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A Study Of The Effects Of Niacin Upon Sleep And Brain Monoamines.

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A STUDY OF THE EFFECTS OF NIACIN UPON SLEEP AND BRAIN MONOAMINES

By

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A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology and Biophysics in the Graduate School of the University of Alabama in Birmingham

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TO ALL THE HUMANS WHO GAVE THEIR TIME

AND

TO ALL THE MICE WHO GAVE THEIR LIVES

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LIST OF ABBREVIATIONS

REM	rapid eye movement
SWS	slow wave sleep (Stages I-IV)
5-HT	5-hydroxytryptamine or serotonin
5-HIAA	5-hydroxyindoleacetic acid
NE	norepinephrine
PCPA	p-chloro-phenylalanine
NREM	non-rapid eye movement
AMPT	α-methyl-p-tyrosine
5-HTP	5-hydroxytryptophan
DA	dopamine
CA	catecholamine
COMT	catechol-O-methyl-transferase
MAO	monoamine oxidase
AADC	aromatic amino acid decarboxylase
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
EEG	electroencephalogram
EOG	electro-oculogram
EMG	electromyogram
TRT	total recording time
TST	total sleep time

List of Abbreviations (continued)

CB	cerebellum
TE	telencephalon
D-M	diencephalon-mesencephalon
LP-M	lower pons-medulla
OPT	ortho-phthaldialdehyde
EDTA	ethylenediamine tetraacetate (tetra sodium)
MAOI's	monoamine oxidase inhibitors
AM-5-HTP	α-methyl-5-hydroxytryptophan
AMPA	α-methyl-phenylalanine
CSF	cerebrospinal fluid

CHAPTER I

INTRODUCTION

Over the last 300 years neurochemistry has been developing and yet its scope or limits are not easily defined. There has been a close correlation of its development with that of neurophysiology and biochemistry. Chemistry did not exist in ancient times. Later, alchemy laid a foundation for the rise of true chemistry. Head injuries were studied in 1600 B.C. Contralateral innervation was recognized as early as 370 B.C. By the second century A.D. the anatomy of the central nervous system was known in detail. The brain was recognized as the seat of intelligence, voluntary movement and sensation. Motor and sensory nerves were distinguished. The effects of sections and crude ablations at various levels were experimentally established. Fifteen hundred years later the anatomy and much of the physiology had not been developed any further. Physiologic chemistry had no such striking precursors, however. Much of ancient knowledge derived from philosophy rather than experiment. With the coming of the Renaissance, communication and trade broadened and free thought developed. From the seventeenth century onward developments came very, very rapidly. In the rise of neurophysiology the developments in electrophysiology came much

earlier than in neurochemistry. By the beginning of the 19th century neurophysiology was coming of age.

Biochemistry paralleled the developments in neurophysiology. Chemistry in the 17th century began with the study of air, of combustion and oxidation and of its importance to life. Neurochemistry developed more slowly. The complexity of the chemistry of the nervous system proved baffling and lagged behind similar approaches in other areas of animal physiology. The 19th and 20th centuries brought rapid progress in neurochemistry which has persisted to the present time (Tower, 1957).

J. L. W. Thudichum initiated work on neurochemistry, but the flame died when clinical neurology and Freudian psychiatry took over. Then in the 1920's researchers began to show that nervous tissue had a metabolism. The imagination of researchers was fired when substances such as anticonvulsants, chemical transmitters of nerve impulses, tranquilizers and serotonin were found. A part of the story began with the discovery of serotonin (5-hydroxytryptamine, 5-HT) in 1947 and its presence in large amounts in the brain (Page, 1957). Yet the story of 5-HT and other neurotransmitters is far from complete despite some very early beginnings. The road to understanding through chemistry may sometimes be shunned from an unconscious fear that it casts doubt upon the religious nature of man. Page (1957) makes a most eloquent plea to the scientist and the small part he plays in shaping man's future.

> I personally see nothing to persuade me that the functions of the brain are not the functions of

> protoplasm and that these functions encompass both the material and the transcendant; that there is the necessity to include in the philosophy of biology both those material attributes which are our science and those immaterial attributes which are our values. It is the amalgamation of the two that will close the abyss, which has so destructively separated science from humanity as to make it appear the enemy of man and the enemy of God. In our hearts we know it is neither.

Although not forgetting the above, this research deals with only the science of the physiology involved in sleep, neurotransmitters and chemical behavior.

CHAPTER II

REVIEW OF LITERATURE

Introduction

Beaton et al. (1974a, b and 1975) working with the trans-methylation hypothesis in schizophrenia found that nicotinamide when administered alone to mice increased rapid eye movement (REM) sleep. The animals were injected with 250 mg/kg of nicotinamide once daily for 21 days prior to recording of EEG activity. Using both C57 and Swiss mice all showed a 25% or more increase in percentage REM.

In addition to REM sleep there are four other stages of sleep simply called slow wave sleep (SWS). These are classified I-IV according to the criteria of Rechtschaffen and Kales (1973). There is a preponderance of evidence in the literature indicating that 5-HT is a major neurotransmitter in the sleep process. Whether it acts alone or in concert with other neurotransmitters such as norepinephrine has not been fully established for the classical four stages of slow wave sleep (SWS) or for the stage of REM sleep. Nevertheless, the role of nicotinamide in sleep is an intriguing one since the amino acid tryptophan can not only be converted to 5-HT but also, along another metabolic pathway, to niacin.

Molecular Basis of Sleep

In the mammalian sleeping brain, two states of sleep can be easily recognized by polygraphic techniques. The first state is characterized by the sleeping posture, the eyes closed and the pupils myotic. A degree of tonus remains in some muscle groups. Electrical activity of the cortex is characterized by spindles and slow waves.

Jouvet (1967 and 1969) has called the second stage of sleep paradoxical sleep, now more commonly called REM sleep. Cortical activity is similar to that of waking, is associated with regular theta rhythm of the hippocampus, and electromyographic activity is decreased. Rapid eye movements (50-60 per minute) occur in a pattern which differs from that of waking. They have cortical and subcortical components of activity, ponto-geniculo-occipital activity. High voltage waves can be recorded from the reticular formation of the pons, from the lateral geniculate and from the occipital cortex. These waves always precede REM sleep by about 30-45 seconds and accompany REM at about 60 per minute.

Stern and Morgane (1974) have very appropriately made a distinction between mechanism and function of REM sleep. This can be made by examining whether upon withdrawal of the drug treatment (which decreased REM time) there occurs a subsequent REM rebound. If there is no rebound then the drug fulfilled the function of the REM sleep by neurochemical action. This study deals with the

mechanism of REM sleep and SWS as opposed to the function served by REM sleep.

Since the monoamines do not cross the blood brain barrier, various precursors have been used to study the relationship of the amines of sleep. It has been shown by various studies (Griffiths et al., 1971; Ursin, 1974) that 5-hydroxytryptophan increases slow wave sleep both in animals and man. Because the injection of dihydroxyphenylalanine, which restores the catecholamine concentration following the administration of reserpine, leads to the reappearance of REM sleep, the catecholamines were implicated in REM sleep (Jouvet, 1967). This piece of evidence, however, is disputable since Tissot (1965) found that the administration of reserpine, 3-4 mg, increased paradoxical sleep from 22% to 41% of the total sleep time. This occurred at the expense of Stage III and IV and not Stage II.

Monoamine oxidase inhibitors suppress REM sleep and increase SWS in the cat even when the animal is REM deprived (Jouvet, 1967 and 1969).

By pharamacological and neuroanatomical manipulations certain areas of the brain have been shown to be responsible for the observable electrical phenomena in the various stages of sleep. Para-chloro-phenylalanine (PCPA) selectively decreases the concentration of serotonin in the brain without altering the concentration of norepinephrine or dopamine by inhibiting trytophan hydroxylase (Jouvet, 1969 and 1973; Rechtschaffen et al., 1973; Dement et al., 1973; King, 1974; Ursin, 1974). The administration

of the drug produces complete wakefulness. This effect can be reversed by injecting 5-hydroxytryptophan which readily crosses the blood brain barrier and by-passes the tryptophan hydroxylase step in synthesis of serotonin. Sleep then returns to normal. Dement et al. (1973) found that in the cat after continued administration of PCPA and maintainence of maximum serotonin depletion, substantial amounts of sleep returned--an unexplained phenomenon.

Sinha (1973) found that after ten minutes of non-REM (NREM) sleep or SWS, 5-HT content of the cerebral cortex decreased from the awake value. Other brain areas demonstrated the same trend. The 5-HT values of all areas approximated the awake levels after five minutes of REM sleep. He suggests that a transient decrease in serotonin content within a time period shorter than its half-life may be due to an increase in the rate of release of the neurotransmitter. The decrease he found may be brought about by a massive release of serotonin, most likely due to an increase in impulse traffic over serotonergic synapses during the specified time.

By destroying the neurons of the raphe system insomnia is produced in proportion to the extent of the destruction of the raphe neurons. Also, a correlation between extent of destruction and a decrease in serotonin was demonstrated (Jouvet, 1967, 1969 and 1973).

The locus coeruleus has been implicated in the phase of REM sleep. Bilateral destruction produces total suppression of REM sleep. These nuclei contain norepinephrine and thus may play a role in REM sleep. One patient with an infarction of the basis

pontis had a decreased sleep time but a normal percentage of REM (Freeman et al., 1974). Alpha-methyl-p-tyrosine (AMPT) which blocks synthesis of catecholamines at the level of tyrosine hydroxylase selectively suppresses REM sleep--a reason for looking at norepinephrine in our animal studies (Jouvet, 1969). However, according to Stein et al. (1974) the inhibition of catecholamine synthesis produces an increase in serotonin (5-HT) turnover. And so the prior studies may lead us back to the serotonin hypothesis of sleep. This study pointed out the fact that AMPT does not suppress REM sleep and is not in agreement with the hypothesis that catecholamine mechanisms are involved in the triggering of this phase of the sleep cycle. They also showed that destruction of the locus coeruleus and subcoeruleus is followed by an increase of 5-HT turnover and is accompanied by a parallel increase in both Stage II and REM. The question is not settled since other data such as that atropine suppresses REM sleep in the cat are available.

The fact that serotonin is necessary for the priming of REM is substantiated by the finding that a minimum of 16% of daily SWS is necessary for the appearance of REM sleep in the cycle (Jouvet, 1973). It may be noted almost nothing is known about what triggers the 5-HT system. Blood tryptophan levels may be involved in the triggering since tryptophan hydroxylase is not a rate limiting step. Tagliamonte et al. (1974) found that in normal subjects the concentration of free (unbound) tryptophan in serum was about 45% higher at midnight than at noon. Conversely, Fernstrom and Wurtman (1974a) found, among human subjects who ate three meals a day,

tryptophan levels highest in late morning or early afternoon and lowest at two to four a.m. Tryptophan is the only amino acid in plasma which is highly bound (about 95%) to serum proteins. Results from Tagliamonte's laboratory show that the concentration of free tryptophan in serum controls the concentration of tryptophan in the brain. The ratio of free to bound in serum is not constant. The free fraction can increase without a change in total tryptophan.

Griffiths et al. (1971) showed that oral administration of l-tryptophan to young adult males caused sedation prior to sleep, decreased total awake time, reduced frequency of brief movement arousals and increased SWS. This was accompanied by a trend toward early onset of REM and significant shortening of the REM to REM cycle. Larger doses produced an increase in percent and absolute amount of REM sleep. Hartmann et al. (1973) also found similar effects.

It has been shown that enhanced biogenic amine synthesis following REM sleep deprivation is due to non-specific stress rather than to loss of REM sleep per se (Stern et al., 1971). The data from the works of Cho-Chung and Pitot (1967 and 1968) show that adrenal cortisol induces the enzyme, tryptophan hydroxylase.

Jouvet and Pujol (1974) have summarized the animal data which strongly favor the 5-HT containing neurons of the raphe system being involved in sleep mechanisms. (1) The inhibition of the synthesis of 5-HT (at the level of tryptophan hydroxylase) with PCPA leads to insomnia which is quickly reversed by small doses of 5-hydroxytryptophan (5-HTP) the immediate precursor of 5-HT.

(2) The destruction of the 5-HT-containing perikarya in the raphe system leads to insomnia. (3) The hypersomnia, as much as 400% increase in REM on the first day after the lesion (Jouvet and Pujol, 1974), which follows the destruction of the norepinephrine bundle at the level of the isthmus is accompanied by an increase of 5-HT turnover.

Other hormones have been implicated in sleep. For example, melatonin, the principal hormone of the pineal gland, appears to be a potent sleep inducer (Cramer et al., 1974).

When monoamines are considered for study there are various components to consider--the effect at the receptor; the effect of the receptor through neural feedback mechanisms on the synthesis and release of amine; the interaction of other amines; and chemical or neural sequences and/or receptor events.

Neuroanatomy of the Monoamine Systems of the Brain Related to Sleep

Before 1958 the Swedish neuroanatomists Dahlstrom and Fuxe, working with the fluorescent microscope, had located the neurons where serotonin (5-HT) and catecholamines are stored. They stated with little doubt that the green and yellow fluorescence was due to the accumulation of a primary catecholamine and of 5-HT, respectively. The fact that reserpine markedly reduced or abolished the fluoroscence was further evidence that the material belonged to the monoamine group (Dahlstrom and Fuxe, 1964).

A large nigro-neostriatal dopamine (DA) neuron system has been characterized. Also, the hypothalamus, the preoptic area and the limbic system are innervated by ascending catecholamine (CA) neuron systems which degenerate after destruction of the CA cell groups in the mesencephalon. The existence of mesencephalo-hypothalamic and mesencephalo-limbic 5-HT neuron systems is firmly established. It has also been concluded that at least a large part of the CA terminals in the limbic lobe, the neocortex, the preoptic area and the hypothalamus belong to CA neurons with their cell bodies in the ventromedial part of the mesencephalon, with their axons ascending in the medial forebrain bundle. Thus, a vast majority of 5-HT and CA terminals in the prosencephalon (telencephalon plus diencephalon) belong to special neurons with their cell bodies in the lower brain stem (Anden et al., 1965).

At least 75% of the ascending norepinephrine (NE) fibers originate in the cell bodies of the pons and medulla oblongata with terminals in the hypothalamus, the preoptic area, the striae terminalis, the septal area, the amygdaloid complex, the hippocampal formation, the gyrus cinguli and the neocortex (Anden et al., 1966). A large portion of the NE system is formed by a group of cell bodies located in the locus coerulus. Lesions here produce a marked decrease in REM sleep (Kostowski et al., 1974).

At least 75% of the 5-HT nerve terminals in the diencephalon and telencephalon arise from 5-HT fibers ascending in the area of the medial forebrain bundle. These fibers originate mainly in the nucleus raphe dorsalis (B7), nucleus raphe medianus (B8) and the

formatio reticularis (B9) of the mesencephalon (Anden et al., 1966). 5-HT terminals were observed in the globus pallidus, the septal area, the amygdaloid complex, the hypothalamus and the gyrus cinguli. Practically no monoamine nerve cell bodies have been detected in or rostral to the medial forebrain bundle.

Lorens and Guldberg (1974) produced a 54% fall in striatial 5-HT by lesions in the dorsal raphe nucleus and a 62% fall in hippocampal 5-HT with lesions in the median raphe nucleus. The larger number of 5-HT fibers seem to originate in the dorsal raphe nucleus, which also appears to send a greater number of its fibers to telencephalic rather than diencephalic structures. The authors suggest that the neurons which predominate in the dorsal raphe nucleus are different from the more numerous cell bodies of median raphe nucleus.

The serotonin systems in the brain primarily comprise the raphe nuclei and their various projections of fibers, in particular the powerful rostral extensions in the medial forebrain bundle (Morgane and Stern, 1973). In the cat, the nuclei raphe obscuris, pallidus and magnus located at the level of the inferior olive and above in the medulla project into the spinal cord with pallidus and magnus also receiving strong afferents from the spinal cord. The nuclei raphe obscuris and pontis project to the cerebellum. The fastigial nuclei of the cerebellum project onto the nuclei raphe magnus and pontis of the posterior and middle raphe groups. The nucleus raphe pontis is found at the level of the locus coeruleus. Fibers originating in the dorsal and ventral nuclei of

Gudden pass through the nucleus centralis superior of Bechterew and through the nucleus raphe dorsalis, via the tegmento-peduncular tract, to the area of the interpeduncular nucleus. This latter is one of the primary nodal points for the confluence of fibers passing rostrally which are tributary to the medial forebrain bundle system. Morgane and his co-workers have shown that lesions of the dorsal and ventral tegmental nuclei of Gudden decrease both serotonin and norepinephrine in the basal forebrain bundle without significant effects on sleep profiles. However, the nucleus centralis superior of Bechtrew through which the fibers from Gudden's nuclei pass, does contain serotonin cell elements and lesions in this nuclear formation produce a raphe lesion-like effect, an increase in waking and a decrease in SWS with reductions only in basal forebrain serotonin. Lesions of midlateral hypothalamic and discrete limbic-midbrain lesions selectively deplete the basal forebrain and antero-lateral hypothalamic area of serotonin (Morgane and Stern, 1973).

Most ascending serotonergic systems arise from the "anterior" raphe area including the nucleus raphe dorsalis, nucleus raphe medialis, the nuclei linearis rostralis and intermedius and the nucleus centralis superior of Bechterew and not from "middle" and "posterior" raphe cellular assemblies.

Lesions of the anterior group appear to correlate with changes in forebrain serotonin and SWS. More caudal lesions do not summate to produce a more severe effect on sleep or lowering of forebrain serotonin.

Morgane and Stern (1973) stated that the noradrenergic input to the raphe from the locus coeruleus may well be a link relating REM to SWS. The locus coeruleus is primarily noradrenergic and may modulate activity rather diffusely in the raphe complex. Although it has not been investigated, it is possible that the medial and posterior raphe complex may interact with the locus coeruleus to affect or trigger REM. Since the locus coeruleus system also innervates the geniculate bodies, this pathway may be involved in the regulation of geniculate discharges found during REM sleep.

Parmeggiani et al., (1974) using cats in which the thalamic structures were heated, concluded that the neurochemical activity of anterior hypothalamic structures may underlie the evolution of fast wave sleep episodes. The possibility exists that the effect of anterior hypothalamic heating on fast wave sleep duration depends on the change in the rate of some chemical reactions; e.g., of those involved in the turnover of monoaminergic transmitters.

Further data implicating the raphe nuclei in REM sleep come from studies done at NIH by Sheu et al. (1974). They used tungsten electrodes implanted in the median and magnus raphe of unrestrained, freely behaving cats and found that these neurons fire slowly during SWS, faster during awake and fastest during REM. Also, during REM the raphe units tended to fire in bursts. Twelve units from the raphe magnus began to discharge more rapidly about 10-20 seconds before the onset of the hippocampal theta rhythm characteristic of REM sleep. This activity decreased concomitantly with cessation of theta activity. DeFrance and others (1973) have found that the

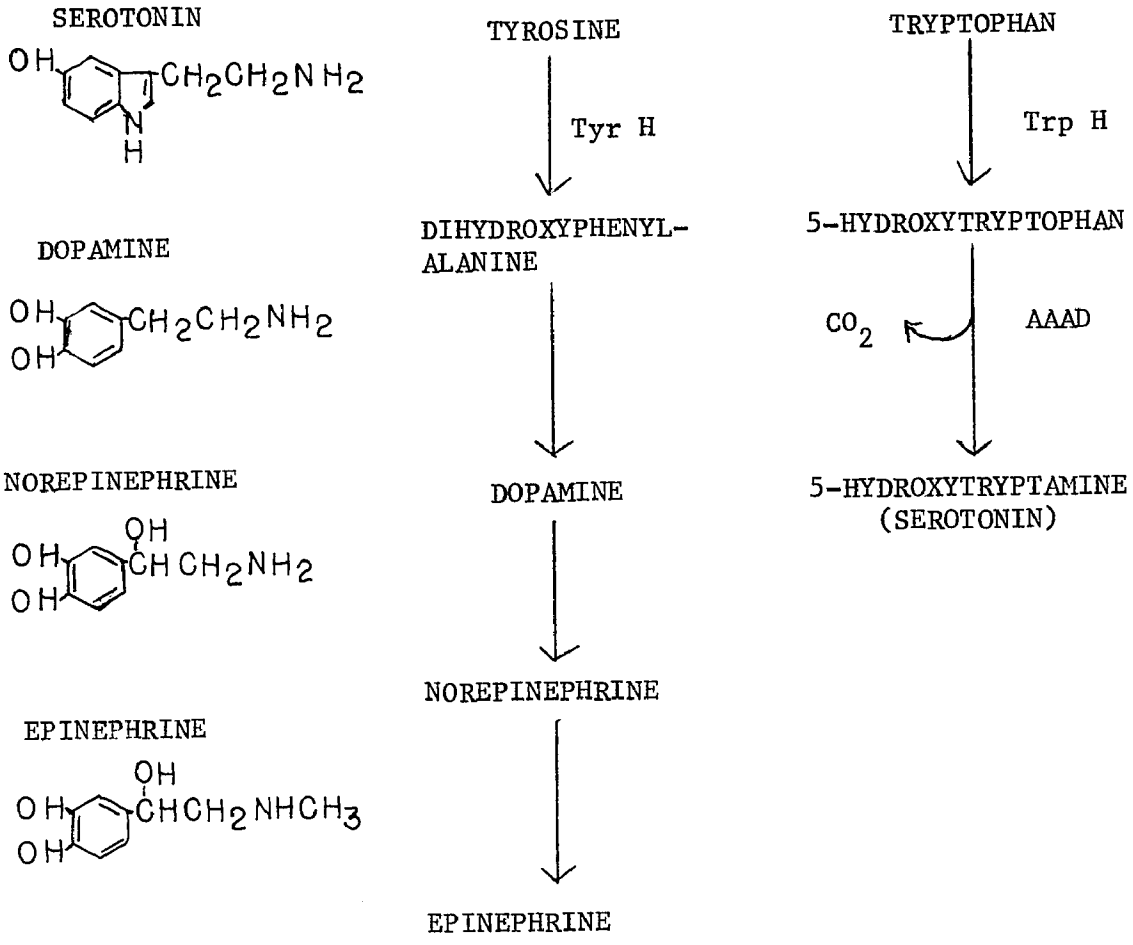
hippocampal inputs to the septal nuclei are mono-synaptically excitatory. By applying 5-HT ipsilaterally to the fimbria, field potentials were recorded in the dorsal part of the lateral septal nucleus. Hence, 5-HT becomes a candidate for being at least one of the transmitters utilized by the hippocampal pyramidal cells.

Biochemistry of Sleep

Four neurotransmitters found in the brain of mammals are serotonin, dopamine, norepinephrine and epinephrine. The structures are shown below. The epinephrine and serotonin pathways are as shown (Fernstrom and Wurtman, 1974b). Two other compounds are reasonably well established as transmitters. They are acetylcholine and gamma-amino butyric acid.

5-Hydroxytryptamine was first discovered in 1948 at the Cleveland Clinic (Fernstrom and Wurtman, 1974b).

Tryptophan from which 5-HT is derived is the least abundant amino acid in dietary protein. It was first discovered in 1901 by Hopkins and Cole in the physiological laboratory at Cambridge. They published an interesting paper in the Journal of Physiology (London). Tryptophan was synthesized in 1907 and 1908 by Ellinger and Flaman in Germany (Price et al., 1965). Rose et al. (1954) established the daily need for tryptophan and for eight other essential amino acids.



Insulin facilitates the uptake by body tissues of all the neutral amino acids except tryptophan, thus increasing the relative concentration of tryptophan in the blood. This produces a relative influx of tryptophan into the brain and a proportionate increase in synthesis of 5-HT (Kolata, 1976; Fernstrom and Wurtman, 1974a). Brain tryptophan does not simply reflect plasma tryptophan but depends also upon the plasma concentration of other neutral amino acids which are not appreciably bound to plasma proteins. Thus,

carbohydrate consumption can increase brain 5-HT. Gessa and Tagliamonte (1974) showed that changes in brain tryptophan were time related and proportional to changes in free serum tryptophan and not in total serum tryptophan.

Tryptophan hydroxylase is a low-affinity enzyme. Only when the substrate concentration is higher than normal in the cells does it function at a maximum rate. In contrast tyrosine hydroxylase is a high affinity enzyme and operates at maximum efficiency even when the concentration of tyrosine is no greater than normal.

Brain, adrenal medulla and sympathetically innervated tissues contain a specific hydroxylase that catalyzes the conversion of l-tyrosine to dopa. This enzyme, dopamine-β-hydroxylase has been isolated and characterized. In 1964, Nagatsu et al. reported a procedure for isolation, purification and characterization of a soluble tyrosine hydroxylase. They found its activity in the brain to be highest in the brain stem. Much less is present in cortex, and little is present in cerebellum in several mammalian species. Dopa, norepinephrine, and related compounds inhibit tyrosine hydroxylase, a manifestation of product inhibition. It is thought that the Km of tyrosine hydroxylase is such that the enzyme is probably saturated with substrate and increasing tyrosine concentration does not increase catecholamine synthesis (Balazs and Cremer, 1972). Many believe that the hydroxylase is the rate limiting step in norepinephrine biosynthesis (Nagatsu et al., 1964). However, Wurtman et al. (1974) presented evidence that treatments which increase or decrease brain tyrosine concentrations can produce

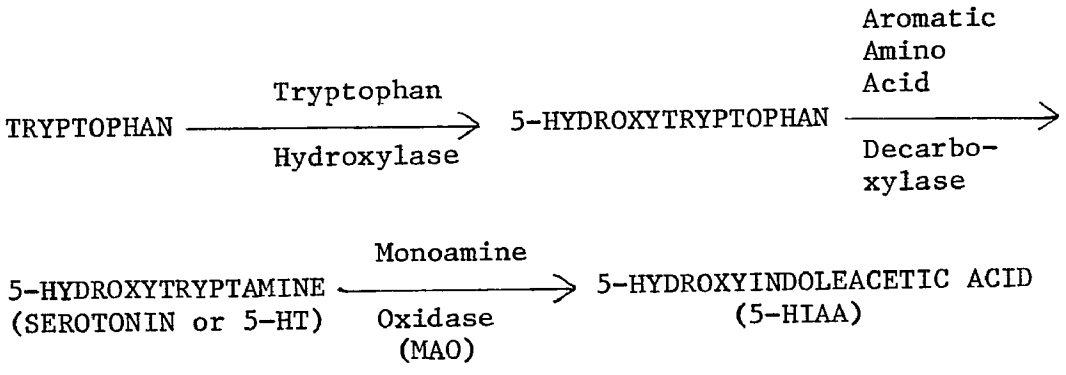
parallel changes in the rate at which the brain synthesizes catecholamines and constitutes an additional controlling factor. Also increasing tryptophan in the brain causes immediate increases in synthesis of 5-HT. Compartments of amino acids, particularly phenylalanine and tryptophan, are present in synaptosomes which have a differing accessibility to amino acids. Integrity of these transport pools depends on supply of metabolic energy.

Enzymatic degradation of catecholamines occurs by way of O-methylation and oxidative deamination catalyzed by the enzymes catechol-O-methyl-transferase (COMT) and monoamine oxidase (MAO), respectively. Axelrod (1965) was involved in much of the early work on metabolism of catecholamines. MAO was shown to catalyze oxidative deamination of amines in 1928 and 1937. MAO is present in almost all tissues but is highly localized in the mitochondria. COMT, in contrast, is located in the cytoplasm. Reuptake and binding protect the amines from enzymatic attack. Axelrod suggested that there are two pools of noreinephrine. One store is rapidly turning over and a second has a slower turnover rate. He further suggests that COMT metabolized the easily released norepinephrine while the more tightly bound is metabolized by MAO.

Stelmasiak and Curzon (1974) found that increased catecholamine secretion peripherally is followed by a rise in unesterified fatty acid concentration which displaces tryptophan from binding to plasma albumin. This results in increased brain tryptophan and increased 5-HT turnover.

Shields and Eccleston (1973) have suggested that 5-HT is synthesized and stored in two separate pools in the rat brain. One pool is quantitatively small and the synthesis responds rapidly to changes in firing rate of neurons. It was suggested that this small pool was situated at the nerve ending and represented that part of 5-HT which was functionally active, being released by the nerve impulse. 5-HT in the second, larger pool was postulated to be synthesized by a separate pathway not controlled by nervous activity. Balazs and Cremer (1972) lent support to this theory. They stated that evidence from PCPA studies shows that there are two species of tryptophan hydroxylases and perhaps these hydroxylases subserve the synthesis of different pools of 5-HT. Tryptophan hydroxylase is partially localized in the synaptosome.

Tryptophan proceeds along the metabolic pathway to 5-hydroxyindoleacetic acid (5-HIAA) as shown below.



5-HIAA is excreted normally in the urine of man as an end-product of tryptophan metabolism via the 5-hydroxyindole route and accounts for an appreciable portion of the dietary tryptophan (Udenfriend et al., 1955). Amine storage of granules and monoamine oxidase are the mechanisms controlling the functional pool of 5-HT and protecting post-synaptic sites from "spill over". Turnover *in vitro* in cultured raphe nuclei from newborn rat increases with a time course paralleling that described for tryptophan hydroxylase *in vivo*. If nicotinamide increases 5-HT, then 5-HIAA may be increased as well.

By inhibiting the aromatic amino acid decarboxylase enzyme (AADC), David et al. (1974) has shown that when tissue levels of tyrosine, phenylalanine or tryptophan are elevated, decarboxylation becomes a major route for their metabolism. These amino acids could be further elevated in heart, kidney, liver and brain by administration of an AADC inhibitor. It appears that the three aromatic amino acids undergo a much greater conversion to amine than has before been suspected.

Many and various drugs affect the brain levels of serotonin. For example, Perez-Cruet et al. (1971) found that repeated administration of lithium salts to rats increased brain 5-HIAA by about 80% and brain 5-HT by 15-20%. The changes were due to an increase in the rate of synthesis of brain 5-HT which increased by about 80%. Curzon (1971) found that adrenal hormones and stress effect brain 5-HT levels. Hydrocortisone, which induces pyrrolase, caused low brain 5-HT. Betamethasone results in similar findings.

Stress of immobilization, through adrenal action, lowers 5-HT synthesis and increases 5-HIAA as well.

The effect of nicotinamide upon brain 5-HT and NE is, however, of particular interest in this study.

Nicotinamide and Brain 5-HT

General

The term niacin is now used as a generic term for both nicotinic acid (pyridine-3-carboxylic acid) and nicotinamide or niacinamide. In 1913 Funk isolated from rice polishings a compound that he identified as nicotinic acid. Elvehjem et al. (1937) proved its efficacy in curing black tongue in dogs. Spies et al. (1939) reported successful treatment and prevention of human pellagra with nicotinic acid. In 1935 nicotinamide was obtained from a coenzyme isolated from the red blood cells of the horse (Goodman and Gilman, 1970).

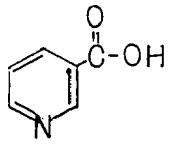
Nicotinic acid is a crystalline solid melting at 234-237°C, while the amide is a crystalline solid melting at 128-131°C. Both are water soluble and extremely stable to heat, air, light and alkalies. In biological tissues, niacin occurs as the amide and also in the form of two dinucleotide coenzymes, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). Good sources are liver, kidney and other meats, fish, legumes, coffee, tea and wheat germ. Tryptophan serves as

an important source of nicotinic acid. About 60 parts of tryptophan can yield one part nicotinic acid in man.

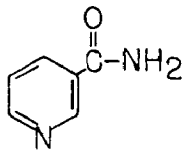
Pyridoxal phosphate (vitamin B_6) serves as a coenzyme in the conversion of tryptophan to nicotinic acid in conjunction with the enzyme kynureninase converting 3-hydroxykynurenine to 3-hydroxy-anthranilic acid (DiPalma, 1971). If there is a pyridoxal phosphate deficiency there is an increase in tryptophan metabolites, particularly xanthurenic acid. The latter is an insulin antagonist and has a diabetogenic effect (Bennink et al., 1975). Pyridoxal phosphate is also a coenzyme of the transaminases and of aromatic amino acid decarboxylases.

Annie Homer published in 1914 what was considered positive evidence as to the "constitution" of natural kynurenic acid and noted its close connection with tryptophan in the metabolism of the dog.

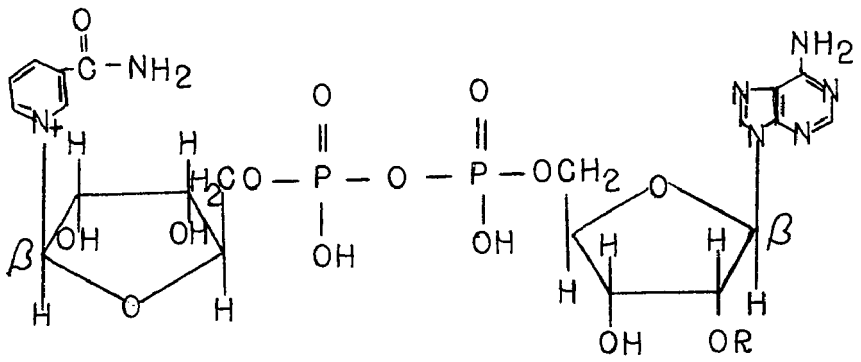
The structures of nicotinic acid, nicotinamide, NAD and NADP are as follows (Goodman and Gilman, 1970).



NICOTINIC ACID



NICOTINAMIDE



NAD and NADP

If R = phosphate group then NADP.

If R = H then NAD.

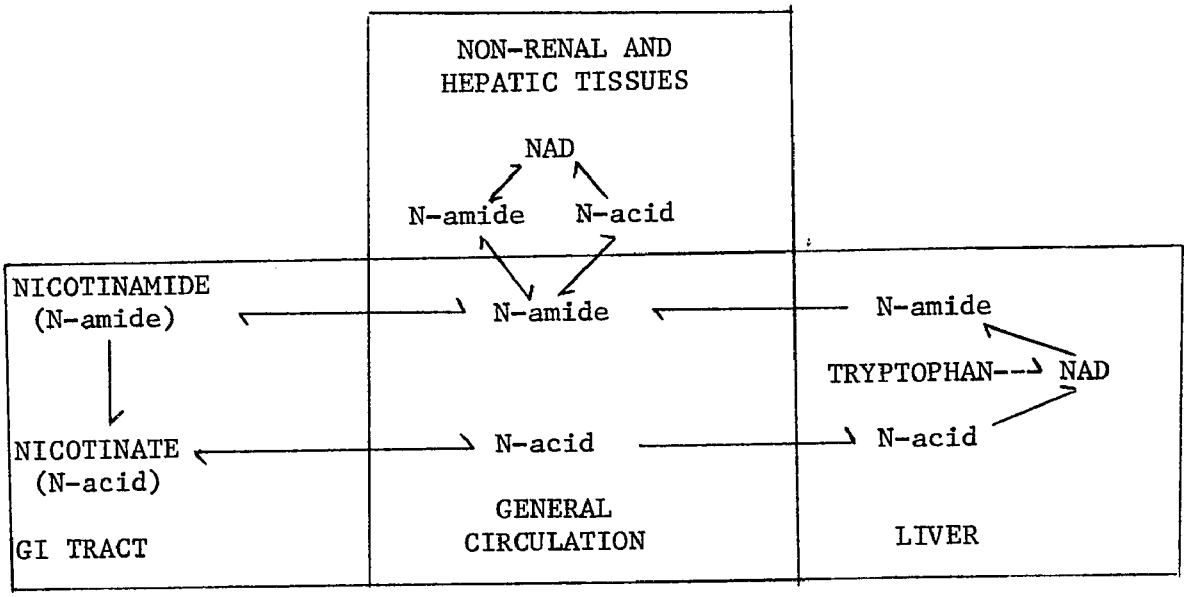
NAD and NADP function as the coenzymes of a large number of dehydrogenase enzymes. These coenzymes along with the appropriate dehydrogenases accept reduction equivalents and hydrogen from the appropriate substrates to become reduced to NADH and NADPH. These then either transfer the hydrogen or reduction equivalent to the riboflavin component of flavoproteins or reduce another substrate with the aid of another dehydrogenase and become oxidized to NAD and NADP. Thus only catalytic quantities of these coenzymes are needed in intermediary metabolism. The recommended daily dietary allowance of niacin is 20 mg or less.

Both forms of niacin are readily absorbed throughout the gastrointestinal tract and from all parenteral sites. The usual

route of metabolism is through N-methyl nicotinamide to N-methyl-4-pyridone-3-carboxamide and N-methyl-2-pyridone-5-carboxamide. In very high dosages much is excreted unchanged.

The metabolic pathway by which nicotinic acid is converted to NAD has been elucidated for a variety of tissues, known as the Priess-Handler pathway. Nishizuka and Hayaishi (1963) showed that 3-hydroxyanthranilic acid is converted to niacin ribonucleotide with several intermediate steps. The acute lethal intravenous dose of nicotinic acid in animals is approximately 4 gm/kg; dogs readily tolerate daily oral administration of 1 gm/kg. Side effects include gastric distress, increased gastrointestinal motility and increased secretion of sebaceous glands (Goodman and Gilman, 1970). Vasodilatation is a common side effect of nicotinic acid particularly.

Dietrich (1971) has described a systemic pyridine nucleotide cycle whereby adequate concentrations of pyridine compounds for NAD synthesis for the cells is guaranteed. It is as follows:



SYSTEMIC PYRIDINE NUCLEOTIDE CYCLE

Actions

Niacinamide is known to have many effects upon various systems of the biological organism. Those which appear specifically related to its activity in the brain are discussed as follows.

Nicotinamide is a competitive inhibitor of S-adenosyl-methionine and a scavenger of labile methyl groups (Cantoni, 1951; Halpern et al., 1971).

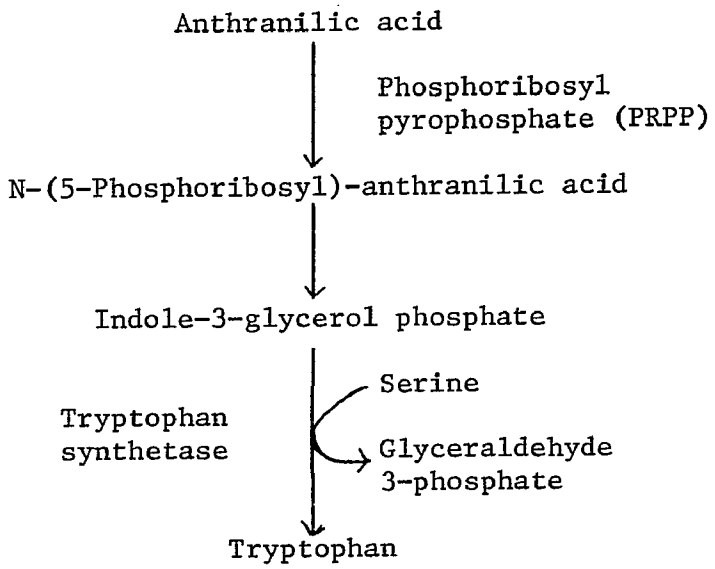
T-RNA methylases are non-competitively inhibited by nicotinamide (Halpern et al., 1971). Thus protein synthesis is inhibited. The inhibitory effect on t-RNA methylase is regulatory in nature.

Nicotinamide blocks the cerebral effects of L-dopa in Swiss albino mice (Cotzias et al., 1973).

In isolated smooth muscle nicotinamide has been shown to antagonize serotonin effects (Woolley, 1958). Nicotinamide caused half-maximal inhibition of contraction in uterine muscle standardized with serotonin. Am amount of nicotinamide which would almost totally inhibit serotonin reduced only slightly the acetylcholine response. Larger amounts of nicotinamide did, however, prevent acetylcholine induced contractions. Nicotinamide is not a specific antagonist to serotonin. It is not competitive.

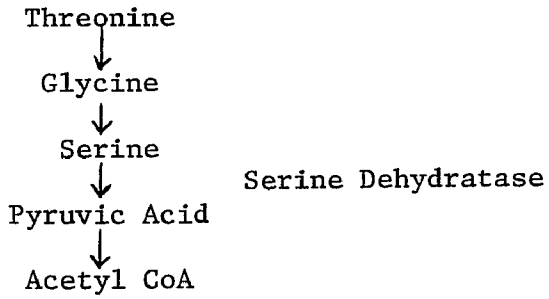
Nicotinic acid influences human purine metabolism by (1) diminishing fractional clearance of uric acid by directly modifying the bidirectional handling of uric acid by renal tubules and (2) by decreasing intracellular phosphoribosylpyrophosphate (Gershon and Fox, 1973).

The biosynthesis of tryptophan from phosphoenolpyruvate plus erythrose 4-phosphate through anthranilic acid is possible in the organism. Details of the pathway from anthranilic acid to tryptophan are shown below (Lehninger, 1970).



Nicotinamide induces tyrosine transaminase activity (Shimoyama et al., 1972). This raises the question of whether more tyrosine could be shunted down this pathway and less down the catecholamine one.

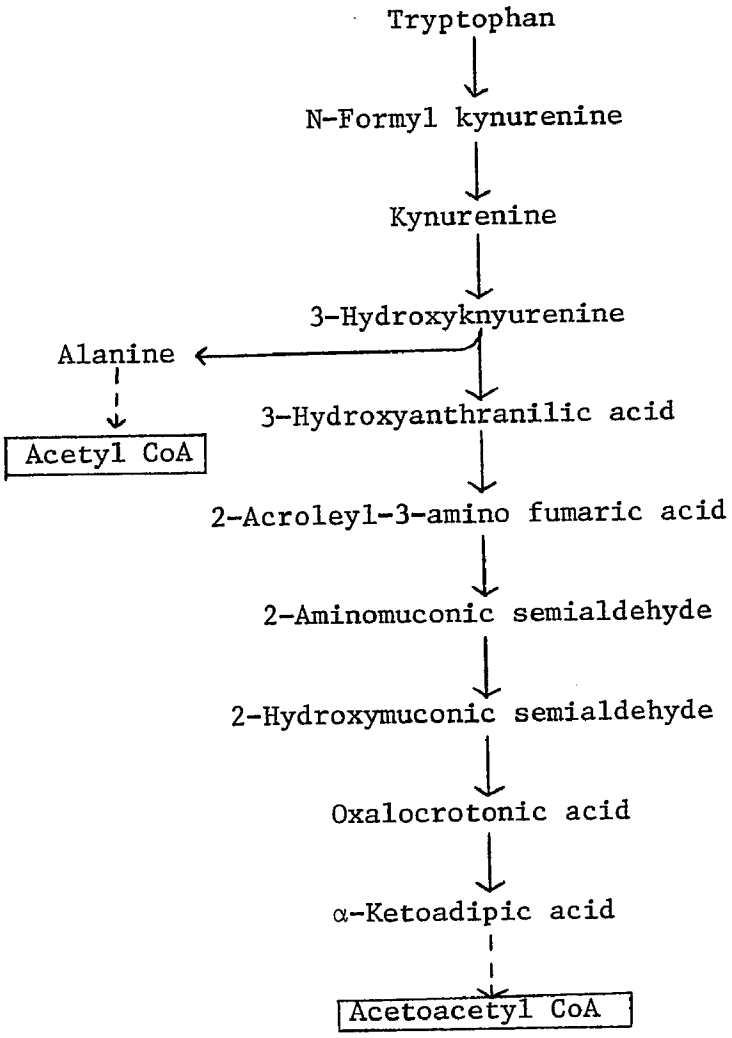
According to Shimoyama, nicotinamide also induces serine dehydratase in the following pathway.



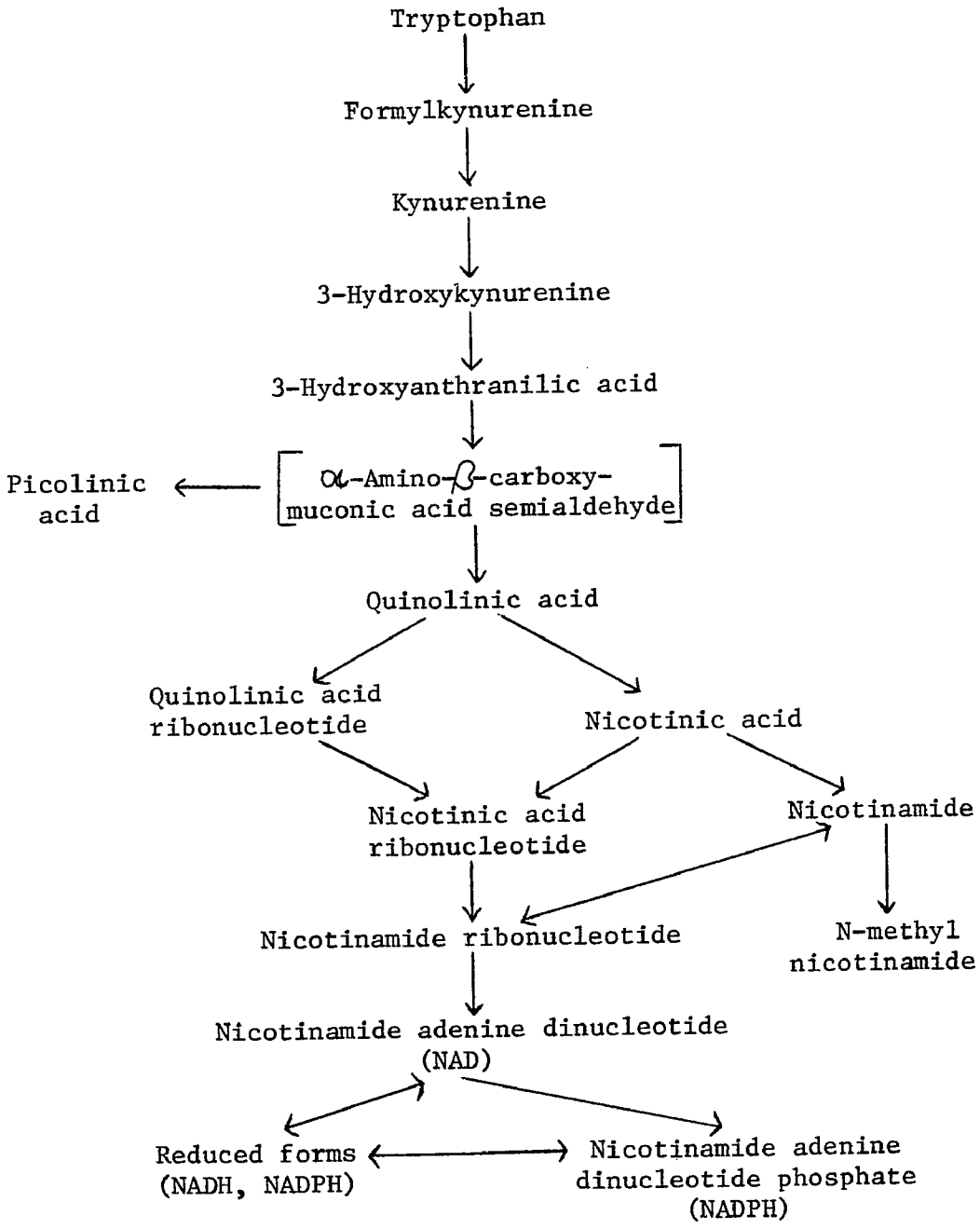
Nicotinamide also inhibits rat liver cAMP phosphodiesterase (Shimoyama et al., 1972). Thus it increases cAMP and the effects of this. Nicotinamide is a potent inhibitor; nicotinic acid inhibits but to a lesser degree. Concentrations required are relatively high compared with nicotinamide normally found in cells. Increasing cAMP in the adrenal cortex leads to the induction of certain enzymes by stimulating secretion of the adrenocortical hormones. It is interesting to note that 60% of brain phosphodiesterase activity may be synaptosomal membrane bound (Thompson and Appleman, 1971).

Probably the most interesting effect of nicotinamide, however, is its effect on tryptophan pyrrolase activity. Nicotinamide is converted in large amounts to NAD. NAD may be a feedback inhibitor of tryptophan pyrrolase which initiates the sequence of reactions leading to complete oxidative degredation of the benzene ring of tryptophan, as well as the pathway to NAD (Yamaguchi et al., 1971). When NAD is high it may be necessary to reduce the flow of tryptophan through these pathways. Induction of tryptophan pyrrolase by tryptophan would operate in reverse. The combined operation regulates flow. The pathway for the conversion of tryptophan to acetyl CoA and acetoacetyl CoA and acetoacetyl CoA is as follows (Lehninger, 1970).

Conversion of tryptophan to acetyl CoA
and acetoacetyl CoA



3-Hydroxyanthranilic acid is a precursor of nicotinic acid. The process is shown below (Calbiochem Metabolic Pathways Map 2: Aromatic Amino Acids, 1976).



The question is then raised, is nicotinamide converted into NAD in sufficient amounts to slow this tryptophan pathway and kick tryptophan into the serotonin pathway?

Cho-Chung and Pitot (1967 and 1968) have done much work with the tryptophan pyrrolase enzyme which catalyzes the first irreversible step in the degradative metabolism of tryptophan. They found the enzyme to be strongly inhibited by NADPH. NADH and nicotinamide are inhibitory to the enzyme but at considerably higher concentrations. Their work, using rat liver, also showed both NAD and 3-hydroxyanthranilic acid were found to be competitive inhibitors of tryptophan pyrrolase. NADPH is an allosteric type of inhibitor, binding on the pyrrolase enzyme surface at a site different from that of the tryptophan binding site. NADPH does not overlap the active site but induces a slight distortion of the site so that the affinity of that enzyme molecule for tryptophan is reduced.

Tryptophan pyrrolase has a dual control mechanism, one by substrate and the other by corticoid hormones. The above authors found that nicotinamide produced a 70-90% inhibition of the substrate elevation of tryptophan pyrrolase while only producing a 10-15% inhibition in the hormonal induction of the enzyme. The evidence suggests that tryptophan stimulates the production of tryptophan pyrrolase at the level of genetic translation; i.e., that of protein synthesis. Nicotinamide or some derivative thereof appears to exert inhibitory effects at the translational step rather than at transcription of the messenger RNA for the enzyme molecule.

Yamaguchi et al. (1971) found nicotinamide much more potent than nicotinic acid in causing increased NAD biosynthesis. Contrary to the above, they found nicotinic acid (more than nicotinamide) to induce tryptophan pyrrolase. They proposed this to be due to nicotinamide and nicotinic acid causing an increased secretion of adrenal cortical hormones. Cho-Chung and Pitot's work appears most well-done, however.

Most of the tryptophan pyrrolase and nicotinamide work was done using rat liver. Application to brain biochemistry must be done cautiously.

For this study, one of the most interesting results of nicotinamide administration comes from the work done at the Max-Planck Institute for Psychiatry by Scherer and Kramer, 1972. After 1 gm/kg administration of nicotinamide to rats a significant increase of 17% in 5-HT levels of the brain was found as compared to saline controls. Sedation previously observed by Woolley (1958) was confirmed. The latter, however, thought that nicotinamide might act as an antagonist to serotonin. He suggested it did not antagonize directly but possibly interfered with the action of calcium ions. Scherer and Kramer proposed that an increase of brain 5-HT after nicotinamide administration resulted from an inhibition of tryptophan pyrrolase in vivo and/or repressed de novo synthesis of tryptophan pyrrolase.

Beaton et al. (1974a, b) found behavioral changes in mice with one-fourth the dose of nicotinamide used by Scherer in rats. After 250 mg/kg the mice showed significant increases in REM sleep.

Niacin and the Mouse

Collins and Chaykin (1972) found that metabolism in the mouse is designed for the use of nicotinamide as a precursor of NAD and nicotinic acid is converted to nicotinamide for that purpose. The Preiss-Handler pathway functions primarily for the purpose of converting nicotinic acid to nicotinamide (Collins and Chaykin, 1971). Liver and intestine serve as centers for conversion. Chaykin et al. (1965) had previously reported that in mice, nicotinamide and nicotinic acid are not metabolically equivalent forms of the vitamin. Mason and Kodicek (1970) showed nicotinic acid cannot be used by the rat, also. It must first be converted to nicotinamide.

CHAPTER III

THE PROBLEM FOR STUDY

What are the effects of nicotinamide upon sleep in the human mammalian organism? What is the relationship of increased levels of nicotinamide to the regional concentrations of brain monoamines, specifically norepinephrine and 5-hydroxytryptamine along with its metabolite 5-hydroxyindoleacetic acid?

To study the problem a two-pronged approach is necessary. Both human and animal work is needed.

From the review of the literature it can be seen that although many facts are known numerous questions remain unanswered. Some events in sleep and brain biochemistry are well established but lack explanation for why they occur.

From the work of Dahlstrom and Fuxe and other neuroanatomists the specific location of cell bodies containing 5-HT, dopamine and norepinephrine is well established. Projection tracts and the major neurotransmitters are also well-defined (Dahlstrom and Fuxe, 1964; Anden et al., 1965 and 1966; Lorens and Guldberg, 1974; Morgane and Stern, 1973).

5-Hydroxytryptamine has been implicated in SWS by anatomical and chemical lesioning of the 5-HT containing neurons in the pons

and mid-brain raphe (Jouvet, 1967, 1969 and 1973). The injection of 5-HTP, a precursor of 5-HT, increases SWS in both animals and man (Griffiths et al., 1971; Ursin, 1974). PCPA when injected into animals blocks tryptophan hydroxylase and selectively decreases 5-HT without altering other amines. There occurs a marked decrease in SWS (Jouvet, 1969 and 1973; Rechtschaffen et al., 1973; Dement et al., 1973; King, 1974; Ursin, 1974). With continued administration of PCPA, Dement found substantial amounts of sleep returned.

The role catecholamines play in the sleep process is more contradictory and remains unestablished. Some researchers have shown that the administration of reserpine which depletes catecholamines increased REM sleep (Tissot, 1965). Others have reported the reappearance of REM sleep when catecholamines have been repleted after reserpine depletion (Jouvet, 1967). Similarly, there is evidence in the literature that AMPT which blocks catecholamine synthesis both suppresses REM sleep (Jouvet, 1969) and does not lower REM sleep (Stein et al., 1974; Morgane and Stern, 1973). Another piece of the puzzle is that MAOI's in Jouvet's laboratory work decreased REM sleep and increased SWS even when the animal was REM deprived (1967 and 1969). Many researchers have found that bilateral destruction of the locus coerulus, where large numbers of norepinephrine containing cell bodies are located, produces total suppression of REM sleep.

As one can readily see, the role of catecholamines and their relationship to 5-HT is far from definitive. There are many established researchers who have found that an inhibition of

catecholamine synthesis produced an increase in 5-HT turnover and, conversely, increasing 5-HT significantly decreased catecholamines, specifically norepinephrine (Stein et al., 1974; Everett, 1974; Morgane and Stern, 1973). Some have postulated that the area postrema is a site likely to have 5-HT receptor function.

Since tryptophan can be converted to niacin via the pyrrolase-quinolinic acid route and tryptophan is the substrate from which 5-HT is obtained in vivo, the relationship of niacin to sleep becomes of major interest. Since Beaton et al. (1974 and 1975) found high dosages of nicotinamide produced significant increases in REM sleep in mice, one wondered what this vitamin would do to human sleep. If there are changes in human sleep, how is it effected? Do neurotransmitter levels change? If so, in what regions of the brain? What can be postulated then about the mechanisms of sleep?

A search of the niacin related literature shows many facts are known concerning its metabolism in the mammalian organisms. Some of these are the following. NADPH strongly inhibits tryptophan pyrrolase. NADH and nicotinamide are inhibitory as well, but at higher concentrations. Both tryptophan and corticoids, by different mechanisms, induce the pyrrolase enzyme (Cho-Chung and Pitot, 1967 and 1968). Nicotinamide does significantly increase total brain 5-HT in rats (Scherer and Kramer, 1972). Nicotinamide induces tyrosine transaminase and could thus reduce catecholamine synthesis (Shimoyama et al., 1972).

It follows from the above that the hypotheses to be tested are

(1) Nicotinamide in large dosages will produce significant changes in human sleep especially REM sleep among both normal sleepers and sleepers with moderate to severe insomnia.

(2) When varying dosages of nicotinamide are injected into mice there will occur significant alterations in brain neurotransmitters.

   (a) 5-HT and 5-HIAA will be increased in at least some regions of the brain and

   (b) Norepinephrine will be decreased significantly in some or all regions of the brain.

CHAPTER IV

MATERIALS AND METHODS

Human Research

Normal Sleepers

Six subjects with normal sleep patterns established by in-laboratory sleep recordings meeting the criteria of Williams, Karacan and Hursch (1974) were studied. Each night's sleep record consisted of an electroencephalogram (EEG), an electro-oculogram (EOG), and an electromyogran (EMG). Electrodes were placed over the right central cortex and left mastoid, mid-eye and outer canthus, and sternocleidomastoid muscle (2). Sleep records were staged according to the criteria of Rechtschaffen and Kales (1973). Each subject was recorded for four nights without drug and served as his or her own baseline or control. After baseline data were obtained, nicotinamide by oral route was begun according to the following schedule: one gram in two divided doses the first day of drug, two grams in two separate doses the second day and three grams (one gram three times a day) on the third through the 23rd day of the drug.

On nights 14, 15, and 16 and on nights 21, 22 and 23 the subjects returned to the laboratory where sleep records were

obtained. The subjects were then withdrawn from medication and returned to the laboratory for sleep recordings on nights 19, 20 and 21 of recovery.

Three men and three women ages 21 to 47 years participated in this aspect of the study. Two of the male subjects did not return for recovery nights of recordings. As is traditionally done, the first nights of each series of 4 or 3 nights of recordings were not used to allow for the subjects' adaptation to the laboratory.

Subjects with Insomnia

Two subjects with moderate to severe insomnia were studied according to the protocol discussed above. The degree of insomnia was established by baseline in-laboratory recordings of sleep. Both subjects were female, ages 40 and 50 years.

All subjects were screened by Dr. John Smythies for history of liver disease and other factors which would make their participation contraindicated. The amide preparation of the vitamin, however, has little known toxicity and few untoward side effects.

The subjects were kept on nicotinamide for three weeks since Smythies and others in personal communications with the author have observed in clinical use that the full effect of niacin is not manifest until approximately the third week of use. The same rationale was used in obtaining recovery sleep records after approximately three weeks of drug withdrawal.

It was felt that the use of placebos in a separate group of sleepers or these sleepers was not necessary. Placebos can increase

sleep time by decreasing sleep latency. The latter was at a minimum in the normal sleepers and the amount of REM in the insomniacs increased far beyond that expected with a simple increase in sleep time. Further, it is doubtful that placebos have any control upon the time spent in the various stages of sleep after sleep onset. Kales et al. (1971) in a 17-night study of 12 insomniac subjects found no significant effects upon sleep induction, sleep maintenance or sleep stages. The mean sleep latency varied by only 3 minutes from baseline. Placebo administration did not produce any significant change in REM percent, REM time, or number of REM periods. Hartmann and Cravens (1973) reported on the use of placebos in normal human subjects and found that during 2 months on placebo that waking time was decreased slightly and that REM time increased by 1.4%. This persisted into the month following withdrawal from placebo. He reports these mean differences from baseline to be significant. However, he does not show clearly whether he is reporting analysis of total sleep time (TST) or of total recording time (TRT).

The participants signed forms giving their consent to be used in the study. Proper approval was gained from the Human Use Committee before the study was begun. An example of both forms can be found in Appendix F.

Animal Research

Random bred Swiss male mice weighing approximately thirty grams each were divided into groups of six. Each animal in each group was injected for 14 days with nicotinamide subcutaneously in the back at

approximately the same time each morning. Each group was treated with one of the following dosages of nicotinamide; 125 mg/kg, 250 mg/kg, 375 mg/kg, or 500 mg/kg. Groups of saline injected animals served as controls.

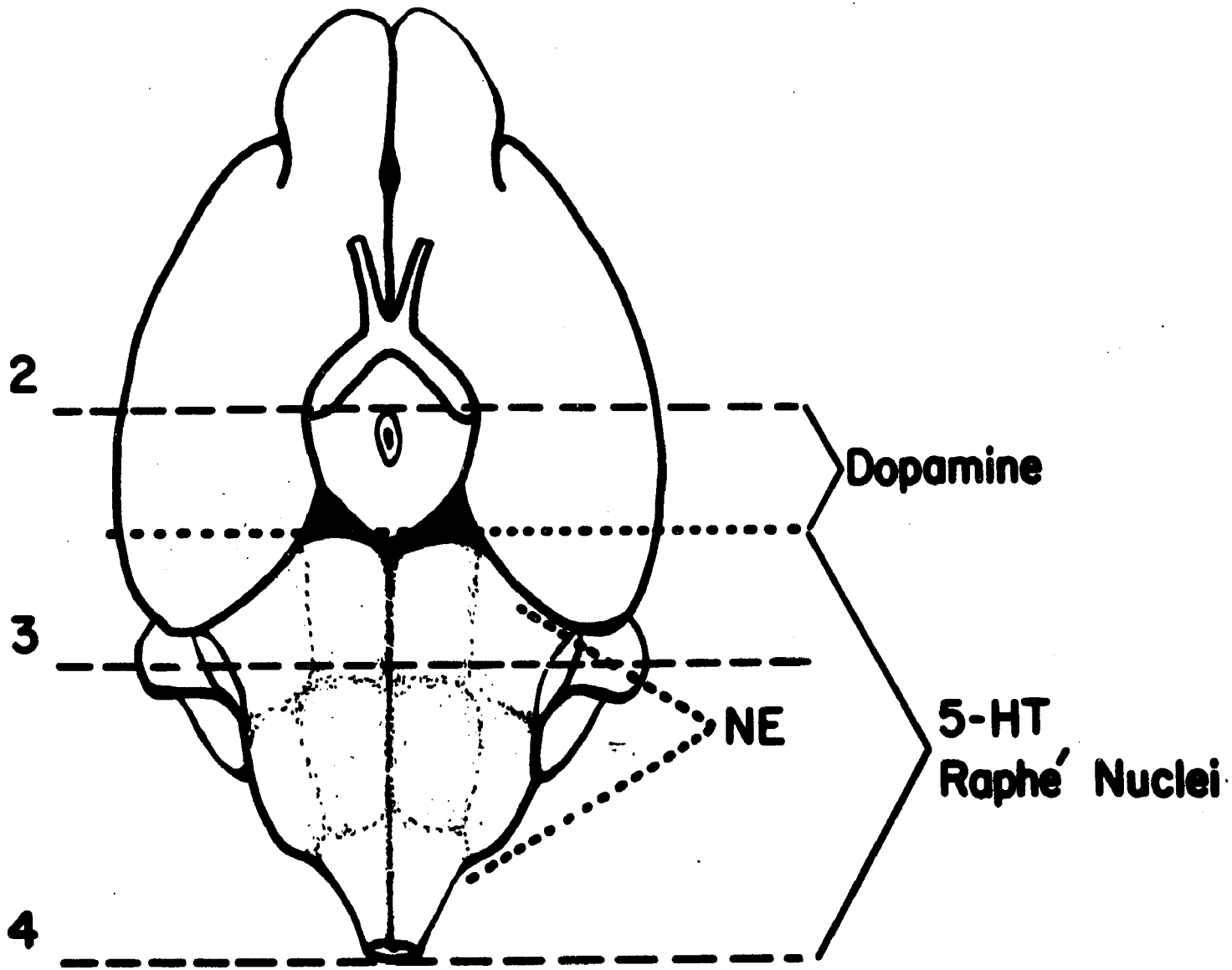
Scherer and Kramer (1972) gave one gm/kg of nicotinamide to rats and found a 17% increase in total brain serotonin which was significant. However, Beaton et al. (1974a, b, and 1975) found significant alterations in sleep records of mice after injections of 250 mg/kg.

Time after the 14th injection to sacrificing the animals seemed crucial. Bonavita and associates (1961) found that maximal levels of NAD in mouse liver after single injections of high doses of nicotinamide were reached 8 hours after injection. Scherer and Kramer (1972), however, found increased levels of 5-HT in the brain in response to nicotinamide, i.p., upon decapitation at 60 minutes after injection. Collins and Chaykin (1972) found that, with normal levels of nicotinamide, radioactivity increased in all tissues including brain from 5 to 30 min. after injection and had begun to decline at 60 min. It appeared, then, that nicotinamide might reach some peak activity level within 60 min. NAD levels probably would be continuously high with daily injections for 14 days.

At one hour after the 14th injection each mouse was sacrificed very quickly by decapitation with a minimum of handling and dissection of the brain performed on a cold glass plate. For regional studies the brain was divided into four sections as follows (Fig. 1).

FIGURE 1

Sectioning of the brain for neurotransmitter assays, ventral view. 1-cerebellum (not shown), 2-telencephalon, 3-diencephalon-mesencephalon, 4-lower pons-medulla. Areas of greatest concentration of neurotransmitters are shown. ------denotes sites of sectioning.


2
3
4
Dopamine
NE
5-HT
Raphé Nuclei

The cerebellum was removed first. Then from the ventral side of the brain a coronal cut was made at the knee of the internal capsule or the caudal visible end of the optic tract. The rostral portion plus the two occipital poles became the telencephalon which contained caudate and putamen as well as other structures such as the hippocampus and amygdala.

The remaining brain was then subdivided by coronal sectioning just below the inferior colliculus on the dorsal side of the brain. The rostral portion of this then was the diencephalon-mesencephalon section and the caudal portion became the lower pons and medulla section.

Sectioning the brain by this method means that the lower pons-medulla section most likely contains 5-HT nuclei B1 through B6, and the locus coerulus with cells high in NE. The mesencephalon-diencephalon section contains 5-HT raphe cell bodies, B7 through B9. One would then expect the former section of control animals to be high in NE and the latter to be higher in 5-HT (Fuxe and Jonsson, 1974). Through our knowledge of projection tracts, the telencephalon section would be high in NE.

The brain sections of 6 animals were quickly transferred to cold test tubes which had previously been weighed. The tubes and tissue were then reweighed and the weight of each tissue sample obtained. The tubes containing brain tissue were then covered and quickly frozen at -72°C until determinations could be done. For a table of brain weights and length of time frozen see Appendix A.

Table 1 shows the dosages of nicotinamide, the number of animals receiving each dose level, and the number of biochemical determinations made at each drug level after pooling six sectioned mouse brains for each determination. The latter category can be further subdivided so that for each region of the brain the number is as shown in Table 2.

TABLE 1

Number of Animals Used and Number of Determinations of Neurotransmitter Levels per Six Animals

Nicotinamide mg/kg	Number of animals injected	Number of determinations of neurotransmitters
0 (Saline)	30	5
125	24	4
250	30	5
375	24	4
500	30	5

TABLE 2

Number of Determinations for Each Region of the Brain

Nicotinamide mg/kg	Cerebellum	Telencephalon	Diencephalon-Mesencephalon	Lower Pons-Medulla
0 (Saline)	5	5	5	5
125	4	4	4	4
250	5	5	5	5
375	4	4	4	4
500	5	5	5	5

Regional determinations of NE, 5-HT and 5-HIAA were done using simultaneous spectrophotofluorometric methods. Regis Chemical Company Kit Number 190020 was obtained and used for the assays. For an outline of the procedure and a list of the solutions see Appendix B.

The value of this method for determining changes in neurotransmitter levels and the metabolite 5-HIAA associated with REM sleep lies in the fact that nicotinamide is a dietary product and has few untoward effects in high doses. Also, the animals being studied are not subjected to nonspecific stress as is often done in REM deprivation studies.

It is best to have a minimum of 280 mg of tissue. Refer to Appendix B for an outline of the procedure. Homogenization of tissues in 10 vols% of acidified n-butanol extracts substances in the aqueous phase. Ansell and Beeson (1968) found higher tissue butanol ratios cause tissue components to precipitate later and also gives rise to higher blanks. The addition of HCl elutes 5-HT and NE. The addition of heptane makes it possible to re-extract 5-HT and NE into aqueous acid. 5-HTP is found in brain in small amounts and is completely removed during the procedure. Heptane also removes lipids which gather at the interface between organic and inorganic phases. The Regis procedure was derived from many authors by whom the above actions were also discovered (Udenfriend et al., 1955; Bogdanski et al., 1956; Maickel et al., 1968; Cox and Perhach, 1973; Smith et al., 1975).

5-HIAA is extracted from the organic phase by a base, specifically sodium bicarbonate.

5-HT and 5-HIAA fractions are made highly fluorescent by the addition of orthophthaldialdehyde (OPT). The 5-HT, 5-HIAA and OPT interactions are not known. Fluorescence depends upon concentration and time of reaction. By heating at 100°C fluorescence peaks at 10 minutes.

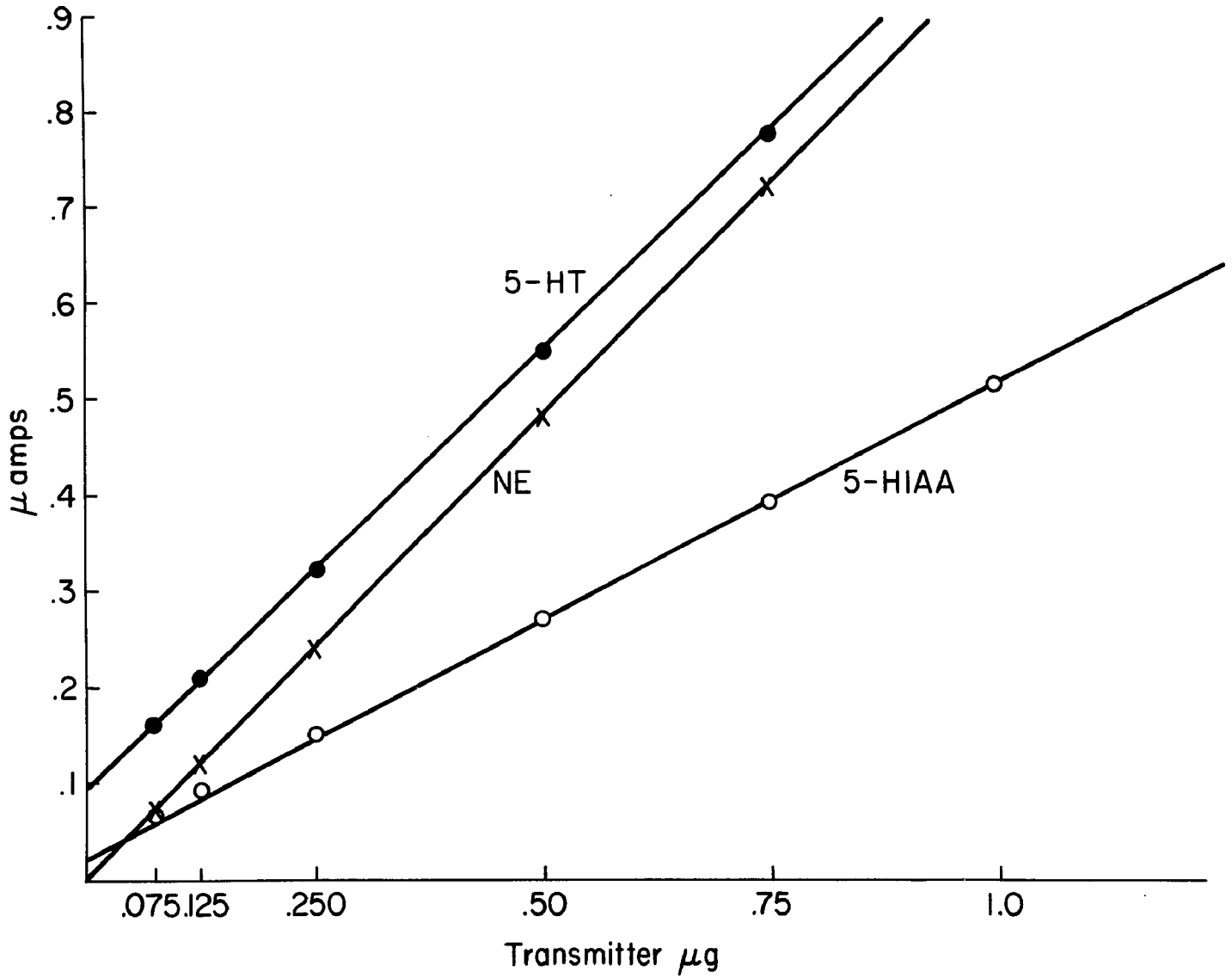
The addition of EDTA to the NE fraction makes recovery more predictable and the fluorphore last longer. Ethanolic iodine oxidizes the NE to form the fluorphore. Alkaline sulfite stops the oxidation process. After a pH adjustment the NE fraction is heated at 100°C for 2 minutes at which time fluorescence peaks. The NE fluorphore is stable for 60 min at room temperature. The 5-HT fluorphore is stable for several days (Komesu and Thompson, 1971; Chang, 1964; Ansell and Beeson, 1968).

Optimum wave lengths in nanometers can be found by scanning. The Farrand Optical Company spectrofluorometer was standardized each day, in so far as possible, with a quartz rod on sensitivity of 10 at 7.5 microamps. Excitation and emission slits were 20 and 10 nm, respectively. Wave lengths for standardization were 500 and 500 for activation and emission, respectively.

A dilution curve was run from the kit standards and was found to be linear by the least squares method in the range from 0.075 to 25 μg. The curve has been plotted in Fig. 2 ranging from 0.075 to 1.0 μg for 5-HT, NE and 5-HIAA. The dilutions with their fluorometric readings can be seen in Appendix C.

FIGURE 2

Dilution curves for the standards (5-HT, 5-HIAA, and NE) in the range from 0-1.0 µg by the method of least squares.


.9
.8
.7
.6
.5
.4
.3
.2
.1
μ amps
5-HT
NE
5-HIAA
.075
.125
.250
.50
.75
1.0
Transmitter μg

After the extractions of 5-HT, 5-HIAA and NE were done and spectrofluorometric readings obtained for tissues, recoveries and blanks, the levels were calculated by the following method of Regis Kit No. 190020.

$$\frac{(X-Y)}{(Z-Y)} \times \frac{1000}{M} = \text{concentration } (\mu g/g \text{ tissue})$$

X = fluorescence reading of sample

Y = fluorescence reading of blank

Z = fluorescence reading of recovery

M = weight of tissue sample in mg.

On each day that an assay was done recovery tubes containing one µg of each standard and blanks containing distilled water were prepared and carried through the extraction procedure with the tissue samples. This corrected for daily variations in cleanliness of glassware, ambient temperature, changes in fluorometer response, and so forth. Extraction efficiency, in the hands of this researcher, varied from 30% to over 60%. Maximum efficiency in the hands of others is about 80%.

There is a problem with high blanks found by all researchers using this and similar procedures, especially with the OPT blanks. Ansell and Beeson (1968) and Cox and Perhach (1973) found the limit of sensitivity is a recovery reading of twice the blank. A ratio smaller than two results in erroneous values. Early in this study problems with high blanks were experienced. As technique improved

high blanks became no longer a problem. Recovery to blank ratios can also be seen in Appendix C.

CHAPTER V

PRESENTATION, ANALYSIS AND INTERPRETATION OF DATA

Human Work

After scoring each night's record for each subject (6 normal sleepers and 2 subjects with insomnia) percent of total recording time and percent of total sleep time were calculated. Averages for each subject for each period of the study and sleep efficiency can be seen in Appendix D.

Subjects with Normal Sleep Patterns

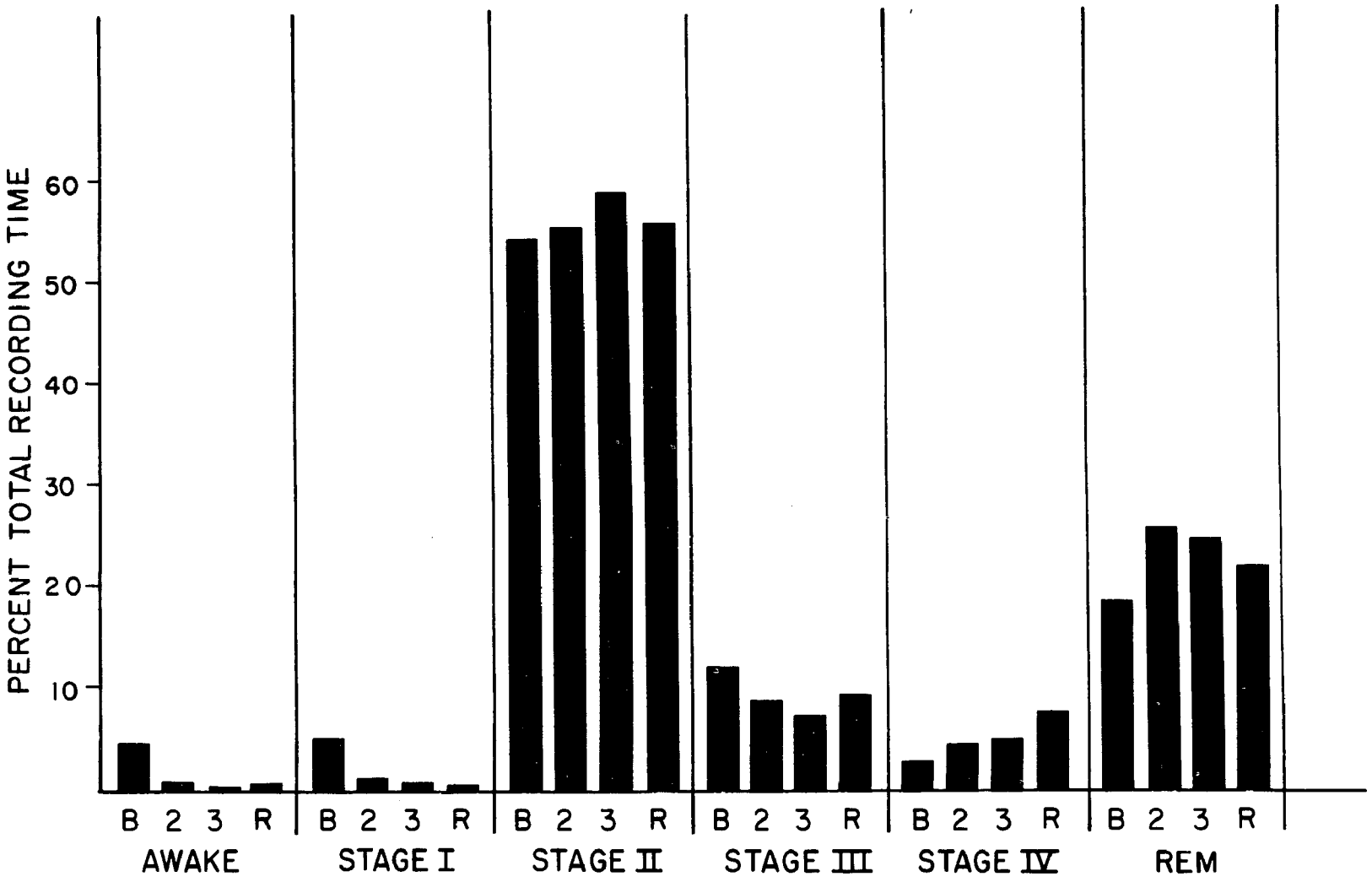
Alterations in percent sleep of total recording time of the normal sleep subjects can be seen in Table 3 and Fig. 3. Data for the second and third week represent averages of nights 15 and 16 and nights 22 and 23 of nicotinamide intake. Recovery data are averages of nights 20 and 21 after the withdrawal of nicotinamide.

For all subjects, by Mann-Whitney U tests, there was a significant increase in REM sleep at the 0.002 probability level, using baseline and recovery as control data and data after two and three weeks as experimental. There was no significant difference between baseline and recovery data. The changes in all other stages of

FIGURE 3

The effects of nicotinamide upon sleep in normal subjects. B=baseline data before drug administration; 2=nights 15 and 16 following two weeks of three grams per day of nicotinamide ingestion; 3=nights 22 and 23 of drug intake; R=nights 20 and 21 after withdrawal of nicotinamide.

THE EFFECTS OF NICOTINAMIDE UPON SLEEP
(NORMAL SUBJECTS)



sleep were not significant when compared with baseline and recovery. Sleep efficiency (the time asleep as percent of total recording time) was not significantly altered.

TABLE 3

Alterations in Percent Sleep of Total Recording Time for All Normal Sleepers

	Stages of Sleep						
	A	I	II	III	IV	REM	N
Baseline	5.3	5.9	55.0	11.7	3.7	18.4	18
2nd Week	1.5	1.8	56.5	9.7	4.8	25.7*	12
3rd Week	0.5	0.8	59.5	8.7	5.2	25.0*	11
Recovery	1.0	0.7	56.7	9.8	8.7	23.1	8

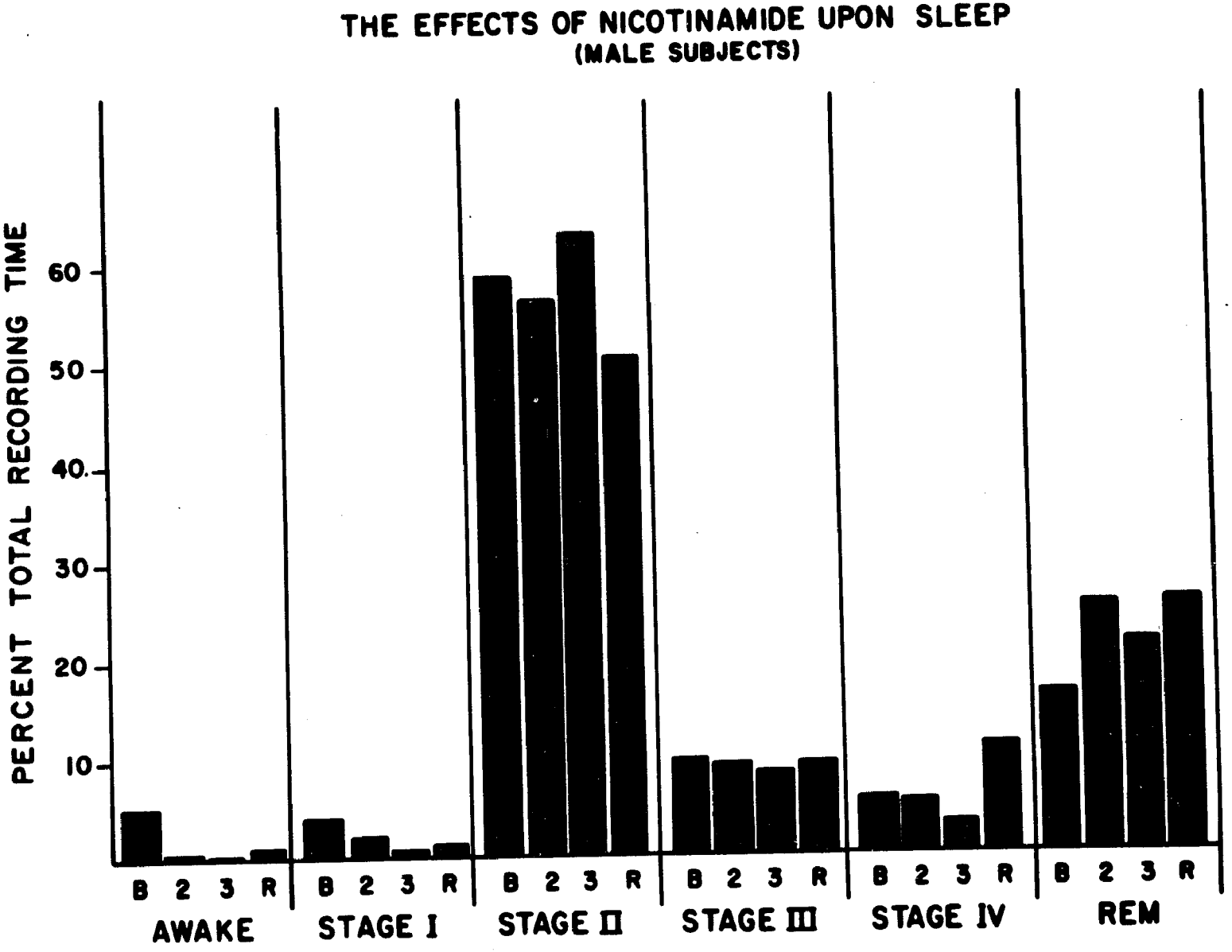
*Significant at $p = 0.002$ over control.

Data were also analyzed in terms of percent of the various stages of total sleep time. Since the normal subjects had little awake time the latter analysis of data was predictable. Again, REM sleep varied significantly from control data at $p = 0.0027$. As in the analysis of total recording time, there were no significant differences among the other stages of sleep of total sleep time.

The effects of nicotinamide upon the three male subjects can be seen in Table 4 and Fig. 4. Upon recovery, the percent REM did not return to baseline. This, however, is not significant since the

FIGURE 4

Percent changes in awake and Stages I-IV plus REM for three male subjects with normal sleep patterns. B=baseline data before drug administration, 2=nights 15 and 16 following two weeks of three grams per day of nicotinamide ingestion, 3=nights 22 and 23 of drug intake, R=nights 20 and 21 after withdrawal of nicotinamide.


THE EFFECTS OF NICOTINAMIDE UPON SLEEP
(MALE SUBJECTS)
PERCENT TOTAL RECORDING TIME
60
50
40
30
20
10
B 2 3 R
AWAKE
STAGE I
STAGE II
STAGE III
STAGE IV
REM

recovery represents only one male subject and two nights of data. The two other male subjects withdrew from the study during the third week of drug. The percent increase in REM for male subjects after two and three weeks of nicotinamide over baseline is significant at $p = 0.05$.

TABLE 4

Alterations in Percent Sleep of Total Recording Time for Normal Male Sleepers

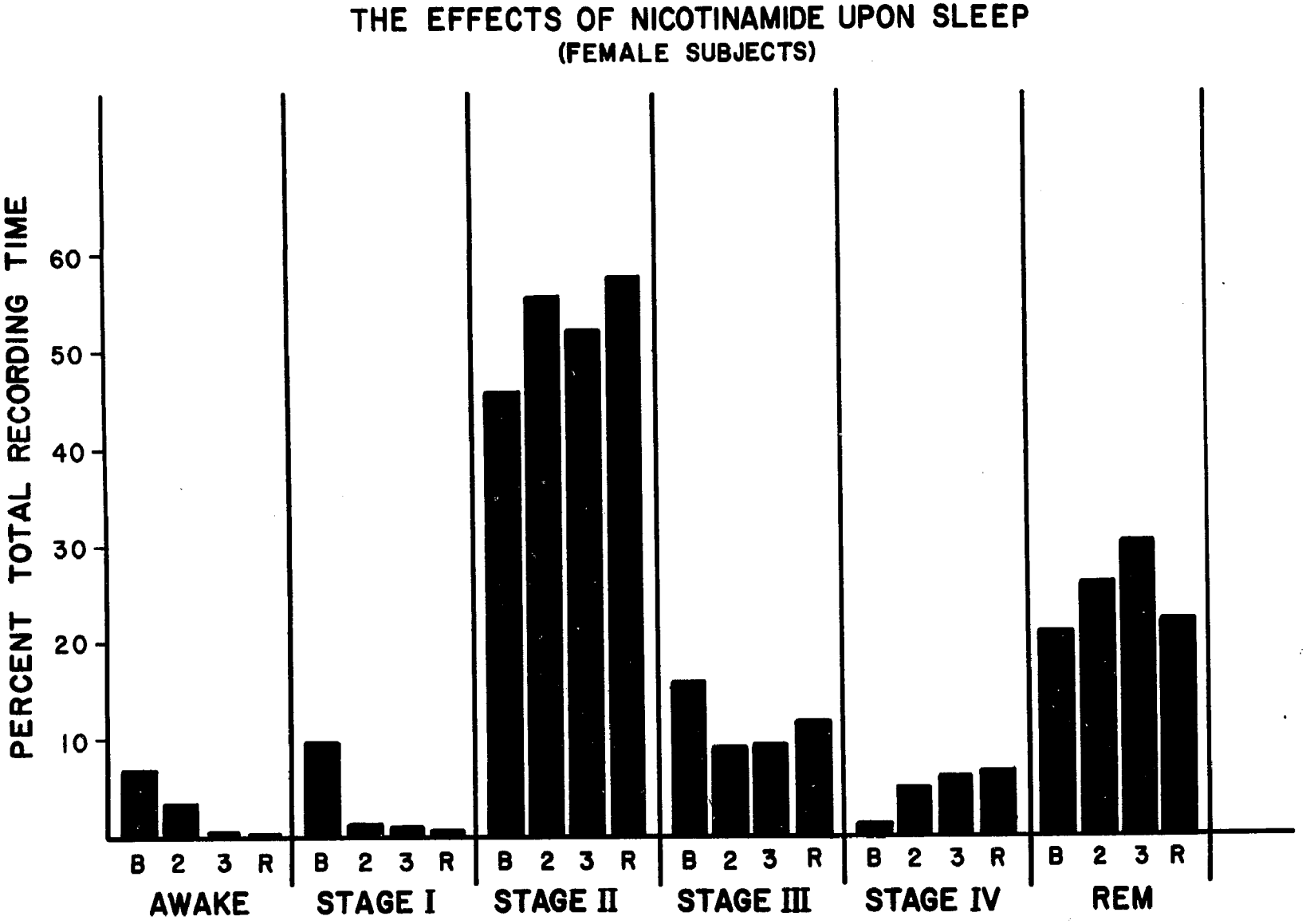
	Stages of Sleep						
	A	I	II	III	IV	REM	N
Baseline	5.3	4.2	58.7	9.8	5.7	16.4	8
2nd Week	0.6	2.2	56.6	9.5	5.5	25.6+	6
3rd Week	0.3	1.1	64.8	8.2	3.3	22.0+	5
Recovery*	1.1	1.4	50.8	9.6	11.3	25.8	2

*Only one subject and two nights of data.
+Significant at $p = 0.05$ over baseline.

Data for the normal female sleepers in Table 5 and Fig. 5 do show REM on recovery returning to near baseline levels. Stage IV for female subjects was significantly increased over baseline at two and three weeks and remained elevated upon recovery at a p value of 0.02. For REM percent of total recording time baseline and recovery data did not significantly differ. Therefore, using baseline and

FIGURE 5

Percent changes in sleep patterns for female subjects with normal sleep patterns before, during and after 23 days of oral intake of three grams of nicotinamide per day. B= baseline data before drug administration, 2=nights 15 and 16 following two weeks of nicotinamide intake, 3=nights 22 and 23 of drug intake, R=nights 20 and 21 after withdrawal of nicotinamide.


THE EFFECTS OF NICOTINAMIDE UPON SLEEP
(FEMALE SUBJECTS)
PERCENT TOTAL RECORDING TIME
60
50
40
30
20
10
B 2 3 R
AWAKE
STAGE I
STAGE II
STAGE III
STAGE IV
REM

recovery as control, REM during two and three weeks was significantly elevated by Mann-Whitney U test at $p = 0.02$.

Awake and Stage I decreased throughout the study and may be indicative of some property of nicotinamide. However, this may reflect adaptation to sleeping in the laboratory, only.

TABLE 5

Averages of Alterations in Percent Sleep of Total Recording Time for Normal Female Sleepers

	Stages of Sleep						
	A	I	II	III	IV	REM	N
Baseline	5.2	7.7	51.2	13.5	1.7	20.5	8
2nd Week	2.3	1.4	56.4	9.8	4.2+	25.5*	6
3rd Week	0.6	0.6	54.2	9.1	7.1+	28.1*	6
Recovery	0.9	0.5	58.6	9.9	7.8+	22.2	6

*Significant at $p = 0.02$ over control.
+Significant at $p = 0.02$ over baseline.

Again, for all six normal subjects and all nights of data, the changes in REM after the second and third week were significantly different from the data gathered during baseline and recovery. The fact that female subjects varied in some aspects of sleep when male subjects did not is not surprising when one considers the variations in hormonal influence and cyclic changes. One general impression is that the male subjects REM less and do not increase absolute REM

time to as high levels as do females. One female subject had a high of 35.9% REM. Another had a high of 34.7% REM. Both occurred after the second week of nicotinamide. Only one male subject had a REM percentage over 30 (31.0%). Conversely, during baseline one male subject had only 9.5% REM. The lowest REM% recorded for a female subject was 13.6%.

Subjects with Insomnia

Two subjects with moderate to severe insomnia were recruited for study using the protocol described in Chapter IV, Materials and Methods. Again, baseline data were obtained (three nights) followed by laboratory recordings after two and three weeks of nicotinamide intake (3 gm/day). Recovery data were gathered three weeks after drug withdrawal. Table 6 and Fig. 6 show the percent of time in each stage of sleep during each aspect of the study.

TABLE 6

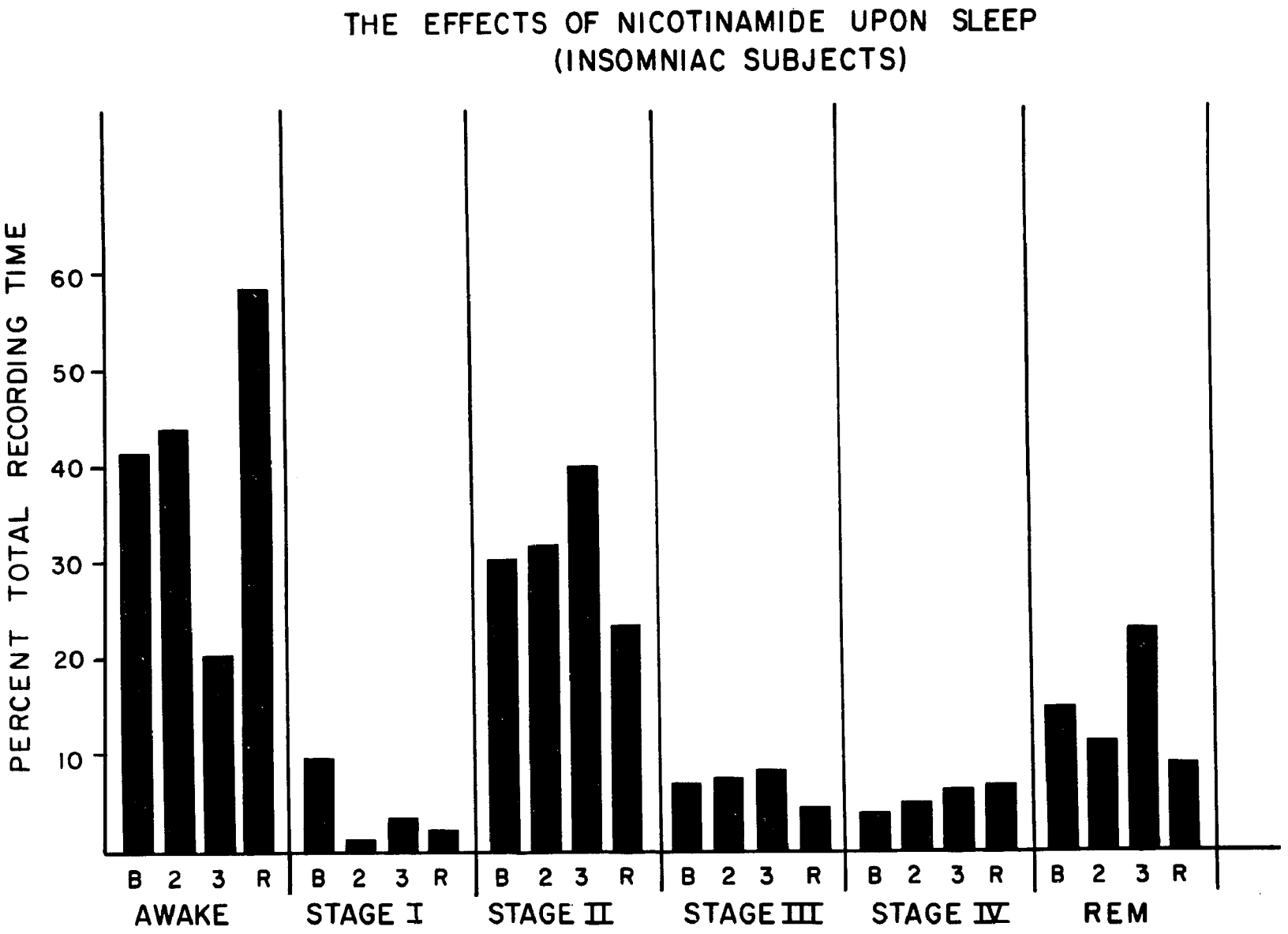
Alterations in Percent Sleep of Total Recording Time for Insomniac Subjects

	Stages of Sleep						
	A	I	II	III	IV	REM	N
Baseline	41.5	2.3	30.5	7.2	4.8	13.7	6
2nd Week	44.3	2.1	32.0	7.3	5.0	9.3	4
3rd Week	20.5+	3.6	40.2	8.5	4.1	23.1*	4
Recovery	58.6	2.6	24.4	4.5	1.6	8.3	4

*Significant at $p = 0.001$.
+Significant at $p = 0.002$.

FIGURE 6

The effects of nicotinamide upon sleep in subjects with insomnia. B=baseline data before drug administration; 2=nights 15 and 16 following two weeks of three grams per day of nicotinamide ingestion; 3=nights 22 and 23 of drug intake; R=nights 20 and 21 after withdrawal of nicotinamide.


THE EFFECTS OF NICOTINAMIDE UPON SLEEP
(INSOMNIAC SUBJECTS)
PERCENT TOTAL RECORDING TIME
60
50
40
30
20
10
B 2 3 R
AWAKE
STAGE I
STAGE II
STAGE III
STAGE IV
REM

For the subjects with insomnia, nicotinamide did not improve sleep within two weeks, but by the third week of drug ingestion, there were significant results. Again, using baseline and recovery data as control, by Mann-Whitney U test, REM sleep was significantly increased after the third week at the 0.001 probability level. Awake time was significantly decreased after three weeks at the 0.002 probability level. No other significant changes in sleep were noted.

Sleep efficiency for both subjects was 58.5% on baseline, 55.7% after two weeks and 79.5% after three weeks. On recovery from nicotinamide, sleep efficiency dropped to a low 41.5%.

It might appear that the increases in REM sleep simply occurred as a result of the total increase in sleep time. Although this is a factor, it was noted that one subject had a 26.8% and 37.1% REM of total sleep time on nights 22 and 23 respectively. The other subject had 32% and 19.9% REM of total sleep time on the same nights. Therefore, it appears that simply increasing total sleep time does not explain the phenomenon entirely. The answer awaits further study, however.

Variance Between Normal Sleepers and Insomniacs

Analysis of variance among baseline sleep records by each day between the normal sleepers and the subjects with insomnia showed percent awake and percent sleep to be significantly different at a p value of less than 0.002. See Table 7.

TABLE 7

A Comparison of Percent Changes in Total Recording Time of Sleep Stages for Normal Sleepers and Subjects with Insomnia

% TRT	Baseline		Nicotinamide					
			Two Weeks		Three Weeks		Recovery	
Sleep Stages	Normal	Insomnia	Normal	Insomnia	Normal	Insomnia	Normal	Insomnia
Sleep	94.7	58.5	98.5	55.7	99.5	79.5	99.0	41.4
Awake	5.3	41.5	1.5	44.3	0.5	20.5	1.0	58.6
Stage I	5.9	2.3	1.8	2.1	0.8	3.6	0.7	2.6
Stage II	55.0	30.5	56.5	32.0	59.5	40.2	56.7	24.4
Stage III	11.7	7.2	9.7	7.3	8.7	8.5	9.8	4.5
Stage IV	3.7	4.8	4.8	5.0	5.2	4.1	8.7	1.6
REM	18.4	13.7	25.7	9.3	25.0	23.1	23.1	8.3

The two groups of sleep subjects were drawn from two very different populations. REM changes behaved differently between the two groups. After two weeks, percent REM of TRT increased significantly in the normal sleepers and decreased (non-significantly) in the insomniac subjects. After three weeks both normal and insomniac sleepers showed significant increases in REM, normal at $p = 0.0027$ and insomniac at p less than 0.001. The p values were, again, obtained by Mann-Whitney U test using baseline and recovery as control data. The latter two did not significantly differ.

Animal Data

Tables containing the fluorescence readings and tables of calculated 5-HT, 5-HIAA, and NE levels in μg/g can be found in Appendix E. Also, contained within these tables are the recovery to blank ratios. Only data having a ratio of two or greater, especially the OPT data, were used in analysis of variance because of the problem discussed in Chapter IV, Materials and Methods.

Means and standard error for each drug level for each of the four brain sections for each neurotransmitter were calculated. Duncan's test was used for analysis of variance. For purposes of this paper data for each transmitter will be discussed separately followed by a discussion of their relatedness to each other.

5-Hydroxytryptamine

The mean levels of 5-HT at varying doses of nicotinamide in the four regions of mouse brain (Table 8) show a general increase

TABLE 8

5-Hydroxytryptamine (µg/g of Tissue Wet Weight)
Means, Standard Errors, and Numbers of Assays for Each Brain Section

Nicotinamide mg/kg	Cerebellum µg/g	Telencephalon µg/g	Diencephalon-Mesencephalon µg/g	Lower Pons-Medulla µg/g
0	0.357 ± 0.10(3)	0.312 ± 0.03(3)	0.982 ± 0.14(3)	0.788 ± 0.17(3)
125	0.865 ± 0.19(4)	0.583 ± 0.05(4)	3.143 ± 0.65(4)	3.495 ± 0.52(4)
250	0.733 ± 0.23(3)	0.555 ± 0.19(3)	1.770 ± 0.68(3)	2.290 ± 1.27(3)
375	0.622 ± 0.24(4)	0.461 ± 0.16(4)	2.132 ± 0.74(4)	2.010 ± 0.74(4)
500	1.010 ± 0.19(4)	0.631 ± 0.08(4)	3.068 ± 0.19(4)	1.920 ± 0.37(4)

in the neurotransmitter. All mean control values are comparable to those found by Ansell and Beeson (1968) and by others using a similar assay method. The standard error was somewhat high in some cases, probably due to the limited number of assays.

5-HT levels of saline injected animals were higher in the diencephalon-mesencephalon and lower pons-medulla sections as expected.

There is a significant difference in nicotinamide action at the various sites in the brain (Fig. 7). That is, the same dosage decreased the 5-HT levels in one region of the brain and increased them at another site. This nicotinamide-nicotinamide interaction is significant at $p = 0.0241$. Not unexpectedly, the site did significantly influence the drug interaction. The drug-site interaction is significant at $p = 0.001$.

All dosages of nicotinamide increased 5-HT in all regions of the brain over saline control animals. See Fig. 7.

Duncan's Multiple Range Test.--In the cerebellum (CB) nicotinamide 500 mg/kg produced the highest levels followed by 125, 250, and 375 mg/kg. However, at the 95% confidence level the variations of any drug level from any other drug level or from saline controls were not significant in this brain section.

The changes in 5-HT levels in the telencephalon (TE) behaved in a manner relatively like those in the cerebellum (Fig. 8). The only difference was that the concentration of 5-HT is less than in the cerebellum. In fact 5-HT is lower in the telencephalon than in all other areas of the brain under all experimental conditions.

FIGURE 7

The effect of increasing dosages of nicotinamide upon mean 5-HT levels in four different sites or regions of the mouse brain. (Transmitter levels are measured in μg/g of wet tissue weight and nicotinamide in mg/kg of body weight.)

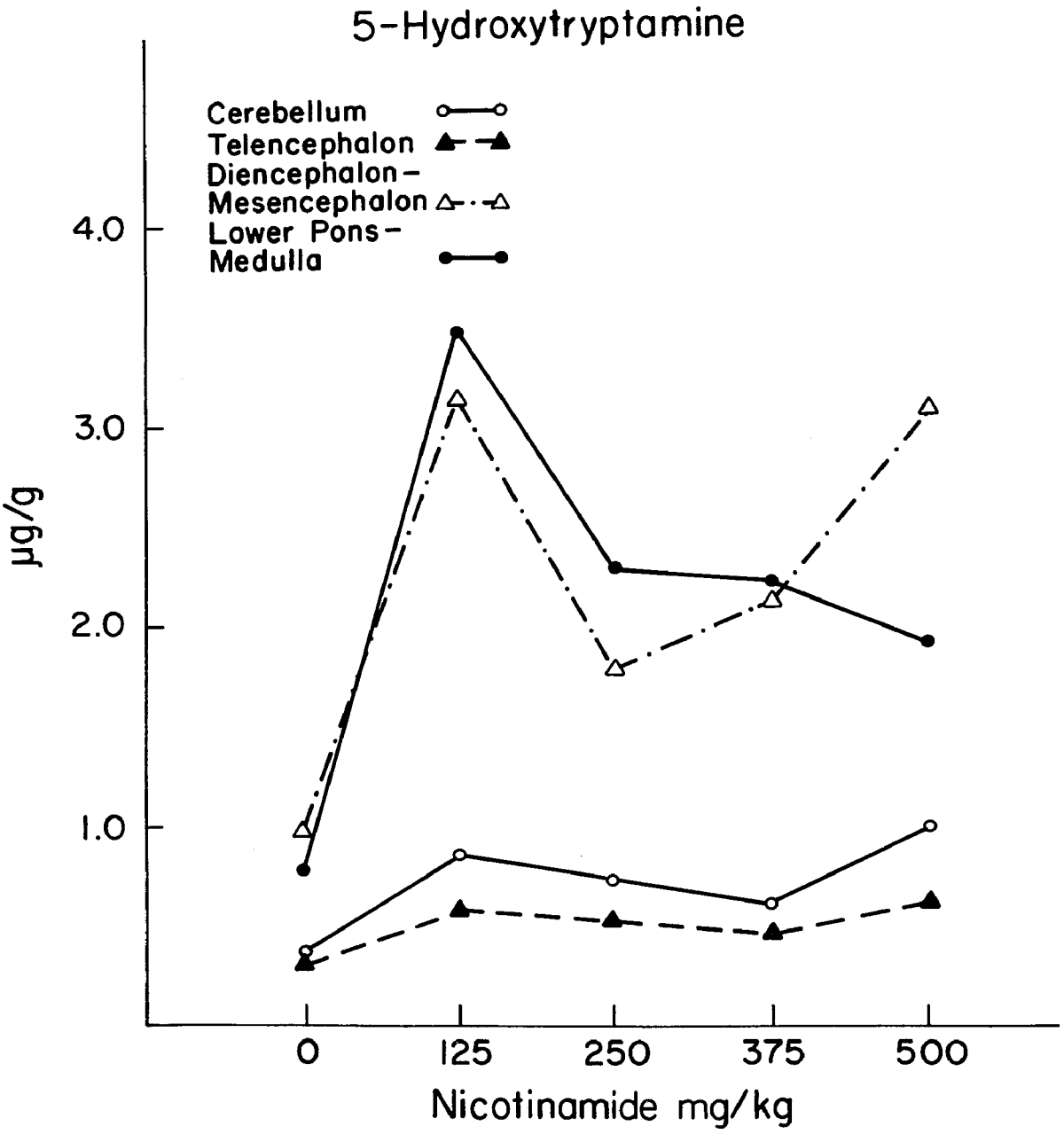
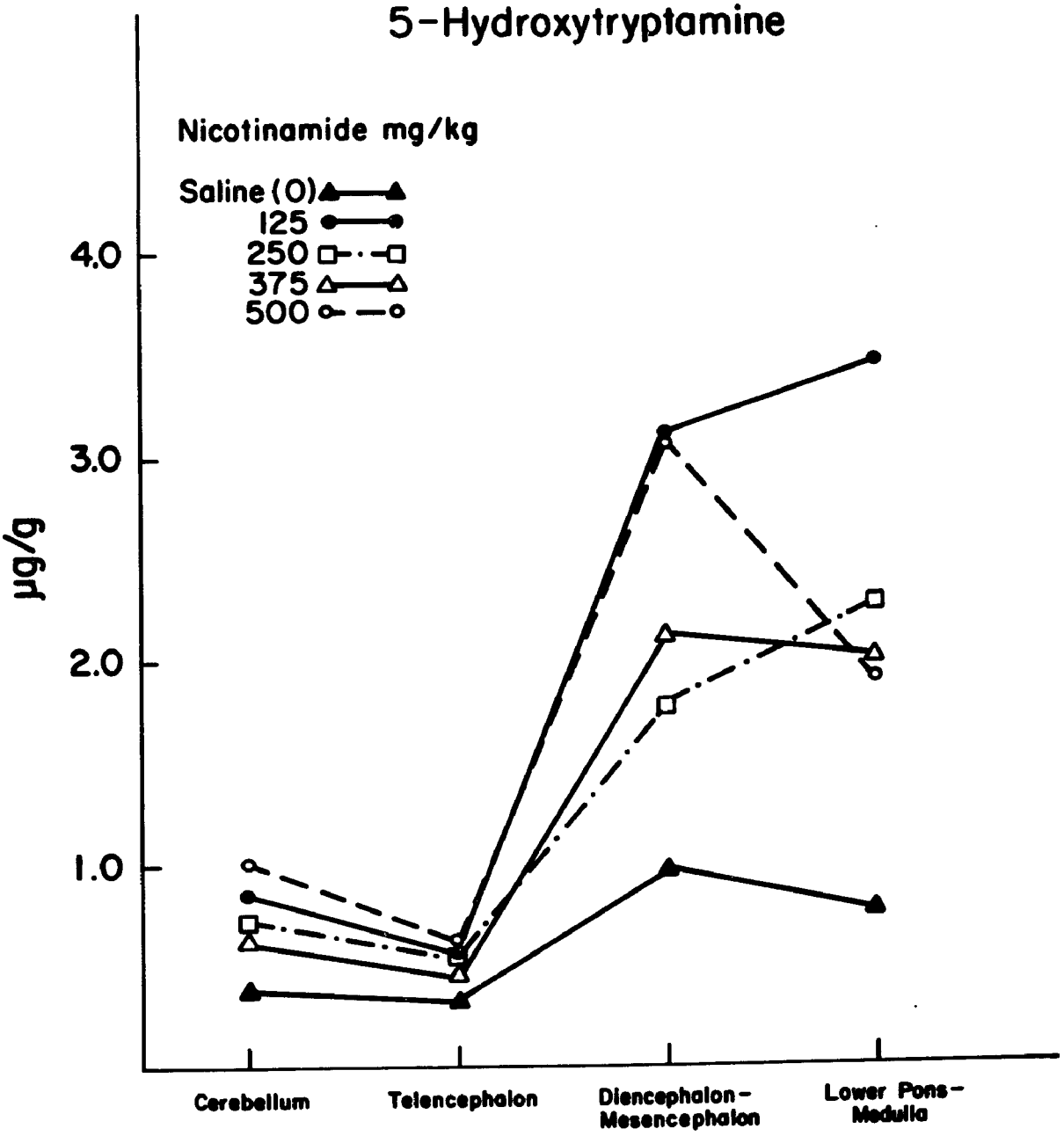

5-Hydroxytryptamine
Cerebellum
Telencephalon
Diencephalon–
Mesencephalon
Lower Pons–
Medulla
4.0
3.0
2.0
1.0
μg/g
0
125
250
375
500
Nicotinamide mg/kg

FIGURE 8

Changes in mean levels of 5-HT in each of four regions of the mouse brain as dosages of the drug are increased.


5-Hydroxytryptamine
Nicotinamide mg/kg
Saline (0)
125
250
375
500
μg/g
4.0
3.0
2.0
1.0
Cerebellum
Telencephalon
Diencephalon-
Mesencephalon
Lower Pons-
Medulla

Variations in 5-HT in the diencephalon-mesencephalon (D-M) portion of the brain presented a somewhat more complicated situation (Table 8 and Figs. 7 and 8). The effect of nicotinamide upon 5-HT concentrations was greatest at the 125 mg/kg level followed in descending order by 500, 375, 250, and 0 or saline. From the saline control animals the lowest concentration of 5-HT was obtained. The high levels produced by 125 and 500 mg/kg were significantly different from saline control at $p = 0.05$. The 5-HT values obtained from 375 and 250 mg/kg fell in the middle and did not differ from each other, from saline controls or from 125 and 500 mg/kg values.

Activity in the lower pons-medulla (LP-M) section varied in yet another way; again refer to Figs. 7 and 8. The 125 mg/kg dosage again produced the highest concentration of 5-HT and was significantly different from the 375, 500, and 0 values (lowest three) but did not vary significantly from the 5-HT values obtained from 250 mg/kg (second highest). The lowest three were not different from each other or from control. Something interesting and somewhat perplexing happened in this portion of the brain and not seen in other sections. Dosages of nicotinamide and 5-HT concentration are related inversely, that is, the smallest dose of nicotinamide produced the greatest increase in 5-HT and the largest dose brought values closer to control. All dosages between (250 and 375) progressively lowered the values.

One can only speculate upon reasons for the above, based on the present data. It may lie within the field of enzyme kinetics. There may be a build-up of products from the enzyme induction by

lower dosages of nicotinamide (or some product thereof) which accumulates at progressively higher dosages. For example, lower dosages of nicotinamide (or some metabolic substance thereof) may induce an enzyme with the resulting increase in some product which at higher dosages then inhibits the enzyme activity. Or the increasing dosages of nicotinamide may, through another pathway, progressively produce products which inhibit enzymes in the 5-HT pathway. There may be any number of other possible explanations. In any event this research is not designed to answer that question.

An examination of the 5-HT data from another perspective was meaningful, again using Fig. 7 and the Duncan's test. This inspection was done by taking each dosage of drug alone and determining its activity at the various sites. There were no significant differences in mean 5-HT concentration among the four regions of mouse brain in the saline control animals. However, on the basis of the anatomy and physiology of the specific regions in question, 5-HT was predictably higher in the diencephalon-mesencephalon section, only slightly lower in the lower pons-medulla section and much lower in the cerebellum and telencephalon. The latter two contained almost equal amounts. This confirms much other research in the literature.

At the 125 mg/kg nicotinamide level (Fig. 8), 5-HT concentration was significantly higher in the diencephalon-mesencephalon and lower pons-medulla than in the cerebellum and telencephalon. Neither the D-M and LP-M nor the CB and TE differed significantly from each other. Again, this was predictable from anatomy and physiology of

the region. Nicotinamide 250 mg/kg resulted in the same significance though mean concentrations were lower in each site. Although means varied, the same significance obtained at the 375 mg/kg level.

The variance found in data generated by 500 mg/kg of nicotinamide deviated from the above pattern. This dosage significantly increased the 5-HT concentration in the diencephalon-mesencephalon section over the lower pons-medulla section and over the cerebellum and telencephalon sections. At the same time the concentration in the lower pons-medulla section (second highest) was significantly lower than both the D-M and in the CB and TE. The latter two were significantly lower than both the D-M and the LP-M but did not differ from each other. So, 500 mg/kg produced a three way variance showing that this drug level was producing the same type of changes but dramatically increased the mean level over the other dosages of nicotinamide.

5-Hydroxyindoleacetic Acid

Means and standard errors of 5-HIAA data for all nicotinamide levels and all regions of the mouse brain, Table 9, show no consistent changes in either elevating or decreasing the metabolite. Contrary to the 5-HT findings, nicotinamide at the various dosages acted similarly on all portions of the brain ($p = 0.3613$). For example, when a dosage of 500 mg/kg increased the 5-HIAA in the diencephalon-mesencephalon section it was not significantly different from the changes produced by other dosages or by the saline injections. There is no nicotinamide-nicotinamide interaction as appeared in the 5-HT data.

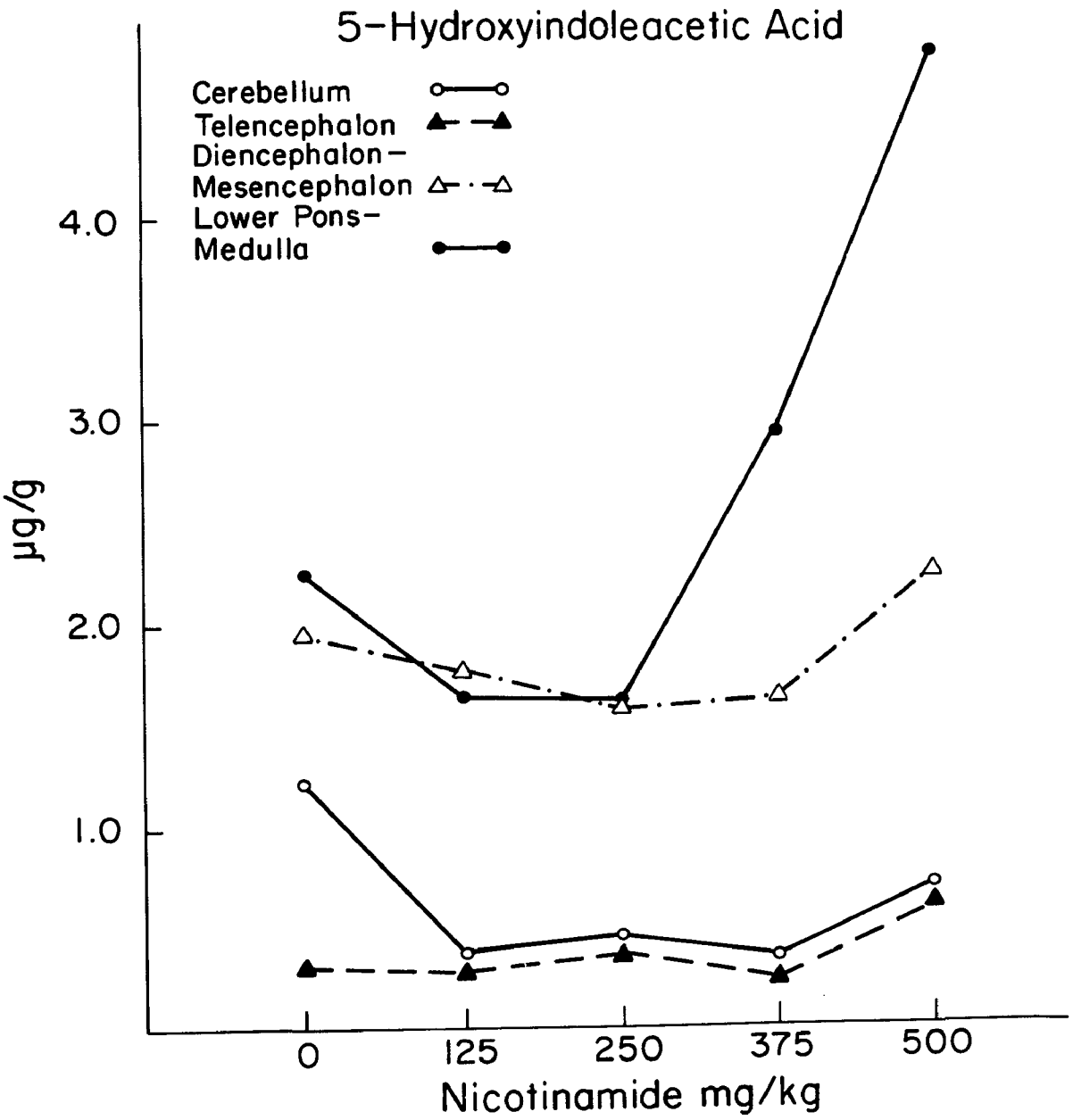
TABLE 9

5-Hydroxyindoleacetic Acid (µg/g of Wet Tissue Weight)
Means, Standard Errors, and Number of Assays for Each Brain Section

Nicotinamide mg/kg	Cerebellum µg/g	Telencephalon µg/g	Diencephalon-Mesencephalon µg/g	Lower Pons-Medulla µg/g
0	1.220 ± 0.44(3)	0.312 ± 0.06(3)	1.952 ± 0.05(3)	2.251 ± 0.27(3)
125	0.393 ± 0.05(4)	0.296 ± 0.02(4)	1.775 ± 0.17(4)	1.655 ± 0.23(4)
250	0.456 ± 0.21(4)	0.353 ± 0.11(4)	1.594 ± 0.55(4)	1.624 ± 0.70(4)
375	0.349 ± 0.08(4)	0.240 ± 0.04(4)	1.646 ± 0.39(4)	2.965 ± 1.21(4)
500	0.714 ± 0.33(3)	0.600 ± 0.24(3)	2.248 ± 0.59(3)	4.825 ± 2.21(3)

FIGURE 9

The effects of increasing dosages of nicotinamide upon mean 5-HIAA levels in four different sites or regions of the mouse brain. (The metabolite is measured in µg/g of wet tissue weight and nicotinamide in mg/kg of body weight.)


5-Hydroxyindoleacetic Acid
Cerebellum
Telencephalon
Diencephalon–
Mesencephalon
Lower Pons–
Medulla
4.0
3.0
2.0
1.0
µg/g
0
125
250
375
500
Nicotinamide mg/kg

Some sites, however, did show significant differences in the level of metabolite when compared with other levels in other sites, Fig. 10. This is discussed further below.

Duncan's Multiple Range Test.--For the variable 5-HIAA this test showed that the lower pons-medulla section responded significantly ($\alpha = 0.05$) differently from both the diencephalon-mesencephalon and from the cerebellum and telencephalon. The latter two did not vary from each other. Also, the D-M levels were significantly different from both the LP-M and from the CB and TE portions of brain. Thus, there was a three-way significance in difference in the drugs at these sites. Some of this significance can be explained upon the basis of knowledge of anatomy and physiology of the brain which was sectioned to gain this maximum difference.

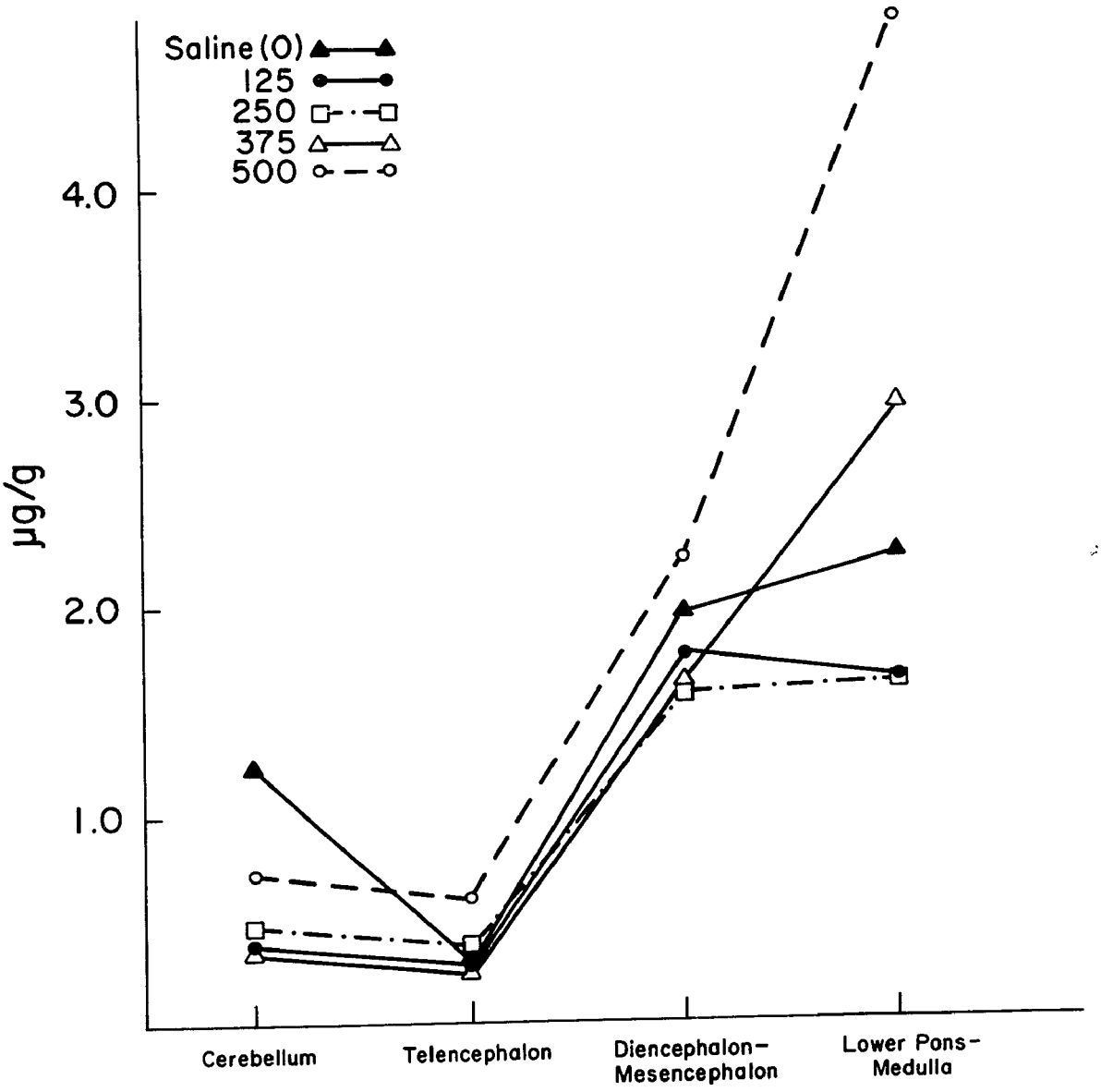
It is readily noticeable in Fig. 10 that there were other differences. These are as follows.

(a) Nicotinamide did not change the levels of 5-HIAA in the cerebellum significantly. All levels were lowered but not meaningfully, Fig. 9 and 10. In the telencephalon there was the same lack of response of 5-HIAA levels to the nicotinamide dose response curve. The diencephalon-mesencephalon section showed higher levels both control and experimental but there were no significant changes related to the drug injections.

(b) The nicotinamide dosage of 500 mg/kg produced a highly significantly different level in the lower pons-medulla section beyond that of saline and all lower dosages of nicotinamide. The

FIGURE 10

Changes in mean levels of 5-HIAA in each of four regions of the mouse brain as dosages of nicotinamide are increased.


5-Hydroxyindoleacetic Acid
Saline (0)
125
250
375
500
4.0
3.0
2.0
1.0
µg/g
Cerebellum
Telencephalon
Diencephalon–
Mesencephalon
Lower Pons–
Medulla

5-HT levels in this brain section produced by 500 mg/kg were not significantly different from controls. 5-HT was in fact higher in the LP-M section at the 125 mg/kg dosages discussed above. 5-HIAA was accumulating much more rapidly than 5-HT in the same region, LP-M.

Again, site differences at the various drug levels individually were examined. Control animals had significantly higher 5-HIAA levels in the lower pons-medulla from that level found in the telencephalon. Neither of the latter two sites varied meaningfully from the other two. Dosages of 125 and 250 did not change 5-HIAA levels in any brain section significantly from any other section. The 125 mg/kg level brought all levels down except in the telencephalon and thus reduced the differences to being non-meaningful. The 250 mg/kg was little different from the above (Fig. 10). However, at the 375 mg/kg level other changes were seen. The LP-M section varied significantly from the CB and the TE. Although 375 mg/kg increased the levels of 5-HIAA in the D-M section, this portion of the brain did not vary significantly from either the sections with higher 5-HIAA. Finally, at 500 mg/kg, 5-HIAA levels were elevated in the lower pons-medulla sections significantly over all other sites which did not vary from each other (Fig. 10).

Norepinephrine

As was seen in the 5-HT data the drug, nicotinamide, acted significantly differently on NE content of the various brain regions (Fig. 11). The drug-drug interaction was significant at

TABLE 10

Norepinephrine (µg/g)
Means, Standard Errors, and Numbers of Assays for Each Brain Section

Nicotinamide mg/kg	Cerebellum µg/g	Telencephalon µg/g	Diencephalon-Mesencephalon µg/g	Lower Pons-Medulla µg/g
0	1.657 ± 0.88(3)	1.230 ± 0.83(3)	2.050 ± 0.63(3)	2.089 ± 0.52(3)
125	1.433 ± 0.64(4)	0.668 ± 0.21(4)	4.018 ± 0.48(4)	4.763 ± 1.11(4)
250	1.033 ± 0.33(4)	0.279 ± 0.07(4)	1.376 ± 0.52(4)	2.267 ± 0.90)4)
375	1.243 ± 1.22(2)	0.138 ± 0.11(2)	1.772 ± 1.63(2)	2.322 ± 2.12(2)
500	1.721 ± 0.47(4)	0.359 ± 0.10)4)	2.145 ± 0.61(4)	2.551 ± 0.45(4)

FIGURE 11

The effect of increasing dosages of nicotinamide upon mean NE levels in four different sites or regions of the mouse brain. (NE levels are measured in μg/g wet tissue weight and nicotinamide in mg/kg of body weight.)

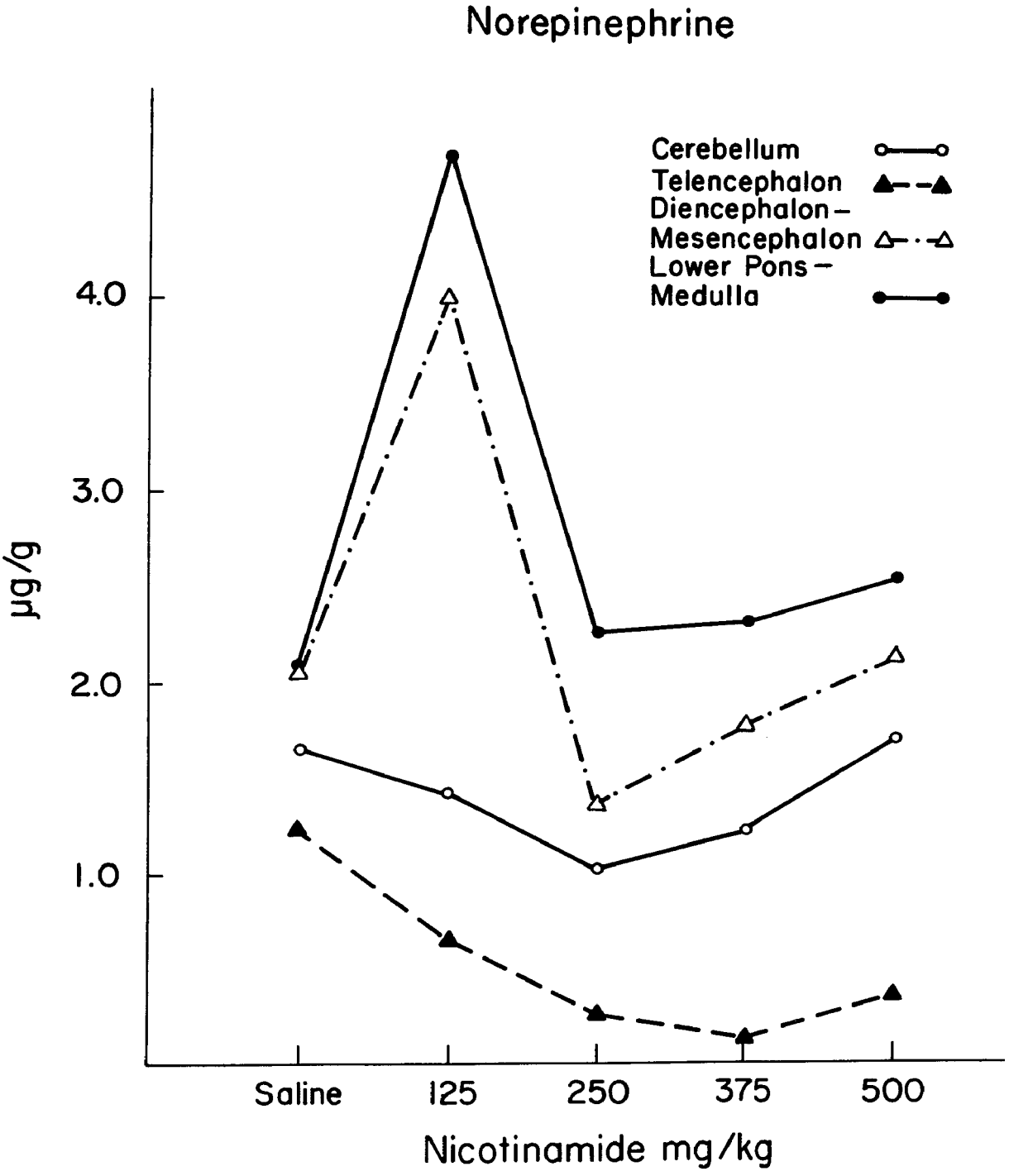
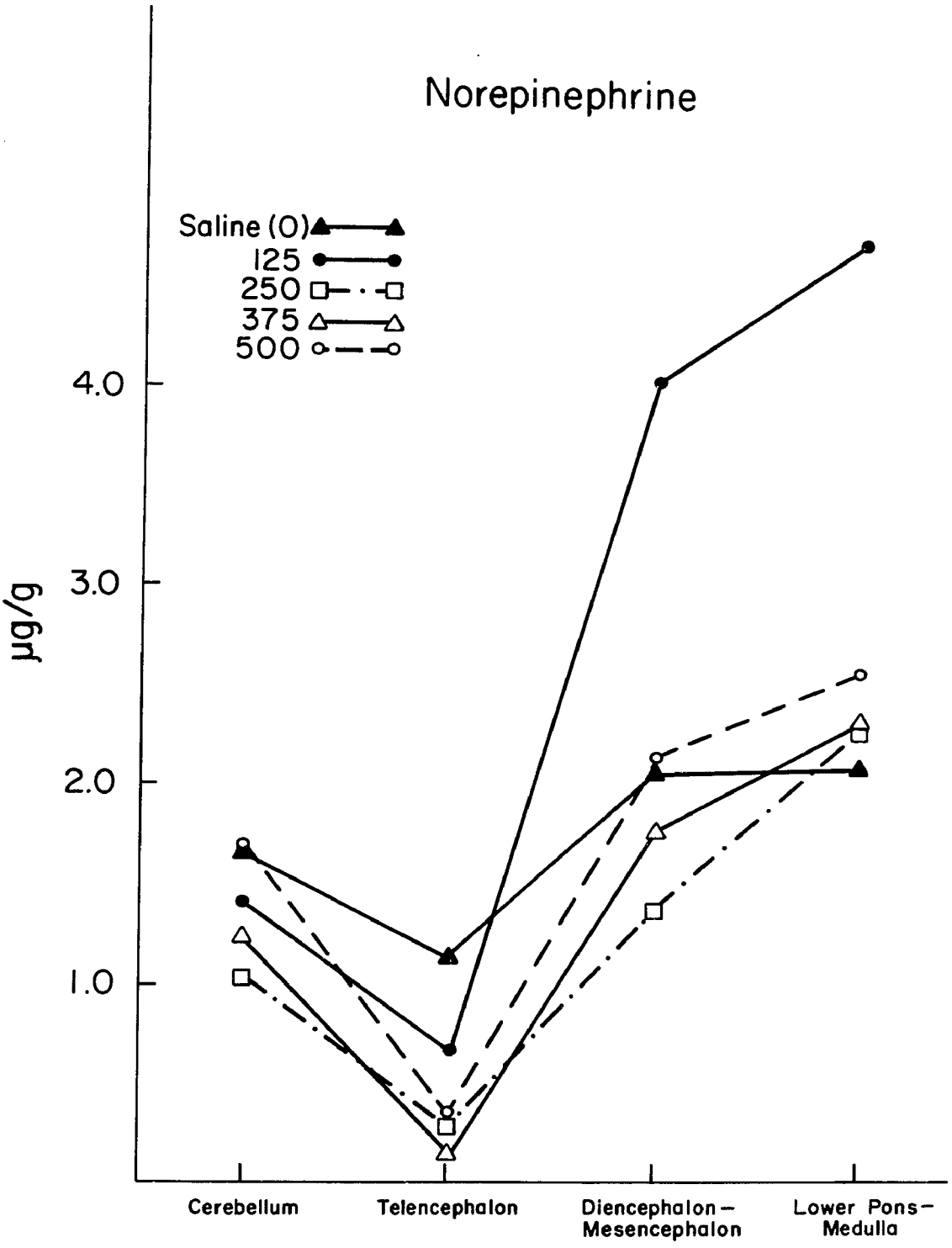

Norepinephrine
Cerebellum
Telencephalon
Diencephalon–
Mesencephalon
Lower Pons–
Medulla
4.0
3.0
2.0
1.0
µg/g
Saline
125
250
375
500
Nicotinamide mg/kg

FIGURE 12

Changes in mean levels of NE in each of four regions of the mouse brain as dosages of nicotinamide are increased.


Norepinephrine
Saline (0)
125
250
375
500
4.0
3.0
2.0
1.0
µg/g
Cerebellum
Telencephalon
Diencephalon–
Mesencephalon
Lower Pons–
Medulla

$p = 0.04$. Again, the sites did influence the differences found in levels of NE, $p = 0.0001$. Table 10 presents mean and standard errors in all regions and all drug levels.

Duncan's Multiple Range Test.--The test for variations in NE showed that in the cerebellum at $\alpha = 0.05$ there were no changes in NE levels at any drug level (Fig. 11). In the telencephalon section no changes were significant, again seen in Fig. 11. However, the diencephalon-mesencephalon section displayed a very high value of NE at the lowest dost of nicotinamide, 125 mg/kg. This was significantly greater than controls and other dosages of drug. Why a smaller dose produced a greater response was speculated upon in the discussion of 5-HT where a similar response was obtained though not significantly in the D-M section but significantly in the LP-M section. One can see in Fig. 11 that the same events occurred in the lower pons-medulla section for NE. The dosage, 125 mg/kg, produced a highly significantly greater level of NE in this portion of the brain.

At this time it must be pointed out that the researcher was considerably more skilled in the extraction procedure when the animals injected with 125 mg/kg were studied. However, since the 125 mg/kg extractions resulted in lowered NE levels, some below controls in some brain sections, the data are then probably not influenced entirely by researcher skills. Some real physiological event is occurring but not definable by this research design.

Continuing to use the Duncan's multiple range test for variation but looking at site variations from single drug levels one finds the following in NE levels (Fig. 12).

Control animals (or 0 drug) showed that NE did not vary significantly among the four sites. NE in baseline animals is higher in the diencephalon-mesencephalon and lower pons-medulla sections as predicted (Fig. 11). At 125 mg/kg NE rose to significantly higher levels in the lower pons-medulla and diencephalon-mesencephalon sections respectively above the cerebellar and telencephalon levels. The former two did not differ from each other meaningfully nor did the latter two differ from each other. Again, the 125 mg/kg of nicotinamide showed the phenomena discussed above.

At 250 mg/kg the mean NE level of 2.267 µg/g in the lower pons-medulla was significantly elevated over the 0.2785 µg/g in the telencephalon. The two remaining sites fell in-between and did not differ meaningfully from the highest or the lowest levels in the former sites (Fig. 12 and Table 10). The same events occurred at the 375 mg/kg level discussed immediately above. It was found, however, that at 500 mg/kg the NE telencephalon levels fell significantly lower than in all other sites in the brain, the latter not varying significantly from each other.

Interactions of 5-HT, 5-HIAA, and NE

Correlation Coefficients.--These were done by using mean transmitter levels and correlating each region of the brain with the

changes in each transmitter. Each region, for each transmitter and the metabolite, 5-HIAA, was correlated with every other region of the brain and the latter's mean changes in each transmitter due to nicotinamide action. This was done to get some indication of whether changes in transmitter levels in any region can be said to cause changes in other transmitters at that or other sites (Fig. 13, 14 and 15).

The highest correlation of changes in transmitter due simply to the changes in drug levels was found in the lowering of NE in the telencephalon. The correlation coefficient was -0.83 and the probability was -0.08, not quite significant (Fig. 14).

For two of the brain sections (TE and D-M), as 5-HT increased the 5-HIAA did so as well, though not significantly. In the remaining two sections (CB and LP-M), as 5-HT increased, the 5-HIAA decreased though again not significantly.

In every region of the brain as mean 5-HT was increased due to the increasing dosages of nicotinamide the cerebellar 5-HIAA, though not significant, showed a negative correlation. In the lower pons-medulla section, as the 5-HT increased the cerebellar 5-HIAA correlation was -0.81 and the probability 0.09 (Fig. 13).

One of the most interesting trends in the correlations, however, was that of the influence of changes in 5-HT levels upon NE. Increased in 5-HT levels due to increasing drug levels in all regions of the brain including the telencephalon were all negatively correlated with NE levels in the TE. Though none of these

FIGURE 13

Changes in mean levels of 5-HIAA and in 5-HT as dosages of nicotinamide are increased.

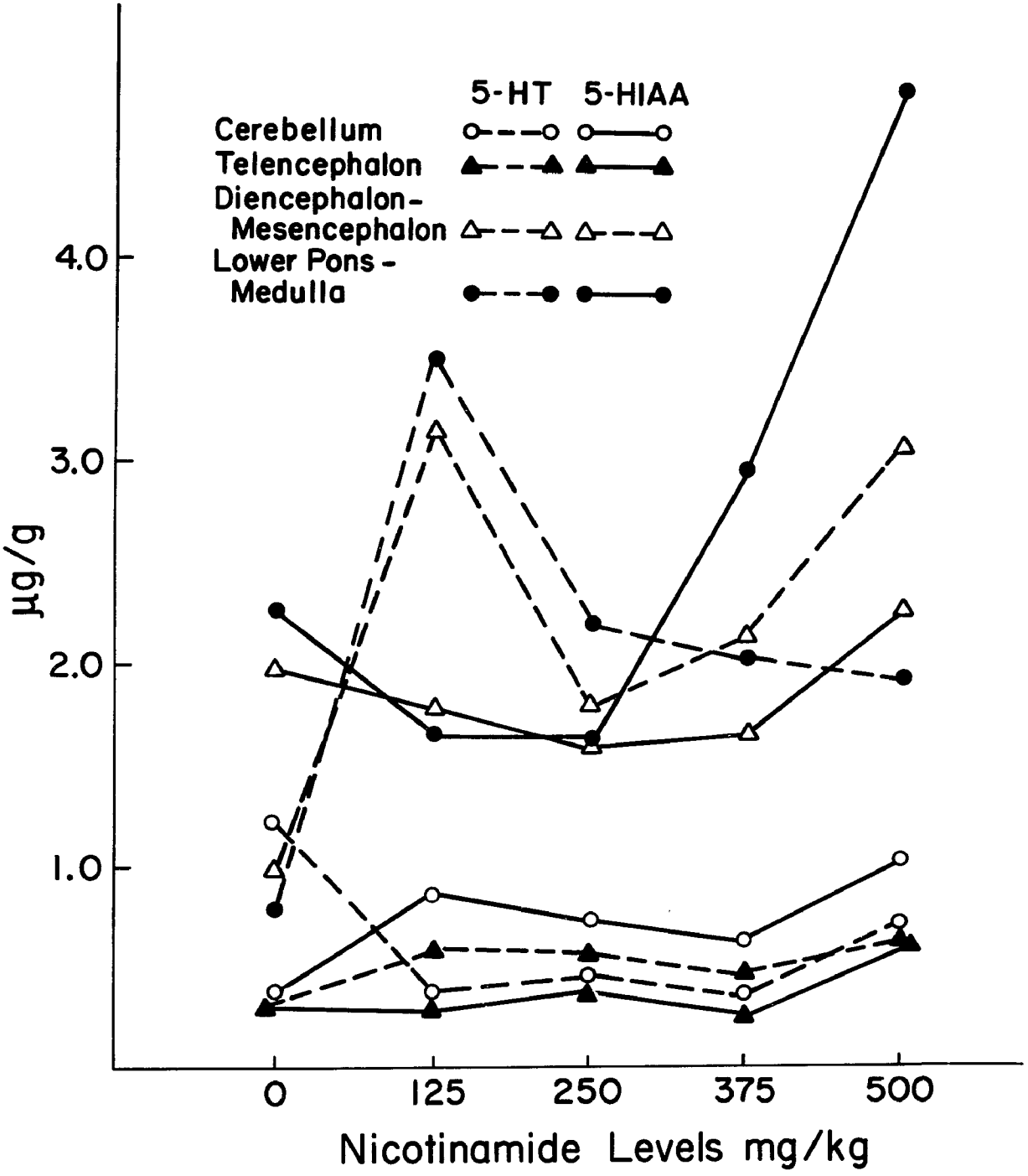

5-HT 5-HIAA
Cerebellum
Telencephalon
Diencephalon-
Mesencephalon
Lower Pons-
Medulla
4.0
3.0
2.0
1.0
μg/g
0
125
250
375
500
Nicotinamide Levels mg/kg

FIGURE 14

Changes in mean levels of 5-HT and in NE as dosages of nicotinamide are increased.

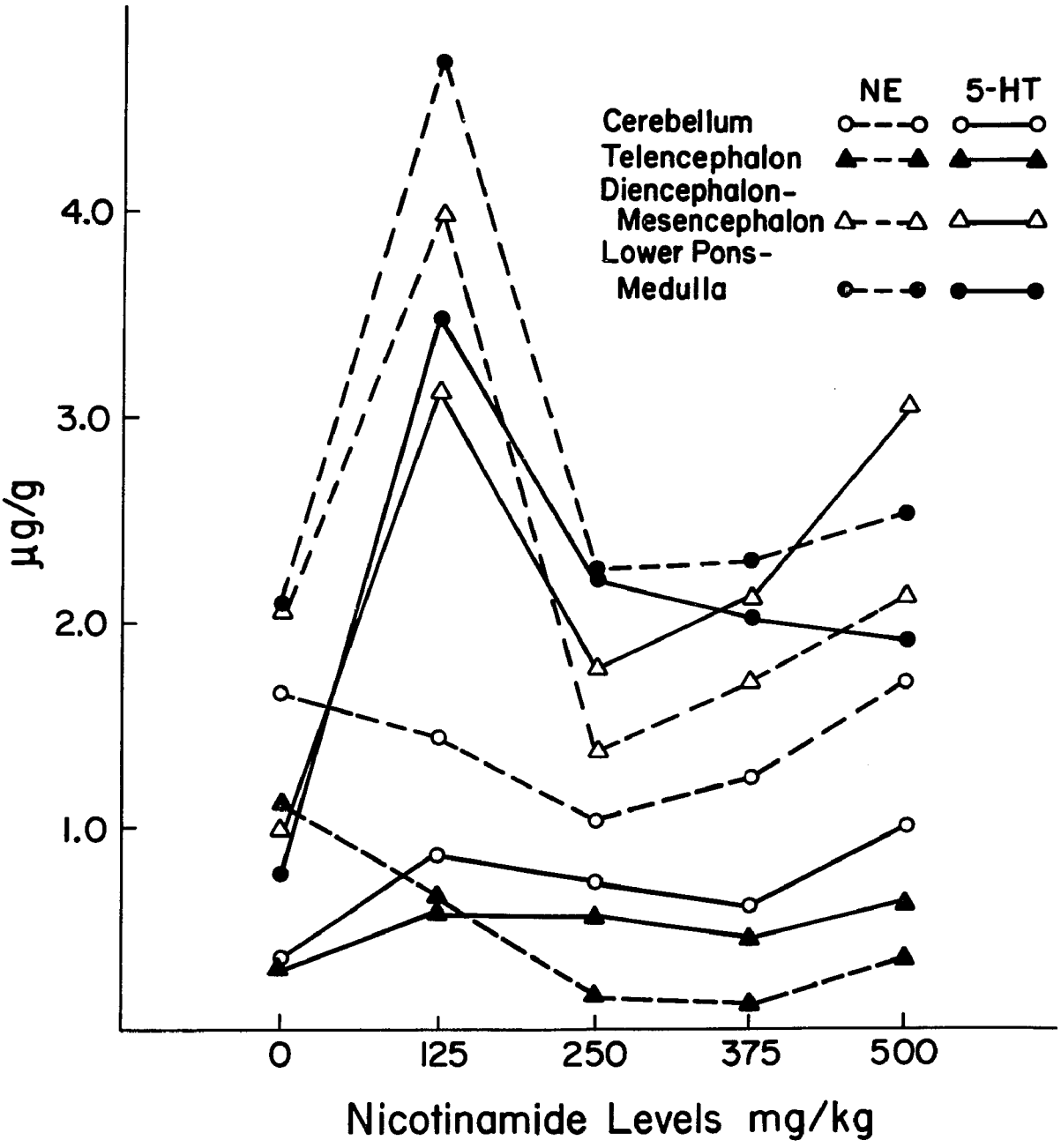
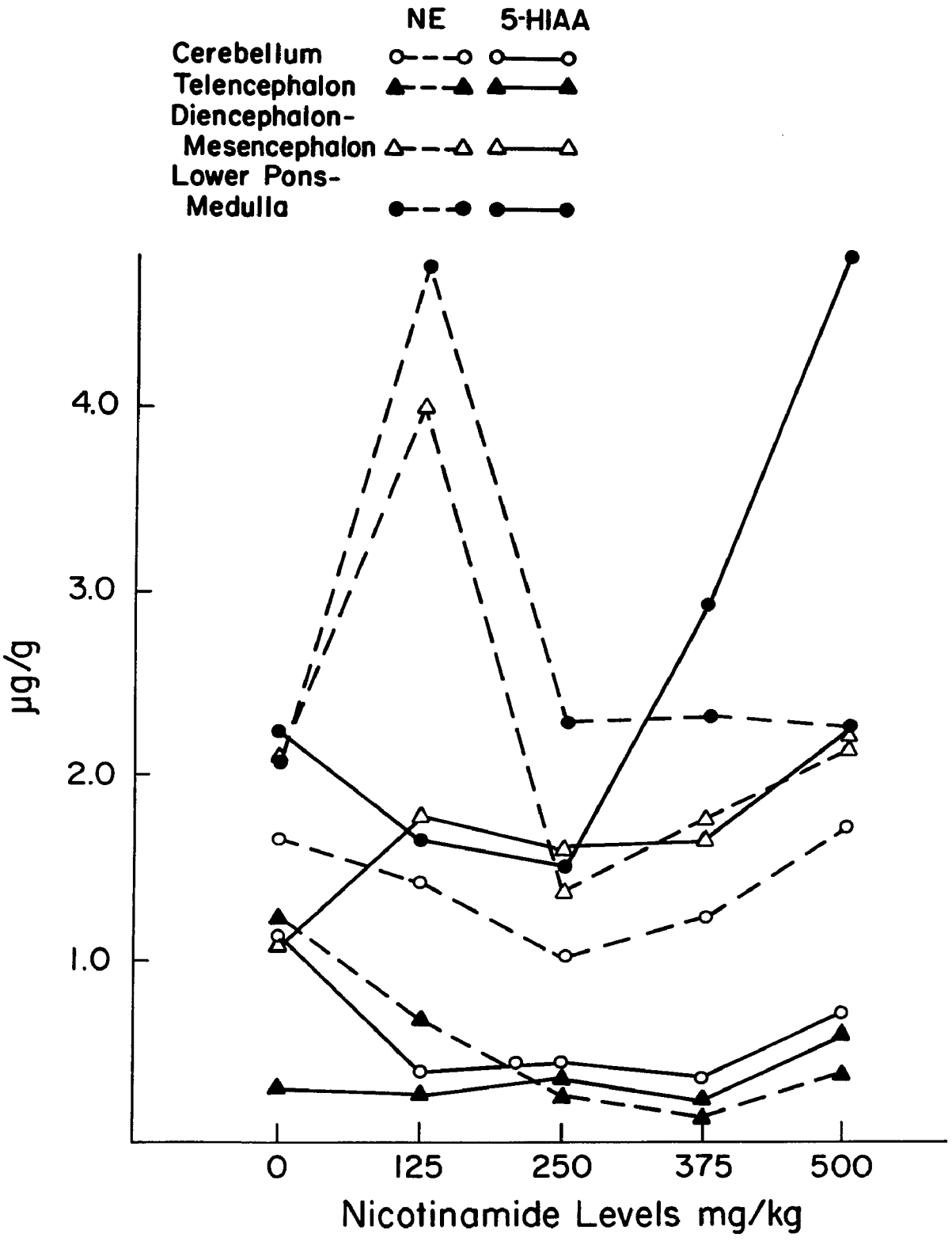

NE
5-HT
Cerebellum
Telencephalon
Diencephalon-
Mesencephalon
Lower Pons-
Medulla
4.0
3.0
2.0
1.0
µg/g
0
125
250
375
500
Nicotinamide Levels mg/kg

FIGURE 15

Changes in mean levels of 5-HIAA and in NE as dosages of nicotinamide are increased.


NE
5-HIAA
Cerebellum
Telencephalon
Diencephalon-
Mesencephalon
Lower Pons-
Medulla
4.0
3.0
2.0
1.0
µg/g
0
125
250
375
500
Nicotinamide Levels mg/kg

correlations were of significant probability, the fact that all were negatively correlated is of much interest (Fig. 14).

Alternatively, an increase in TE 5-HT correlated negatively with CB and TE norepinephrine but positively with NE in the D-M and LP-M sections. Increasing 5-HIAA in the TE was also negatively correlated with NE in the TE, the D-M and the LP-M.

An increase in 5-HT in the D-M and LP-M sections was positively correlated with NE in each of their own sections and in the other section as well. However, increasing 5-HIAA in the D-M was positively correlated with NE in its own section but negatively with NE in the LP-M. Also, as 5-HIAA increased in the LP-M section the NE was lowered in the D-M and LP-M sections (Fig. 15).

It appears then that increasing 5-HT in all regions of the brain does influence NE levels. In the TE, NE is consistently lowered by increasing 5-HT in general. Increased 5-HT in the D-M and LP-M does not lower NE in these sections. Increased 5-HIAA in the D-M does lower NE in the LP-M, and increasing 5-HIAA in the LP-M lowers NE in the D-M and LP-M regions.

In summary, it appears from this study that 5-HT has more influence over the TE norepinephrine and that 5-HIAA influences more the NE levels in the lower brain regions (D-M and LP-M).

Discussion

Koella (1974) suggested that 5-HT acts as a feedback control on the NE system of the brain and stated that the area postrema was

a likely candidate for this 5-HT receptor function. When he severed the posterior brain stem from the forebrain, 5-HT induced signs of arousal only, in the cat. In intact cats injections of 5-HT induced a shift toward cortical synchrony without a phase of arousal. Topical application of 5-HT to the posterior region of the fourth ventricle produced an increase in recruiting responses, an appearance of spindle bursts and an increase in slow wave output.

Wyatt (1972) in long-term studies of human sleep found that low dosages of monoamine oxidase inhibitors (MAOI's) and α-methyl-5-hydroxytryptophan (AM-5-HTP) increased REM sleep. This indicated to him that REM ablation was not caused by a fall in 5-HT metabolites. α-Methyl-phenylalanine (AMPA) which decrease brain catecholamines increased REM sleep while L-DOPA, a catecholamine precursor, decreased REM sleep. The combined finding that 5-HT concentrations are directly correlated and that catecholamine concentrations are inversely correlated with REM sleep indicates that there is an interaction of the adrenergic system with the serotonergic system in man.

Stern and Morgane (1974) hypothesize that one function of REM sleep may be to maintain the availability of catecholamine neurotransmitter (in a select sub-sample of catecholamine-containing neurons). Wakefulness generates the need for REM sleep. Excessive fatigue of catecholamine systems produces a compensatory increase in REM. REM deprivation would then produce depressed activity of the catecholamine systems. It is an interesting hypothesis and

supported in part by this research, though this study is concerned with the mechanism of REM sleep and not function.

One of the values of the methodology used in this research is that it deals with REM increase and not REM deprivation. There is no nonpharmacological procedure for REM deprivation which does not also induce non-specific stress resulting in myriads of physiological changes.

Just how the nicotinamide injections produced differences in the brain levels of monoamines cannot be determined from this study. Maitre et al. (1974) found no significant increase in the levels of NAD in mouse brain after injection of adenosine and nicotinamide (130 mg/kg and 400 mg/kg). Levels of ATP and phosphocreatine were significantly elevated and reached maximum values after 45 min. It is interesting that Maitre et al. found no sedation of mice when nicotinamide alone was injected. A general observation from this present research was that it did not produce sedation in our mice either, contrary to earlier literature.

Raghuramulu et al. (1965) found that the build-up of quinolinic acid in the tryptophan pyrrolase pathway to NAD is inhibitory on nucleotide synthesis. This may result in increases in substrate concentration of tryptophan for the hydroxylase pathway.

Another explanation may be found in the work of Wurtman et al. (1974). They presented evidence that changes in brain tyrosine concentrations could produce parallel changes in the rate at which the brain synthesizes catechols. Tyrosine (50 mg/kg) produced an 81% increase in brain tyrosine and a 13% increase in dopa. The same

dose of tryptophan produced an 18% fall in brain tyrosine and a 32% decrease in dopa accumulation. They suggest tryptophan might have inhibited tyrosine hydroxylase.

Everette (1974) found that large doses of 5-HTP when given to mice increased 5-HT levels markedly, DA levels remained at control and NE showed a significant decrease. The decrease in NE may involve displacement and/or decreased synthesis in the presence of 5-HTP of 5-HT. Kotowski (1974) indicated that there may be changes in the metabolism of NE following destruction of 5-HT pathways.

Morgane and Stern (1975) have recently found electrophysiological evidence that catecholamine neurons directly inhibit the activity of the anterior raphe cells.

In conclusion, for most types of evidence favoring any one hypothesis there has been either contradictory evidence or reasonably alternative interpretations. Something much more complex than neurotransmitter levels, oscillations or turnover is likely responsible for sleep, REM sleep, and wakefulness.

Application

Possible applications of the research data to everyday situations are, of course, myriad. "Serotonin depression" has been called a biochemical subgroup within the affective disorders by Asberg et al. (1976). These people, the authors suggested, have a disorder in 5-HT turnover, and in this group cerebrospinal fluid (csf) concentrations of 5-HIAA are low. The csf concentrations of

5-HIAA are, however, bimodal. The people in the high 5-HIAA mode have a negative correlation with metabolites of NE. From our work it may be that the two depressions are the same. Maybe a low 5-HT or 5-HIAA cannot coexist with a low NE level in the brain. Maybe a high 5-HIAA level leads to a decrease in NE; and, thus, the two "serotonin depressions" are alike in this respect.

MacSweeney (1975) suggested that L-tryptophan and nicotinamide when given together (3 gms of each daily) are superior to electro-convulsive therapy (ECT) administered twice weekly in the treatment of unipolar depression. He suggests these people may have abnormally high levels of tryptophan pyrrolase and that nicotinamide on its own might act as an antidepressant agent.

Others have suggested that depression may be due to the "kynurenine shunt" produced by inadequate levels of NAD. There follows an increase in tryptophan pyrrolase activity which leads to an overall decreased level of brain 5-HT (Mangoni, 1974).

In cerebral infarction disordered central monoamine metabolism contributes to the adverse pathophysiological effects such as decreased cerebral blood flow, progression of infarction, and altered cerebral metabolism (Meyer et al., 1974; Wurtman and Zervas, 1974).

CHAPTER VI

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Briefly, it can be concluded from this research that nicotinamide does influence human sleep by significantly increasing the percent of both total sleep time (TST) and total recording time (TRT) spent in REM sleep. It also significantly decreases the amount of time to sleep onset in the subjects studied who had moderate to severe insomnia.

The mouse data present clear evidence that nicotinamide alters the levels of the neurotransmitters, norepinephrine (NE) and 5-hydroxytryptamine (5-HT), and the metabolite, 5-hydroxyindoleacetic acid (5-HIAA). In general 5-HT is increased in all regions of the brain over control and NE is decreased in the telencephalon (TE) section, whereas, increases in 5-HIAA in the diencephalon-mesencephalon (D-M) correlate negatively with NE in the lower pons-medulla (LP-M) section but not within its own section. There is a significant difference in how the drug behaves within the four different regions of the brain in relation to NE and 5-HT levels.

Since the TE section of the brain contained the hippocampal theta rhythm producing areas of rapid eye movement (REM) sleep, it may be concluded that an increase in 5-HT and a decrease in NE result in the increased amount of time spent in REM sleep seen in

polygraphic recordings. Whether one transmitter level can change without a resulting change in the other can not be determined from this study. Recent literature (Wurtman et al., 1974) suggests that an increase in 5-HT is always followed by a decrease in NE. The reverse or mirror image of this phenomenon has not been studied.

Further study in the area should certainly include the addition of pyridoxal phosphate to the nicotinamide system to determine augmentation of effects since B_6 is a coenzyme in aromatic amino acid dehydrogenases and in transaminases. Dopamine assays can easily be added to the methodology and may give further data concerning the effects upon the adrenergic system in the brain.

Quinolinic acid, a known inhibitor of NAD synthesis, and other intermediates in the tryptophan pyrrolase pathway may, when given in large doses to mice, show whether or not 5-HT can be increased by inhibiting the pyrrolase step and increasing the substrate, tryptophan.

Labeled tryptophan and tyrosine studies could be done to determine the effect of nicotinamide upon the transport of these amino acids into the brain. It is known that leucine decreases brain 5-HT and this can be reversed by nicotinamide administration (Krishnaswamy and Raghuram, 1972).

Since L-dopa markedly decreases brain 5-HT in mice (Everett and Borcherding, 1970), one might give large doses of L-dopa and record the sleep to see if any or what changes occur as the result of decreasing 5-HT.

Since acetylcholine receptors are located in the hippocampus and other areas of the brain, this neurotransmitter should be studied. In the area of sleep very little research has been done in the acetylcholine system. It is known that acetylcholine is increased in the neocortex during REM sleep and waking and that application of cholinomimetic drugs to the brain stem or hypothalamic area induces REM sleep in normal cata (Stern and Morgane, 1974). Anticholinergic drugs produce synchronization of the cortical electroencephalogram (EEG).

Adenosine may be added to the nicotinamide system for study. When both are given to mice the EEG changes from a slow rhythm with high voltage to a faster one with low voltage and protects from audiogenic seizures (Maitre et al., 1974). There may be alterations in sleep EEG as well.

The riddle of sleep mechanisms is far from solved.

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APPENDIX A

1. Table of Brain Weights.

2. Days of Sacrifice, Days of Assays and Number of Days Frozen.

BRAIN WEIGHTS-SIX ANIMALS/ASSAY

Nicotinamide	N	Brain Section							
		CB	Average	TE	Average	D-M	Average	LP-M	Average
Saline	1	300	353	1549	1564	333	424	295	338
	2	385		1430		550		385	
	3	360		1630		385		370	
	4	370		1550		440		325	
	5	350		1660		410		315	
125 mg/kg	1	400	401	1670	1608	385	433	310	305
	2	345		1615		440		325	
	3	400		1555		390		300	
	4	420		1595		515		285	

BRAIN WEIGHTS-SIX ANIMALS/ASSAY (continued)

250 mg/kg	1	272	345	1513	1519	509	474	279	317
	2	365		1370		500		340	
	3	380		1545		490		280	
	4	375		1590		460		310	
	5	335		1575		410		375	
375 mg/kg	1	345	379	1675	1636	490	450	320	288
	2	365		1590		500		270	
	3	400		1670		440		280	
	4	405		1610		370		280	
500 mg/kg	1	317	344	1209	1518	506	436	210	278
	2	355		1545		400		280	
	3	365		1590		445		360	
	4	315		1575		380		375	
	5	370		1670		450		165	

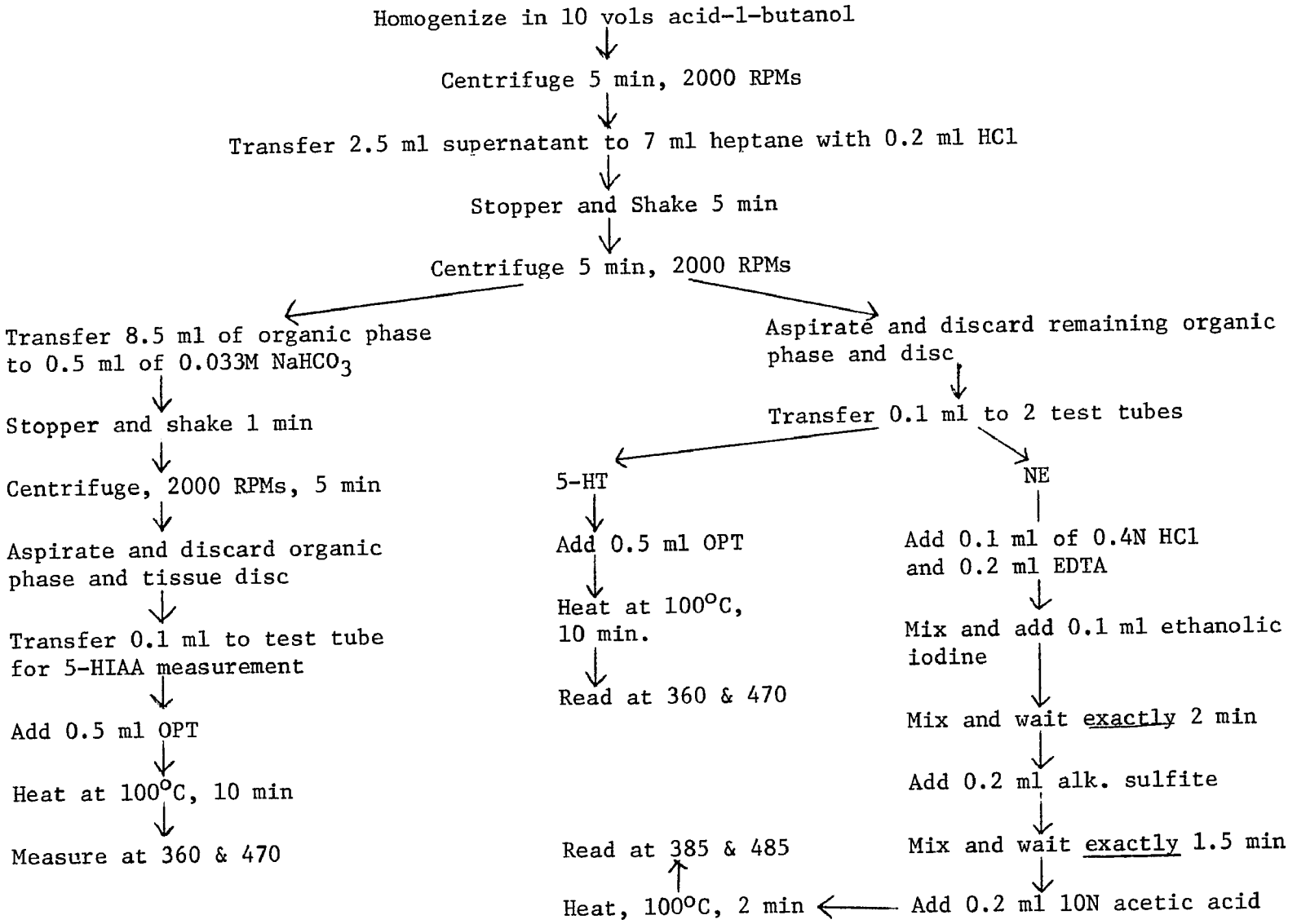
DAYS OF SACRIFICE, DAYS OF ASSAYS AND NUMBER OF DAYS FROZEN

		Sacrificed	Assayed	Days Frozen
Saline	1	9/17/75	10/3/75	26
	2	2/16/76	2/25/76	9
	3	3/16/76	6/8/76	84
	4	4/28/76	6/22/76	63
	5	4/28/76	6/23/76	56
125	1	5/13/76	7/13/76	61
	2	5/13/76	7/14/76	62
	3	5/19/76	7/15/76	57
	4	5/19/76	7/16/76	58
250	1	10/22/75	12/8/76	47
	2	2/17/76	2/27/76	10
	3	3/17/76	6/10/76	85
	4	4/29/76	6/24/76	56
	5	4/29/76	6/30/76	62
375	1	5/6/76	7/7/76	62
	2	5/6/76	7/8/76	63
	3	5/12/76	7/9/76	58
	4	5/12/76	7/10/76	59
500	1	10/26/76	12/15/76	49
	2	2/18/76	3/1/76	12
	3	3/17/76	6/11/76	86
	4	5/5/76	7/1/76	56
	5	5/5/76	7/2/76	58

APPENDIX B

1. Outline of Assay Procedure.

2. List of Solutions for Assay.


Homogenize in 10 vols acid-1-butanol
Centrifuge 5 min, 2000 RPMs
Transfer 2.5 ml supernatant to 7 ml heptane with 0.2 ml HCl
Stopper and Shake 5 min
Centrifuge 5 min, 2000 RPMs
Transfer 8.5 ml of organic phase to 0.5 ml of 0.033M $NaHCO_3$
Stopper and shake 1 min
Centrifuge, 2000 RPMs, 5 min
Aspirate and discard organic phase and tissue disc
Transfer 0.1 ml to test tube for 5-HIAA measurement
Add 0.5 ml OPT
Heat at 100°C, 10 min
Measure at 360 & 470
Aspirate and discard remaining organic phase and disc
Transfer 0.1 ml to 2 test tubes
5-HT
Add 0.5 ml OPT
Heat at 100°C, 10 min.
Read at 360 & 470
NE
Add 0.1 ml of 0.4N HCl and 0.2 ml EDTA
Mix and add 0.1 ml ethanolic iodine
Mix and wait exactly 2 min
Add 0.2 ml alk. sulfite
Mix and wait exactly 1.5 min
Add 0.2 ml 10N acetic acid
Heat, 100°C, 2 min
Read at 385 & 485

List of Solutions for Assays

1. n-Butanol (acidified - 99 ml. n-Butanol and 1 ml. of 0.1N HCl)
2. n-Heptane (Reagent Grade)
3. 0.1N HCl
4. 0.4N HCl
5. 10N Acetic Acid
6. 0.1N Ethanolic Iodine (0.1N Iodine in 100 ml. Ethanol)
7. EDTA (0.1M Tetrasodium EDTA in 1 M Na acetate)
8. Alkaline Sulfite (Sodium Sulfite 0.5g anhydrous in 2 ml. H_2O, add 9 ml. of 5N NaOH. Must be mixed immediately prior to use)
9. 0.033M $NaHCO_3$
10. O-phathaldialdehyde (OPT - 10mg% in 10N HCl)

APPENDIX C

1. Raw Spectrophotofluorometric Readings for Dilution Curve.

2. Spectrophotofluorometric Readings for Dilution Curve by Method of Least Squares.

DILUTION CURVE

Raw Fluorometric Readings

Dilutions µg	Fluorometric Readings µamps		
	5-HT	5-HIAA	NE
25.0	2.60	1.90	8.20
12.5	2.50	1.30	2.80
1.0	1.10	0.60	1.00
0.075	0.70	0.40	0.69
0.500	0.59	0.30	0.44
0.250	0.34	0.16	0.24
0.125	0.17	0.08	0.14
0.075	0.09	0.04	0.08

Fluorometric Readings By Method of Least Squares

Dilutions µg	5-HT	5-HIAA	NE
1.00	1.01	0.517	0.966
0.750	0.78	0.394	0.724
0.500	0.55	0.272	0.483
0.250	0.32	0.149	0.241
0.125	0.21	0.087	0.120
0.075	0.16	0.063	0.072

5-HT $y = 0.92\ (x) + 0.09$

5-HIAA $y = 0.535\ (x) + 0.014$

NE $y = 0.966\ (x) + 0$

APPENDIX D

1. Averages for Each Subject During Each Period of the Study.

2. Sleep Efficiency for Normal and Insomniac Subjects.

AVERAGES FOR EACH NORMAL SUBJECT FOR EACH PERIOD OF THE STUDY
IN PERCENT TOTAL RECORDING TIME AND PERCENT TOTAL SLEEP TIME

Subject	Stages	Baseline						Two Weeks					
		A	I	II	III	IV	REM	A	I	II	III	IV	REM
A	%TRT	7.4	12.9	44.0	15.1	1.1	20.0	4.2	1.9	49.5	8.8	7.4	27.2
	%TST		11.7	47.5	16.3	1.2	21.5		1.9	51.6	9.2	7.8	28.4
B	%TRT	6.2	6.2	47.3	16.0	0.9	22.2	0.9	1.1	62.2	8.6	2.4	24.7
	%TST		6.5	50.5	17.0	1.0	23.5		1.1	63.9	8.9	2.5	25.2
C	%TRT	2.2	3.8	56.0	11.9	8.0	18.3	0.7	3.0	58.1	9.2	5.1	23.9
	%TST		4.0	57.1	12.1	8.2	18.7		3.0	58.5	9.2	5.1	24.0
D	%TRT	10.4	4.0	53.3	10.6	6.5	15.4	0.5	1.3	50.8	11.2	9.4	26.7
	%TST		4.4	59.6	11.8	7.4	16.7		1.3	51.1	11.3	9.5	26.9
E	%TRT	3.4	4.7	66.9	7.0	2.6	15.4	0.7	2.2	60.9	8.2	2.0	26.2
	%TST		4.8	65.3	6.5	2.1	20.6		2.2	61.2	8.2	2.0	26.4

AVERAGES FOR EACH NORMAL SUBJECT FOR EACH PERIOD OF THE STUDY
IN PERCENT TOTAL RECORDING TIME AND PERCENT TOTAL SLEEP TIME (continued)

F	%TRT	2.1	4.0	62.2	9.3	3.2	19.2	1.9	1.3	57.6	12.1	2.6	24.7
	%TST		6.0	68.1	7.9	2.7	15.0		1.2	58.7	12.3	2.7	25.1
Mean %TRT		5.3	6.9	55.0	11.7	3.7	18.4	1.5	1.8	56.5	9.7	4.8	25.7

AVERAGES FOR EACH NORMAL SUBJECT (continued)

Subject	Stages	Three Weeks						Recovery					
		A	I	II	III	IV	REM	A	I	II	III	IV	REM
A	%TRT	0.7	1.0	53.1	7.5	6.1	30.9	0.2	0.1	61.3	10.8	5.7	21.6
	%TST		1.0	53.5	7.6	6.1	31.1		0.1	61.4	10.8	5.7	21.6
B	%TRT	1.0	0.6	51.3	10.9	6.0	30.0	0.6	1.0	54.0	12.6	7.8	23.7
	%TST		0.6	51.9	11.0	6.0	30.3		1.0	54.4	12.7	7.9	23.8
C	%TRT	0.4	1.0	63.7	6.3	1.7	25.6	Includes only night 21. Did not					
	%TST		1.0	64.0	6.3	1.7	25.7	return nights 22, 23 and recovery.					
D	%TRT	0.2	0.9	63.3	10.9	6.0	18.4	Includes nights 21 and 22. Did not					
	%TST		0.9	63.4	10.9	6.0	18.5	return night 23 and recovery.					
E	%TRT	0.3	1.2	67.4	7.3	2.1	21.8	1.1	1.4	50.9	9.6	11.3	25.8
	%TST		1.3	67.5	7.2	2.1	21.9		1.0	51.4	9.6	11.4	26.1

AVERAGES FOR EACH NORMAL SUBJECT (continued)

F	%TRT	0.2	0.2	58.1	9.0	9.3	23.4	1.9	0.4	60.5	6.2	9.9	21.2
	%TST		0.2	58.2	8.9	9.2	23.5		0.4	61.6	6.4	10.1	21.5
Mean	%TRT	0.5	0.8	59.5	8.7	5.2	25.0	1.0	0.7	56.7	9.8	8.7	23.1

SLEEP EFFICIENCY - NORMAL SUBJECTS

	Study Periods			
Subjects	B	2	3	R
A	92.6	95.8	99.3	99.8
B	93.8	99.1	99.0	99.4
C	97.8	99.3	99.6	
D	89.6	99.5	99.8	
E	96.6	99.3	99.7	98.9
F	97.9	98.1	99.8	98.1
Mean	94.7	98.5	99.5	99.0
N	16	12	11	8

AVERAGES FOR EACH SUBJECT WITH INSOMNIA IN PERCENT TOTAL RECORDING TIME AND PERCENT TOTAL SLEEP TIME FOR EACH PERIOD OF STUDY

Subject	Stages	Baseline						Two Weeks					
		A	I	II	III	IV	REM	A	I	II	III	IV	REM
IA	%TRT	51.4	2.7	22.6	7.6	5.8	7.3	54.5	1.9	29.6	7.2	3.5	3.3
	%TST		6.3	45.1	20.1	16.2	12.3		4.3	64.6	15.8	8.1	7.2
IB	%TRT	29.0	1.9	38.3	6.9	3.8	20.1	34.1	2.3	34.4	7.5	6.5	15.2
	%TST		2.7	53.6	9.8	5.3	28.6		3.9	52.7	11.1	9.8	22.5
Mean	%TRT	41.5	2.4	30.4	7.3	4.4	13.7	44.3	2.1	32.0	2.3	5.0	9.2
Mean	%TST		4.0	49.3	14.7	10.7	20.4		4.1	63.4	13.4	8.9	14.4
		Three Weeks						Recovery					
IA	%TRT	17.9	5.2	41.2	9.9	4.0	21.8	71.0	1.0	22.6	3.3	0.0	2.1
	%TST		6.6	49.7	12.6	5.2	26.0		2.5	77.8	9.5	0.0	9.2

AVERAGES FOR EACH SUBJECT WITH INSOMNIA IN PERCENT TOTAL RECORDING TIME AND PERCENT TOTAL SLEEP TIME FOR EACH PERIOD OF STUDY (continued)

IB	%TRT	23.0	2.0	39.3	7.1	4.3	24.3	46.1	4.3	26.1	5.7	3.3	14.5
	%TST		2.6	50.5	9.3	5.6	32.0		7.3	45.9	12.5	6.8	27.5
Mean	%TRT	20.4	3.4	40.3	8.5	4.1	23.1	58.6	2.5	24.4	4.5	1.6	8.2
Mean	%TST		4.6	50.1	10.9	5.4	29.0		5.4	61.5	11.0	3.4	18.4

SLEEP EFFICIENCY - INSOMNIAC SUBJECTS

Subject	Study Periods			
	B	2	3	R
IA	45.9	45.5	82.0	29.0
IB	71.0	65.9	77.0	53.9
Mean	58.4	55.4	78.5	41.4
N	6	4	4	4

APPENDIX E

1. Tables of Calculated Levels of 5-HT, 5-HIAA and NE Under All Experimental Conditions.

2. Tables of Spectrophotofluorometric Readings for 5-HT, 5-HIAA and NE with Recovery to Blank Ratios.

CALCULATED LEVELS OF 5-HYDROXYTRYPTAMINE (μg/g)
UNDER ALL EXPERIMENTAL CONDITIONS

Nicotinamide mg/kg	Assay Number	Brain Section			
		CB	TE	D-M	LP-M
Saline	1	21.666	8.390	36.036	8.463
	2	0.238	0.373	0.970	0.714
	3	0.556	0.263	0.743	0.541
	4	0.278	0.299	1.233	1.110
	5	4.303	2.575	12.488	10.979
125	1	0.900	0.527	3.428	2.968
	2	0.564	0.481	2.557	2.650
	3	1.402	0.723	4.808	5.000
	4	0.595	0.601	1.780	3.363
250	1	6.762	2.066	2.275	7.740
	2	7.306	4.137	13.333	18.626
	3	1.007	0.637	1.338	1.368
	4	0.930	0.843	3.111	4.795
	5	0.263	0.187	0.861	0.706
375	1	0.068	0.056	0.335	0.367
	2	0.442	0.355	1.516	1.553
	3	1.207	0.764	3.214	2.217
	4	0.772	0.670	3.463	3.906
500	1	6.975	3.482	8.327	6.273
	2	1.186	0.726	2.544	2.005
	3	1.469	0.830	3.146	2.445
	4	0.733	0.472	3.442	2.394
	5	0.652	0.496	3.141	0.836

CALCULATED LEVELS OF 5-HYDROXYINDOLEACETIC ACID (μg/g)
UNDER ALL EXPERIMENTAL CONDITIONS

Nicotinamide mg/kg	Assay Number	Brain Section			
		CB	TE	D-M	LP-M
Saline	1	5.833	5.809	54.054	47.390
	2	0.963	0.440	2.020	2.020
	3	2.084	0.204	1.861	1.937
	4	0.614	0.293	1.976	2.797
	5	1.410	0.716	5.032	3.376
125	1	0.321	0.230	1.798	1.572
	2	0.378	0.350	2.224	1.338
	3	0.343	0.290	1.458	1.372
	4	0.529	0.314	1.618	2.339
250	1	5.507	0.826	0.982	11.657
	2	1.076	0.574	3.157	3.571
	3	0.343	0.506	0.599	0.466
	4	0.123	0.150	1.469	1.703
	5	0.282	0.180	1.150	0.755
375	1	0.198	0.109	0.603	0.781
	2	0.329	0.302	1.520	2.222
	3	0.308	0.280	2.072	6.421
	4	0.561	0.267	2.389	2.435
500	1	1.577	0.930	2.472	0.238
	2	1.174	1.024	2.708	9.228
	3	3.083	0.865	6.647	4.514
	4	0.892	0.589	2.957	2.997
	5	0.077	0.188	1.079	2.251

CALCULATED LEVELS OF NOREPINEPHRINE (μg/g)
UNDER ALL EXPERIMENTAL CONDITIONS

Nicotinamide mg/kg	Assay Number	Brain Section			
		CB	TE	D-M	LP-M
Saline	1	2.222	1.291	18.018	1.693
	2	1.300	0.667	2.892	2.951
	3	3.334	2.861	2.444	2.162
	4	0.338	0.161	0.815	1.154
	5	6.666	1.385	10.569	13.758
125	1	1.042	0.449	5.194	4.033
	2	0.644	0.963	3.693	4.786
	3	3.333	1.072	4.273	7.777
	4	0.714	0.188	2.913	2.456
250	1	0.970	0.424	0.630	2.030
	2	9.590	3.467	22.000	36.027
	3	0.817	0.244	1.548	1.478
	4	1.940	0.343	2.767	4.839
	5	0.403	0.103	0.560	0.721
375	1	0.019	0.023	0.143	0.199
	2	2.466	0.252	3.400	4.445
	3	3.750	1.348	7.956	12.495
	4	3.086	1.553	10.812	17.855
500	1	1.073	0.187	1.455	1.797
	2	2.012	0.518	1.929	2.756
	3	21.920	3.145	35.952	27.780
	4	0.889	0.178	1.263	1.920
	5	2.911	0.553	3.931	3.730

5-HYDROXYTRYPTAMINE FLUORESCENCE READINGS (μamps)
WITH RECOVERY, BLANK AND RECOVERY TO BLANK RATIOS

Nicotinamide mg/kg	Assay Number	Brain Section				Recovery	Blank	R/B
		CB	TE	D-M	LP-M			
Saline	1	.190	.315	.295	.11	.08	.06	1.3
	2	.0165	.0445	.043	.0275	.071	.011	6.45
	3	.065	.105	.08	.065	.205	.03	6.83
	4	.0025	.006	.007	.005	.0112	.0015	7.47
	5	.02	.043	.05	.0362	.014	.0075	1.87
125	1	.015	.028	.039	.029	.031	.006	5.17
	2	.015	.036	.0485	.039	.044	.008	5.50
	3	.016	.033	.051	.042	.03	.006	5.00
	4	.015	.032	.031	.032	.033	.009	3.67
250	1	.065	.089	.052	.071	.049	.03	1.63
	2	.0155	.024	.027	.0265	.01	.0075	1.3
	3	.019	.03	.024	.019	.03	.012	2.53
	4	.0025	.0495	.052	.0535	.04	.013	3.10
	5	.15	.325	.375	.30	.925	.075	12.33
375	1	.015	.03	.045	.035	.223	.01	22.3
	2	.018	.043	.055	.034	.07	.008	8.75
	3	.022	.045	.049	.026	.029	.008	3.63
	4	.019	.0435	.05	.044	.041	.009	4.56
500	1	.072	.110	.110	.055	.049	.03	1.6
	2	.019	.039	.036	.023	.0335	.007	5.07
	3	.016	.026	.027	.0295	.022	.0095	2.32
	4	.400	.90	1.450	1.05	1.15	.175	6.57
	5	.014	.031	.048	.011	.036	.007	5.14

5-HYDROXYINDOLEACETIC ACID FLUORESCENCE READINGS (μamps)
WITH RECOVERY, BLANK AND RECOVERY TO BLANK RATIOS

Nicotinamide mg/kg	Assay Number	Brain Section				Recovery	Blank	R/B
		CB	TE	D-M	LP-M			
S	1	.095	.15	.24	.20	.07	.06	1.16
a	2	.012	.0155	.022	.0175	.0205	.0065	3.13
l	3	.08	.055	.078	.078	.095	.035	2.71
i	4	.002	.0025	.0035	.0035	.0037	.0015	2.47
n	5	.0125	.018	.0249	.017	.0165	.0036	1.92
e								
	1	.01	.015	.021	.017	.029	.0075	3.6
1	2	.012	.022	.029	.019	.032	.009	3.56
2	3	.011	.019	.022	.018	.033	.0095	4.4
5	4	.011	.016	.022	.019	.025	.007	3.57
	1	.040	.060	.043	.056	.03	.038	0.79
	2	.012	.0135	.0145	.0165	.015	.0075	2.0
2	3	.013	.019	.0145	.013	.021	.0018	1.78
5	4	.014	.0192	.031	.027	.0398	.0128	3.12
0	5	.10	.15	.20	.15	.34	.075	4.53
	1	.009	.014	.019	.017	.05	.006	8.33
	2	.012	.021	.028	.024	.034	.009	3.78
3	3	.013	.020	.029	.026	.0308	.0105	2.93
7	4	.014	.018	.027	.023	.0293	.0095	3.08
5	5							
	1	.044	.038	.065	.046	.03	.038	0.79
	2	.009	.016	.013	.022	.0125	.006	2.08
5	3	.015	.0165	.026	.018	.0143	.008	1.73
0	4	.25	.48	.55	.55	.506	.15	3.37
0	5	.007	.012	.015	.013	.024	.0065	3.69

NOREPINEPHRINE FLUORESCENCE READINGS (μamps)
WITH RECOVERY, BLANK AND RECOVERY TO BLANK RATIOS

Nicotinamide mg/kg	Assay Number	Brain Section				Recovery	Blank	R/B
		CB	TE	D-M	LP-M			
Saline	1	.135	.12	.16	.105	.11	.10	1.1
	2	.098	.15	.15	.092	.025	.014	1.79
	3	.033	.0135	.004	.004	.0065	.0025	2.6
	4	.003	.0035	.004	.004	.018	.016	2.6
	5	.022	.0219	.028	.03	.0065	.0025	1.13
125	1	.022	.026	.041	.032	.029	.017	1.71
	2	.022	.034	.033	.034	.029	.02	1.45
	3	.017	.019	.019	.023	.015	.009	1.67
	4	.019	.019	.031	.023	.026	.016	1.63
250	1	.026	.048	.042	.064	.079	.026	3.04
	2	.018	.019	.024	.025	.016	.015	1.07
	3	.0185	.021	.025	.02	.0285	.014	2.04
	4	.03	.028	.036	.0385	.033	.022	1.5
	5	.14	.15	.175	.19	.46	.09	5.11
375	1	.015	.02	.025	.024	.171	.014	12.21
	2	.028	.023	.036	.031	.029	.019	1.53
	3	.019	.022	.027	.027	.015	.013	1.15
	4	.022	.027	.033	.037	.021	.017	1.24
500	1	.042	.047	.048	.034	.079	.026	3.04
	2	.0265	.028	.0275	.0275	.0315	.014	2.25
	3	.0245	.023	.0285	.0255	.021	.0205	1.02
	4	.425	.425	.55	.070	.875	.25	3.5
	5	.03	.028	.039	.024	.029	.016	1.81

APPENDIX F

1. Sample of Consent Form Signed by Each Participant in the Human Study.

2. Copy of Approval by Human Use Committee.

University of Alabama in Birmingham

Department of Neurosciences

Sleep Research Authorization

I have been given information concerning the proposed sleep study. I understand that the study is of approximately 6 weeks duration including 13 nights in the laboratory. I also understand that I will be taking the vitamin, nicotinamide, 3 grams per day, for 21 to 23 days. I agree to take the vitamin for the purpose of learning more about its relationship to sleep.

In giving my consent, I acknowledge that my participation in this research is voluntary, and that I may withdraw at any time.

Signed_____

Witness_____

Date_____

COPY

UNIVERSITY OF ALABAMA IN BIRMINGHAM
IDENTIFICATION AND CERTIFICATION OF PUBLIC HEALTH SERVICE APPLICATIONS
FOR SUPPORT OF RESEARCH INVOLVING HUMAN SUBJECTS

This form is applicable to all applications for DHEW research and research training grants, program projects and center grants, demonstration grants, fellowships, traineeships, awards, and other programs involving the use of Human Subjects. This form is not applicable to applications for grants limited to the support of construction, alterations and renovations, or research resources.

The Public Health Service requires assurances from grantee institutions sponsoring research, investigation and care involving human subjects that they will carry out review of all such projects in agreement with the policy and instruction provided in "The Institutional Guide to DHEW Policy on Protection of Human Subjects", December 1, 1971. The Public Health Service also requires certification of the performance of the initial review. This review should be completed prior to submission of relevant proposals, but in any event, it must be done prior to award of funds.

With specific reference to the project numbered _____

entitled "A Study of the Effects of Niacinamide (3 gm/day) upon Sleep in Normal and Insomniac Persons"

under the direction of Vernon Pegram, Ph.D.; John R. Smythies

[ ] 1. This application does not include research involving human subjects, as defined in the DHEW Publication (NIH) 72-102, December 1, 1971.

[X] 2. This application includes research involving human subjects. Our institutional committee has reviewed and approved it on October 28, 1975, in accordance with our assurance approved by the Public Health Service. The Project will be subject to continuing review as provided for in that assurance.

[ ] 3. This application, which includes research involving human subjects, is pending initial review by an institutional committee as provided by our assurance. Certification of completion of the review will be provided as soon as possible. (This certification will be in the form indicated in 2, above, identifying the application by title, investigator, and if known, by number.)

Walter B. Frommeyer

Walter B. Frommeyer, Jr., M.D.
Chairman, Human Use committee

November 3, 1975

FORM I

Revision September 1971
Revision September 1973

REPORT OF PROJECT REVIEW PANEL
UNIVERSITY OF ALABAMA IN BIRMINGHAM

NAME(s) of responsible person(s) Vernon Pegram, Ph.D.; John R. Smythies, M.D.

TITLE OF PROJECT A Study of the Effects of Niacinamide (3 gms/day) upon Sleep in Normal and Insomniac Persons

Type of Project: (XX)Research (___)Demonstration (___)Training
(___)Fellowship (___)Other

This panel reviewed the following items with the investigator: (1) the rights and welfare of the individual(s) involved, (2) the appropriateness of methods used to secure informed consent, (3) the risks and potential benefits of the investigation. On the basis of this discussion, we recommend

(X) Approval

(___) Disapproval

of this project in terms of the University of Alabama in Birmingham Statement of Policy and Procedure Concerning Use of Human Subjects in Investigation.

Remarks: _____

Date of consultation with responsible person(s) October 28, 1975

SIGNED:

_____

_____

_____

_____ Executive Secretary of Panel

cc: _____

_____
Responsible person(s)

UNIVERSITY OF ALABAMA IN BIRMINGHAM
IDENTIFICATION AND CERTIFICATION OF PUBLIC HEALTH SERVICE APPLICATION
FOR SUPPORT OF RESEARCH INVOLVING HUMAN SUBJECTS

Copy

This form is applicable to all applications for DHEW research and research training grants, program projects and center grants, demonstration grants, fellowships, traineeships, awards, and other programs involving the use of Human Subjects. This form is not applicable to applications for grants limited to the support of construction, alterations and renovations, or research resources.

The Public Health Service requires assurances from grantee institutions sponsoring research, investigation and care involving human subjects that they will carry out review of all such projects in agreement with the policy and instruction provided in "The Institutional Guide to DHEW Policy on Protection of Human Subjects", December 1, 1971. The Public Health Service also requires certification of the performance of the initial review. This review should be completed prior to submission of relevant proposals, but in any event, it must be done prior to award of funds.

With specific reference to the project numbered _____

entitled " THE EFFECTS OF NIACIN UPON SLEEP IN NORMAL AND SCHIZOPHRENIC SUBJECTS"

under the direction of Dr. Vernon Pegram

☐ 1. This application does not include research involving human subjects, as defined in the DHEW Publication (NIH) 72-102, December 1,1971.

☒ 2. This application includes research involving human subjects. Our institutional committee has reviewed and approved it on September 30, 1974, in accordance with our assurance approved by the Public Health Service. The Project will be subject to continuing review as provided for in that assurance.

☐ 3. This application, which includes research involving human subject, is pending initial review by an institutional committee as provided by our assurance. Certification of completion of the review will be provided as soon as possible. (This certification will be in the form indicated in 2, above, identifying the application by title, investigator, and if known, by number.)

Walter B. Frommeyer, Jr., M.D.
Chairman, Human Use Committee

October 3, 1974
(Date)

Revision September 1971
Revision September 1973

FORM I

REPORT OF PROJECT REVIEW PANEL
UNIVERSITY OF ALABAMA IN BIRMINGHAM

NAME(s) of responsible person(s) Dr. Vernon Pegram

TITLE OF PROJECT "The Effects of Niacin upon Sleep in Normal and Schizophrenic Subjects"

Type of Project: (X)Research ( )Demonstration ( )Training
( )Fellowship ( )Other

This panel reviewed the following items with the investigator: (1) the rights and welfare of the individual(s) involved, (2) the appropriateness of methods used to secure informed consent, (3) the risks and potential benefits of the investigation. On the basis of this discussion, we recommend

(X) Approval
( ) Disapproval

of this project in terms of the University of Alabama in Birmingham Statement of Policy and Procedure Concerning Use of Human Subjects in Investigation.

Remarks:

Date of consultation with responsible person(s) Sept 30, 1974

SIGNED:

S. T. Epstein
Tom Vaughan

L. Crouch, Executive Secretary of Panel

cc:

Responsible person(s)

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA IN BIRMINGHAM
DISSERTATION APPROVAL FORM

Name of Candidate Connie Ruth Robinson

Major Subject Physiology and Biophysics

Title of Dissertation A Study of the Effects of Niacin Upon Sleep and Brain Monoamines

Dissertation Committee:

G. Vernon Pegram, Chairman

Warren S. Rehm

Director of Graduate Program Warren S. Rehm

Dean, UAB Graduate School

Date 10 December 1976