in liver is a NAD^+ linked enzyme (Craine et al., 1972) suggest the same might be true in the brain. The ability of NADH and reduced biopterin to stimulate TP-hydroxylase (Table XXIV), the high concentration of NADH in the brain and the ratio of NAD^+/NADH (Glock and McLean, 1955; Mahler and Cordes, 1966) all support NADH involvement.in regulation of TP-hydroxylase activity

The data are insufficient to provide a definitive answer at the present time. It is certainly possible that both NADH and NADPH are involved in regulating the short term changes after cycloheximide. The changes after adrenalectomy, steroid injection and stress might also involve availability of reducing equivalents. Adrenalectomy has been shown to decrease and steroid injection restore NADP dependent isocitric dehydrogenase, glutamate dehydrogenase and glutamate-oxaloacetate transaminase activity in brain (Baethmann and Van Harreveld, 1972).

Cold-stress causes first an increase than a decrease in NADPH levels in liver (Yuwiler, 1971). Finally, estradiol injections causes an increase in uterine glucose-6-p dehydrogenase activity in ovariectomized rats by a process mimiced by $NADP^+$ injection and abolish by cycloheximide injection (Moulton and Barker, 1972). In conclusion, the involvement of NADP and possibly NAD in regulation of serotonin biosynthesis in the brain is strongly suspected.

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