Histochemical Localization and Identification of Monoamine-Containing Cell Bodies in the Brain Stem of the Squirrel Monkey (Saimiri Sciureus)

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HISTOCHEMICAL LOCALIZATION AND IDENTIFICATION OF
MONOAMINE-CONTAINING CELL BODIES IN THE
BRAIN STEM OF THE SQUIRREL MONKEY

(Saimiri sciureus)

by

Jack E. Hubbard

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LIFE

Jack Edward Hubbard, Jr., the son of Mr. and Mrs. Jack Edward Hubbard, Sr., was born on March 18, 1944, in Cleveland, Ohio.

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On November 2, 1968, the candidate married the former Kathleen Ann Kroening, R.N. of Wausau, Wisconsin. They have a daughter, Kristin Lynn, who was born on September 1, 1971.
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INTRODUCTION

The monoamines, a term which refers to a group of chemical substances including dopamine, norepinephrine, epinephrine (collectively termed the catecholamines), serotonin, and histamine, have been increasingly associated with neuronal function ever since 1904, when Thomas Renton Elliott first postulated epinephrine to serve as a chemical mediator at sympathetic nerve endings (Elliott, 1904). The literature reveals that these substances are involved with a broad spectrum of behavioral activities. Further, various neurological and psychiatric disorders have been indicated to be associated with the interference of one or more of the monoamines in the central nervous system. For example, an important discovery concerning the treatment of Parkinsonian syndromes reveals the significance of a monoamine -dopamine- for motor control. Parkinsonism, clinically described as a hypokinetic dysfunction of the extrapyramidal system, is characterized by a reduction in the initiation, implementation and facility of movement. In investigating a possible relationship between deficiency of dopamine in the striatum and Parkinsonian symptomatology, Hornykiewicz and his co-workers (Birkmayer and Hornykiewicz, 1961) report that administered Levo-dihydroxyphenylalanine (L-dopa), precursor to dopamine, produces a complete abolition or substantial reduction of Parkinsonian akinesia. Behaviorally, those drugs (e.g. amphetamine) which elevate levels of available catecholamines produce behavioral excitation, while those agents which lower catecholamine levels in the brain (e.g. reserpine) produce sedation or depression (Schildkraut and Kety, 1967). Further, hallucinogenic drugs such as lysergic acid
diethylamide (LSD) have been found to influence the metabolism of monoamines. In animal studies, LSD has been reported to elevate levels of brain serotonin (Freedman, 1961, 1963) and to decrease the levels of norepinephrine in the brain (Freedman, 1963). These findings are further supported by reports that hallucinogenic drugs are able to specifically inhibit firing of the serotonin-containing raphé neurons found in the midline of the brain stem (Aghajanian et al., 1968, 1970). In man, urinary excretion of dopamine and metanephrine (a metabolite of epinephrine) is decreased after LSD administration while the excretion of norepinephrine and its metabolite normetanephrine is increased, according to some authors (Hoffer and Osmond, 1967).

Turning to another behavioral parameter, certain aspects of sleep have been suggested to be based upon monoaminergic mechanisms. Serotonin-containing cells in the raphé nuclei appear to be related to the production of slow wave sleep while noradrenergic cell bodies in the pontine tegmentum are necessary for paradoxical sleep (Jouvet, 1967, 1969). Other behavioral and clinical indications of the significance of monoamines in the function of the central nervous system include studies reporting: lateral hypothalamic adrenergic control of food intake (Grossman, 1960, 1962); brain self-stimulation facilitated by intraventricular injection of norepinephrine (Wise and Stein, 1969); aggressive biting behavior in rats following administration of L-dopa (Yen et al., 1970); involvement of the catecholamines and serotonin in morphine dependence (Schwartz and Eidelberg, 1970; Eidelberg and Schwartz, 1970); a relationship between serotonin concentrations and sexual differentiation of the brain (Ladosky and Gaziri, 1970); catecholamine facilitation and serotonin-inhibition of the electroencephalographic arousal response.
(Takagi et al., 1968); possible relationship between brain dopamine levels and symptomatology of Gilles de la Tourette's disease (Snyder et al., 1970); induction of stereotype behavior in the cat from injections of dopamine into the caudate nucleus (Cools and van Rossum, 1970); and a possible involvement of an adrenergic component in the memory mechanisms (Roberts et al., 1970). Thus from this brief account, one may appreciate that the monoamines are quite important to a wide range of functional activities of the brain of laboratory animals as well as of man.

In the early 1960's, a fluorescent histochemical method for the intra-neuronal identification of catecholamine and serotonin neurotransmitters was reported (Falck, 1962; Falck et al., 1962). This elegant technique has provided, for the first time, a highly sensitive and specific means for the demonstration of a chemical mediator within the neuron. The anatomical and physiological significance of this method lies in the ability to map out specific monoaminergic pathways in the central nervous system, thus helping to bridge the gap between form and function. By knowing that a certain pathway utilizes a catecholamine or serotonin to transmit a nerve impulse to the post-synaptic elements, valuable information is added to our knowledge of the functional organization of the central nervous system. Pharmacologically, the effects of a specific drug treatment which interferes with or enhances the monoamine metabolism in these pathways, thus producing functional alterations, may be evaluated on the basis of increased or decreased intensity of fluorescence as measured by a microspectrofluorometer (van Orden, 1970).

Anatomical, physiological, and pharmacological studies utilizing this technique have been performed mostly on the rat with no significant work
reported using primates. Due to the large phylogenetic distance between rat and man, and considering the behavioral and clinical significance of the monoamines in the functioning of the brain of man, it was felt by this candidate that an in-depth investigation of a sub-human primate using this technique was highly warranted.

In order to provide a base-line study for possible future work on monoaminergic pathways, this study has investigated the distribution of monoamine-containing cell bodies in the brain stem of the squirrel monkey (Saimiri sciureus). The brain stem (medulla, pons, midbrain) was chosen since this region of the brain was reported to include most of the monoamine-containing cell bodies of the central nervous system in the rat (Dahlström and Fuxe, 1965). As described in the results of this dissertation, the fluorescing cell bodies were identified by their monoamine content (catecholamine or serotonin) and distribution as well as general cytological characteristics such as size, number of apparent processes, Nissl substance pattern and nuclear appearance. These findings were compared to the findings in the rat by Dahlström and Fuxe (1965).
REVIEW OF THE LITERATURE

I. Biochemistry of the Monoamines

In order to review the biochemistry of the monoamines for the reader, a brief description of their synthetic and catabolic pathways will be presented.

The catecholamines, dopamine and norepinephrine, are derived from the same amino acid, tyrosine. Tyrosine undergoes hydroxylation at the third carbon atom of the phenyl ring to form dihydroxyphenylalanine (dopa). This step is catalyzed by the enzyme tyrosine hydroxylase and appears to be the rate limiting step in catecholamine synthesis (Levitt et al., 1965). The carboxyl group is removed from the amino side chain of dopa by a non-specific decarboxylase to form dopamine (dihydroxyphenylethylamine). Norepinephrine is formed by hydroxylation of dopamine at the beta position of the side chain by dopamine-beta-hydroxylase. These steps for catecholamine synthesis are summarized in text-figure 1.

The synthesis of serotonin is basically similar to that of dopamine and norepinephrine. Instead of tyrosine, however, the precursor amino acid is tryptophan. Tryptophan hydroxylase, which appears to be the rate-limiting step in the synthesis of serotonin in the brain (Jéquier et al., 1967), converts tryptophan to 5-hydroxytryptophan by hydroxylation at the fifth carbon of the phenyl ring. 5-hydroxytryptophan, in turn, is decarboxylated by a non-specific decarboxylase to form 5-hydroxytryptamine, or serotonin. This synthetic pathway for serotonin is indicated in text-figure 2.

The catabolic pathways for the catecholamines and for serotonin are slightly different. The catecholamines follow two enzymatic pathways:

\[\text{catecholamines} \rightarrow \text{dopa} \rightarrow \text{dopamine} \rightarrow \text{norepinephrine} \]

\[\text{noradrenergic system} \rightarrow \text{epinephrine} \]

The serotonin receptor pathway:

\[\text{serotonin} \rightarrow \text{5-HT} \rightarrow \text{5-HT receptor} \]

\[\text{serotonergic system} \]
oxidative deamination of the side chain catalyzed by monoamine oxidase (MAO) and O-methylation of the phenyl ring catalyzed by catechol-O-methyl transferase (COMT). These two phases of catecholamine catabolism may occur in any order, but result in the same inactive substances. Dopamine may first be deaminated by monoamine oxidase to form 3,4-dihydroxyphenylacetic acid which then undergoes O-methylation by catechol-O-methyl transferase to form homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid). The other possible pathway involves an initial methoxylation by catechol-O-methyl transferase to form 3-methoxytyramine and then deamination of 3-methoxytyramine by monoamine oxidase forming homovanillic acid. Norepinephrine undergoes a similar form of metabolic inactivation, either first being deaminated to 3,4-dihydroxymandelic acid and then methoxylated to 3-methoxy-4-hydroxymandelic acid or first being methoxylated to normetanephrine (3-methoxy-4-hydroxyphenylethanolamine) and then deaminated to 3-methoxy-4-hydroxymandelic acid. These pathways are summarized in text-figure 3. Serotonin inactivation appears to follow primarily the oxidative deamination pathway, forming first 5-hydroxyindolylaldehyde and then 5-hydroxyindolacetic acid (text-figure 2).
Text-figure 1. Pathway for the synthesis of dopamine and norepinephrine.
Text-figure 2. Pathway for the synthesis and catabolism of serotonin.
Text-figure 3. Pathways for the catabolism of dopamine and norepinephrine.
II. Occurrence and Localization of Monoamines in the Brain

As discussed in the introduction of this dissertation, the monoamines are significantly involved in the functioning of the brain. Early attempts to localize these substances in the central nervous system relied upon biochemical determinations. These studies reveal a preferential distribution of the monoamines in different portions of the brain. Norepinephrine, first described in the brain during the late 1940's (von Euler, 1946; Holtz, 1950) has been reported to be highest in the hypothalamus and area postrema in the brain of the dog (Vogt, 1954). In a more recent study (Bertler and Rosengren, 1959), the concentration of norepinephrine, expressed as micrograms per gram of tissue, in whole brain homogenates as well as in homogenates of various parts of the brain, was found to be similar in such diverse species as the dog, sheep, pig, and cat. These authors determined that although norepinephrine is present throughout the entire brain, the substance shows much higher concentrations in certain areas such as the hypothalamus, midbrain, and pons. In man, norepinephrine levels are reported to be highest in the anterior and intermediate portions of the hypothalamus with slightly lower levels in the mesencephalon (Bertler, 1961). Dopamine, the other catecholamine of significance in the brain, was first demonstrated in the central nervous system by Montagu (Montagu, 1957). Unlike norepinephrine, substantial dopamine concentrations are more strictly localized in the extrapyramidal motor centers, i.e., the caudate and putamen nuclei and the substantia nigra in the brain of both man (Bertler, 1961) and of the lower mammals (Bertler and Rosengren, 1959). An interesting aspect of these studies is the finding that although dopamine is a precursor in the synthesis of norepinephrine,
sites which are rich in norepinephrine (e.g., the hypothalamus) contain little dopamine, while in those regions which contain high levels of dopamine (e.g., caudate nucleus) only small amounts of norepinephrine are found.

Serotonin, or 5-hydroxytryptamine, was first described in the central nervous system during the middle 1950's (Twarog and Page, 1953; Amin et al., 1954). In the brain of the dog, the distribution of serotonin is similar to that of norepinephrine, being found highest in concentration in the hypothalamus, area postrema, and the midbrain (Amin et al., 1954). In man, the concentration of serotonin is also highest in the hypothalamus, but unlike norepinephrine levels, the thalamus has been reported to contain relatively high concentrations of serotonin (Bertler, 1961).

Thus, these early biochemical determinations provided a solid basis in terms of the regional distribution of the monoamines. They revealed that: (1) the monoamines are found in the brain in significant concentrations; (2) the distribution of monoamines and their concentrations are not uniform throughout the brain; (3) dopamine and norepinephrine appear to be concentrated in mutually exclusive sites; and (4) a certain parallelism exists between the distribution of norepinephrine and that of serotonin.

While these studies yielded valuable information as to the regional distribution of the monoamines in the brain, more data were needed to specify the morphological localization of these substances. A few histochemical methods were available such as the potassium-iodate reaction (Hillarp and Hökfelt, 1955), but they required large amounts of the monoamines such as found in chromaffin cells or the adrenal medulla. Their low sensitivity and non-specificity did not lend them to be satisfactorily applied to a study of
the central nervous system.

With the advent of electron microscopy in the early 1950's arose the concept that neurotransmitters are closely associated with or stored within structures termed synaptic vesicles (DeRobertis and Bennett, 1954) located in the nerve endings. The monoamines have become identified with a particular type of synaptic vesicle, namely the dense-cored or granular vesicle characterized by a central dense granule. Early studies such as on the hypothalamus (Pellegrino de Iraldi et al., 1963) using osmium fixation, described two different populations of dense-cored vesicles; one form is larger (800-900A in diameter with a dense granule 500A in diameter) than the other form (500A in diameter with a granule 280A in diameter). With this finding a controversial point arose as to which vesicle represented the storage form of the monoamines. Some investigators feel that the two populations characterize the same type of monoamine storing vesicle, but containing different amounts of the neurotransmitter substance (Pfeifer et al., 1968).

However, Hökfelt, in a series of papers (Hökfelt, 1968, 1969; Hökfelt and Jonsson, 1963) using potassium permanganate staining (Richardson, 1966), which appears to be specific for monoamines, concludes that the smaller vesicles are the monoamine storage forms in the pre-synaptic terminal. This conclusion is based upon pharmacological analysis combined with ultra-structural studies of regions of the central and peripheral nervous systems which are known to contain high levels of monoamines. Consequently, the function of the larger dense-cored vesicles, which may represent protein-laden organelles (Bloom and Aghajanian, 1968), remains to be elucidated.

Although electron microscopy has proven to be an important tool in the
study of the central nervous system, a description of the monoamine content of a large area of the brain is technically quite difficult due to the small sample size (1mm.) of each specimen block necessary for electron microscopic examination. This problem is coupled with the fact that although the dense-cored vesicles appear to represent a storage form of the monoamines, they may only demonstrate a portion of the total monoamine content of the neuron. This point is based upon the observation that although monoamines have been described in neuronal cell bodies (Dahlström and Fuxe, 1965) the small granular vesicles have not been found in those nerve cell bodies known to contain monoamines (Hökfelt, 1968; Richards and Tranzer, 1970). This observation is further supported by a study (Fisher and Snyder, 1965) on the subcellular distribution of noradrenaline in sympathetic ganglia which indicates that the norepinephrine in cell bodies, in contrast to the nerve endings, is found mainly in the soluble fraction of brain homogenates rather than in the microsomal fraction.

A more successful approach to the problem of the intracellular localization of monoamines was the observation that monoamines, when treated with formaldehyde, are converted to fluorescent products (Eröö, 1932). This finding enabled Eränkö (1952, 1955) to describe fluorescing norepinephrine-containing cells in the adrenal medulla. However, these early studies employed liquid formaldehyde and were not able to achieve the desired degree of sensitivity.

The high degree of specificity and sensitivity of the technique in its present form originated from a spectrophotofluorimetric method for the determination of tryptamine (Hess and Udenfriend, 1959). In attempting to
adapt this method to catecholamines, Hillarp discovered that these substances could be converted to strongly fluorescent compounds after treatment with formaldehyde gas with the reaction taking place in a dry protein layer. This finding subsequently enabled Hillarp, with the assistance of Falck (Falck, 1962; Falck et al., 1962), to first describe the localization of monoamines in neurons of freeze-dried tissue (see Corrodi and Jonsson, 1967; Björklund et al., personal communication, for a further discussion of the development of this method).

III. The Fluorescent Histochemical Technique for the Localization of Monoamines

The fluorescent histochemical technique for the localization of monoamines as first described by Falck and Hillarp and later in more detail by Falck and Owman (Falck and Owman, 1965), has been extensively investigated in terms of its methodology and chemistry. In view of the extensive literature concerning this technique, a review of the experimental and theoretical bases of the technique is important to consider at this time.

A. Methodology

Briefly, the method includes the following steps: first, the desired tissue is rapidly excised from the animal and freeze-dried in vacuo. When the tissue is thoroughly dehydrated, it is brought slowly to room temperature and exposed to vapors from paraformaldehyde at 80 °C for one hour within a closed glass vessel. Afterward, the specimen is embedded in paraffin and sectioned; the sections are mounted, deparaffinized and covered with a coverslip. Microscopic analysis involves use of a high pressure mercury lamp to serve as the ultraviolet light source which is filtered through a heat ab-
sorbing filter and an excitation filter; the emitted light from the specimen is filtered through a barrier filter. When viewed, sites containing catecholamines appear green while serotonin-containing regions are yellowish.

With this method, the following factors in step-by-step sequence should be considered:

1. The time interval between the moment of death of the animal and the actual freezing of the tissue may affect the degree of fluorescence. Although an interval of one hour without deleterious effects has been reported (Falck and Owman, 1965), a more definitive study is necessary before fluorescent investigations of autopsy material may be seriously considered. In the only known study of human material with this technique (Constantinidis et al., 1969), the authors indicate that when more than an hour had elapsed between death of the patient to freezing of the tissue, the resulting diffusion of the monoamine fluorescence rendered specific and precise localization quite difficult. Although no difference in fluorescence was reported by others (Dahlström and Fuxe, 1965a; Fuxe, 1965b), some authors (Csillik and Erulkar, 1964) claim that a more wide-spread intraneuronal distribution of monoamine fluorescence is obtained in sections taken from living animals under anesthesia than in tissues from dead animals.

2. Freezing of the tissue should be completed as rapidly as possible to a very low temperature. Ultra-rapid cooling produces several principal effects (Moline and Glenner, 1964): (a) all chemical reactions cease; (b) rapid fixation is achieved; (c) diffusion of the highly soluble monoamines is restricted, due to the increased tissue viscosity; and (d) ice crystal artifacts are minimized. This last point is important for preservation of
tissue integrity and cell structure. As discussed by Bell (1952), the larger the ice crystals formed in frozen material, the larger the distance that materials in the tissue will be moved. Ice crystal size (i.e., growth rate) may be reduced by producing a large number of nuclei of crystallization. Studies by Fisher and co-workers (1949) have shown that the more rapidly a critical temperature range of -33 to -43 °C is passed, the greater the degree of nucleation and the finer the resulting ice crystal structures. A limiting factor to rapid cooling or quenching is the thermal conductivity of the cooling bath. Liquid nitrogen (b.p.: -195.8 °C), as discussed by Meryman (1956), is relatively inefficient for rapid cooling. During the transfer of heat from the specimen to the liquid nitrogen, the liquid vaporizes forming an insulating layer of nitrogen gas. Other liquified gases such as propane (b.p.: -42.17 °C) do not produce this gaseous envelope due to their higher boiling points, thus facilitating heat transfer. Hence, the most effective method of ultra-rapid cooling is to plunge the tissue into a bath of liquified gas such as propane, cooled by liquid nitrogen.

3. The next step in this procedure entails dehydration of the frozen specimens. This stage is necessary because, as will be indicated later in this discussion, the histochemical reaction is catalyzed by a dry protein layer. More significantly, however, since the monoamines are quite water soluble, all water must be removed before the tissues are allowed to thaw. During lyophilization, water molecules are removed from their crystal lattice in the frozen tissue by sublimation under vacuum. This process involves three essential steps (Meryman, 1960): (a) introduction of heat to supply the energy necessary for sublimation; (b) transfer of water vapor from the
ice crystal through the dried shell of the specimen; (c) removal of the water vapor from the surface of the tissue. Freeze-drying in vacuo necessitates a considerable amount of time and control; a small piece of tissue usually requires at least a week of lyophilization. If the preparation is not sufficiently dry, the resulting fluorescence will be extremely diffuse with poor intracellular localization of the amines.

4. Upon exposure of the specimen to the formaldehyde vapors, two parameters will affect the fluorescence: (a) the pH of the tissue and (b) the humidity present during the reaction. For optimal results, the pH of the tissue should be 7.5-8.0 (Corrodi et al., 1966). Since mammalian tissue exists at a pH range 7.2-7.4, the above investigators suggest maintaining the specimen in an atmosphere of ammonia gas for a short interval before exposing it to the paraformaldehyde. The humidity, however, presents a more serious problem. Water in the formaldehyde reaction stems from three sources: (a) the air within the vessel, (b) the tissue itself, and (c) the paraformaldehyde. If too little humidity is present, the resulting fluorescence will be weak, for water appears to increase the buffer capacity of the tissue protein, providing a more favorable pH (Corrodi et al., 1966). However, if too much water is present, excessive diffusion of the fluorescence results due to the solubility of the monoamines. Moisture from the first two of the above-mentioned sources may be easily controlled by (a) placing the vessel opened in an atmosphere of dry air until use and by (b) adequate tissue freeze-drying. The moisture of the paraformaldehyde, most of which is absorbed water (Hamberger et al., 1965), however, is of critical importance and needs to be standardized for consistent results, with the
optimal relative humidity of the paraformaldehyde being 50-80% (Hamberger et al., 1965) or 50-70% (Falck and Owman, 1965). A constant humidity of the paraformaldehyde may be maintained by storing the chemical in desiccators with an open vessel containing sulfuric acid of a specific density for 5-7 days before use (Hamberger et al., 1965). In a study using multiple exposures of paraformaldehyde containing different water contents, it was found (Fuxe and Jonsson, 1967) that if dehydrated tissues are first treated with paraformaldehyde of 70% humidity at 80 C for one hour followed by a second treatment of paraformaldehyde with a relative humidity of 90-97.5% for 1-2 hours at the same temperature, a significant increase in the intensity and number of monoamine-containing nerve terminals results. Other factors influencing the quality and intensity of monoamine fluorescence include exposure time and temperature to the formaldehyde vapors and the neuronal monoamine concentration. These points will be discussed later in this literature review.

The fluorescence method as originally described is hampered by two disadvantages: (1) the time-consuming procedure of freeze-drying a block of tissue, requiring an expensive freeze-dryer and (2) the inability to correlate histochemical studies of various enzyme systems from the same block due to the necessity for paraffin embedding. To overcome these limitations, investigators have devised techniques whereby the frozen tissues are directly sectioned on a cryostat and the sections, in turn, are freeze-dried (Hamberger and Norberg, 1964; Nelson and Wakefield, 1968; Placidi and Masuoka, 1968). The obvious advantages of this method are the ability to perform correlative histochemical studies from the same block and shortened time required to
freeze-dry a section as compared to a block of tissue (several hours versus 7-10 days). Some investigators have eliminated freeze-drying in vacuo completely by leaving the sections in the cryostat for several hours until dry (Hamberger and Norberg, 1964), by placing the sections in a vessel containing phosphorous pentoxide and maintaining them below -30 C until dry (Csillik and Kálmán, 1967; Spriggs et al., 1966) or by merely rapidly air drying the sections at room temperature (Csillik and Erulkar, 1964; El-Sadawi and Schenck, 1967). In a study on melanomas, cryostat sectioning was eliminated by simply pressing the desired tissue on a glass slide, drying the slide and exposing it to formaldehyde vapors; the "imprinted" amines then fluoresce (Ehinger et al., 1969). In response to these modified techniques, the innovators of the fluorescence method and their co-workers warn of the high risk of diffusion of the amines, rendering intracellular localization impossible (Falck and Owman, 1965) and cite the illustrations in one particular article as an example (Csillik and Erulkar, 1964).

B. Chemistry

The chemical basis of the fluorescence method is well known, having been studies in vivo and in vitro. Essentially, the fluorescence phenomenon is based upon the principle that when certain substances are excited by light of one wave-length, they emit light of a different, usually longer, wave-length. The fluorescent localization of monoamines employs ultraviolet light (short wave-length, 410mµ) passed through the formaldehyde-treated material which, in turn, emits light in the visible spectrum (longer wave-length 480-525mµ). The observed fluorescence is present only as long as the ultraviolet light excites the treated tissue.
Thus, the basis of the technique lies in the reaction of the monoamines with heated formaldehyde gas. The chemistry of this reaction has been revealed from extensive studies on protein layer models and in tissues (Falck et al., 1962; Jonsson, 1966, 1967a, 1967b; Björklund et al., 1970; Corrodi and Jonsson, 1965a, 1965b; Corrodi and Hillarp, 1963, 1964; Corrodi et al., 1964; see also Corrodi and Jonsson, 1967) and has been described as occurring in three phases. The first phase, a Pictet-Spengler reaction, involves the condensation of an amine with formaldehyde; this portion of the reaction proceeds rapidly, even at room temperature. The reaction for catecholamines, indicated in text-figure 4, yields a 6,7-dihydroxy-1,2,3,4-hydroisoquinoline derivative. The initial product in the condensation of serotonin with formaldehyde is a 6-hydroxy-1,2,3,4-tetrahydro-beta-carboline as indicated in text-figure 5. An unexpected dehydrogenation of the tetrahydro-isoquinoline and -beta-carboline derivatives resulting in a 6,7-dihydroxy-3,4-dihydroisoquinoline and a 6-hydroxy-3,4-dihydro-beta-carboline, respectively, occurs as the next step; the dehydrogenation, as indicated in text-figure 6, is catalyzed by a dry protein layer. In the last phase of the reaction, a pH-dependent equilibrium is established between the 6,7-dihydroxy-3,4-dihydroisoquinoline and a tautomeric quinoidal form as shown in text-figure 7; apparently the dihydro-beta-carboline form is stable and is not affected by pH. At the lower pH range, the protonated non-quinoidal form predominates, but is only weakly fluorescent. At pH 5, the equilibrium shifts to the right to the more strongly fluorescent quinoidal form until maximal fluorescence is reached at pH 7-8.

These investigations have also revealed limitations to which sub-
Text-figure 4. Reaction of dopamine and norepinephrine with formaldehyde vapors to yield a tetrahydrideisquinoline derivative.

Text-figure 5. Reaction of serotonin with formaldehyde vapors to yield a tetrahydride-beta-carbeline derivative.

Text-figure 7. A pH-dependent equilibrium between the protonated non-quinoidal and tautomeric quinoidal forms of the dihydriosequinoline derivative formed from the catecholamines.
stances are identified with this method; i.e., the compound must be either a primary or secondary aromatic amine and a 6-hydroxy or 6,7-dimethoxy group is necessary for strong fluorescence. Of the catecholamines, only 3-hydroxy-or 3,4-dihydroxyphenylethyl amines and their beta-hydroxylated or alpha-methylated analogues and corresponding amino acids (dopa and alpha-methyl dopa) will react. Epinephrine and alpha-methyl-dopa, however, are relatively less reactive than the other amines possibly due to a greater energy requirement for the dehydrogenation step (Jonsson, 1967b). Of the indolethylamines, serotonin as well as 6-hydroxy tryptamine and 5,6-dihydroxytryptamine (Jonsson and Sandler, 1969) produce a specific yellow formaldehyde-induced fluorescence. Fortunately, the 3-O-methylated and deaminated metabolites of the monoamines as well as tertiary amines, amides, tyrosine, tyramine, melatonin, and tryptophan show weak if any fluorescence and, consequently, will not interfere with identification of the desired monoamines. The fluorescent reaction products are relatively water insoluble as compared to the unreacted monoamines. Originally, investigators postulated a chemical bond (e.g. methylene bridges) between the reaction products and the surrounding protein, but more recent evidence (Jonsson, 1967b) suggests that the dihydroisoquinolines and -beta-carboline are enclosed within or absorbed on a protein network formed by the tanning action of the formaldehyde.

C. Specificity and Sensitivity

When viewed with a fluorescence microscope, the catecholamine and serotonin-containing sites may be differentiated by their respective green and yellow color. The difference in color is due to the spectral characteristics of the emitted light; i.e., the catecholamine reaction product emits
a shorter wave-length light (480µm) than does the fluorescence from serotonin-containing sites (525µm). Various methods have been developed to distinguish monoamine fluorescence from autofluorescence, the catecholamine-containing neurons from the 5-HT-containing neurons, and to even differentiate the various catecholamines. These techniques may be classified under three categories: physical conditions, chemical treatment, and pharmacologic methods.

Physical conditions: When the reaction time, temperature and humidity, are varied, catecholamine- and serotonin-containing sites may be distinguished from each other (Jonsson, 1967b). Treatment with paraformaldehyde under mild conditions will produce fluorescence of mostly catecholamines. Optimal visualization of serotonin requires more humid paraformaldehyde treatment for a one to two hour period. In addition, serotonin fluorescence disappears much faster under ultraviolet light than does catecholamine fluorescence. The yellow fluorescence decreases rapidly within the first minute upon exposure of the specimen to ultraviolet light and is much reduced after 10-15 minutes, whereas the green fluorescence does not show evident fading before 45-90 minutes of exposure.

Chemical treatment: A rapid and efficient check to determine whether the observed fluorescence is due to reacting monoamines or autofluorescence utilizes sodium borohydride (Corrodi et al., 1964). This substance reduces dihydro-isoquinoline and -beta-carboline to the corresponding non-fluorescent tetrahydro compounds. Thus a fluorescence which does not disappear shortly after sodium borohydride treatment is not considered as being due to serotonin or catecholamines. In another chemical test, the treatment of
specimens with dry hydrochloric gas allows differentiation of catecholamines from serotonin by characteristic catecholamine spectral shifts (Corrodi and Jonsson, 1967).

Pharmacologic methods: Drugs act upon the nervous system in various ways to interfere with monoamine synthesis, storage and release. These metabolic pathway alterations with examples of the corresponding agents include:

1. inhibition of monoamine precursor formation; e.g.-
   a. Hz4/68 (a methyl ester of alpha-methyl-p-tyrosine) inhibits tyrosine hydroxylase formation.
   b. Hz2/54 (alpha-propyldopacetamine) inhibits tryptophan hydroxylase formation.

2. blockade of monoamine uptake into the dense-core vesicle granules; e.g.-
   a. reserpine produces a long-lasting block of the ATP-Mg²⁺-dependent uptake-storage mechanism.
   b. tetrabenzazine produces a rapid, short-lasting block of the ATP-Mg²⁺-dependent uptake-storage mechanism.

3. displacement of the monoamines by analogues which can not be metabolized; e.g.-
   a. alpha-methyl-dopa displaces catecholamines.
   b. H75/12 (4-methyl-alpha-ethyl-meta-tyramine) displaces both catecholamines and serotonin (Carlsson et al., 1969a).

4. inhibition of monoamine oxidase; e.g.-
   a. nialamide (isonicotinyl-benzylcarbamethylydrazine).
   b. MO-911 (N-benzyl-N-methylpropynylanine).
   c. iproniazid (1-isonicotinyl-2-isopropyl hydrazide phosphate)

5. disruption of the "membrane-pump" mechanism which concentrates extracellular monoamines into the neuron; e.g.-
   a. chlorimipramine.
   b. desimipramine.

6. stimulation of monoamine release from the nerve ending; e.g.-
   a. amphetamine.
   b. lithium salts (Corrodi et al., 1967a).

The net result of the pharmacologic actions is a decrease, an increase, or no change in the specific fluorescence depending upon the effect of the drugs
upon the available reactive monoamines.

Use of these drugs correlated with fluorescent studies have revealed in addition to the site of action and mechanism of the drugs, differences in neuronal localization of serotonin and the catecholamines. Pharmacologically, perhaps the most common means of differentiating serotonin-containing neurons from catecholamine-containing neurons combines reserpine and nialamide. When an animal is sacrificed after administration of reserpine, no significant fluorescence is visible from either catecholamine-containing or serotonin-containing neurons (Carlsson et al., 1964; Dahlström and Fuxe, 1964a; Dahlström et al., 1965; Fuxe, 1965a, 1965b; Fuxe et al., 1966b). If, however, nialamide is administered to the reserpinized animal, only the yellow fluorescence of serotonin-containing neurons is prominent (Dahlström and Fuxe, 1965a; Fuxe, 1965a, 1965b; Fuxe et al., 1966b).

Monoamine oxidase inhibitors such as MO-911 (Fuxe, 1965a, 1965b; Fuxe et al., 1966b), iproniazid (Dahlström and Fuxe, 1965a), 2-phenylcyclopropylamine (Bartonicek et al., 1964), beta-phenylisopropylhydrazine (Bartonicek et al., 1964), isocarboxazide (Bartonicek et al., 1964) and pargyline (Hillarp et al., 1966) may also be administered without prior reserpinization, resulting in increased serotonin fluorescence with slight or no appreciable increase in catecholamine fluorescence. Use of amine synthesis inhibitors will differentially affect serotonin-containing or catecholamine-containing neurons; when the drug H22/54 is administered to an animal, only serotonin-containing neurons are depleted (Corrodi et al., 1967b; Fuxe and Gunne, 1964) while only catecholamine-containing neurons lose their fluorescence when H44/68, a tyrosine hydroxylase inhibitor, is used (Andén et al., 1966c; Corrodi et al.,
1967b; Fuxe and Ungerstedt, 1966; Fuxe and Jonsson, 1967). Blockade of the "membrane-pump" mechanism for concentrating monoamines into the neuron by chlorimipramine appears to be specific for serotonin-containing neurons, decreasing their yellow fluorescence, while catecholamine regions are left unaffected (Carlsson et al., 1969b). Amphetamine (Carlsson et al., 1966, 1967; Fuxe et al., 1966a; Fuxe and Ungerstedt, 1966) and beta-phenylethylamine (Fuxe et al., 1967) decrease fluorescence in catecholamine-containing neurons but not in serotonin-containing neurons.

In addition, the amino acids meta-tyrosine and alpha-methyl-meta-tyrosine cause depletion of catecholamines but not serotonin, possibly by a displacement mechanism (Andén, 1964; Björkland, 1968; Carlsson et al., 1962a, 1964; Carlsson and Lindqvist, 1967; Dahlström and Fuxe, 1964a, 1965a; Fuxe, 1965a, 1965b; Hillarp et al., 1966).

By using combinations of drugs, the primary catecholamines - noradrenaline and dopamine - may be differentiated from each other. When alpha-methyl-dopa pretreatment is followed by either reserpine (Carlsson et al., 1965) or tetrabenazine (Carlsson et al., 1967), only dopamine terminals lose their fluorescence. When lithium is followed by 3H4/68, norepinephrine terminals are depleted significantly so that only dopamine neurons fluoresce (Corrodi et al., 1967a). Only dopamine neurons will fluoresce when norepinephrine uptake is blocked by protriptyline (Carlsson et al., 1966; Hökfelt and Fuxe, 1969) or desipramine (Carlsson et al., 1966; Fuxe et al., 1966a; Fuxe and Ungerstedt, 1966) in animals pretreated with the drug series reserpine+nialamide+L-dopa. RO4-4602, a decarboxylase inhibitor, followed by L-dopa, selectively increases the dopamine concentration and fluorescence.
in the brain (Constantinidis et al., 1967). In addition, a single injection of alpha-methyl-meta-tyrosine reveals a difference in dopamine and noradrenaline activity, for after administration of the drug, dopamine fluorescence recovers first (within 15–24 hours) while norepinephrine neurons require approximately four days to regain their fluorescence (Dahlström and Fuxe, 1965a; Fuxe, 1965a, 1965b; Hillarp et al., 1966). Administration of gamma-butyrolactone has been reported (Aghajanian and Roth, 1970) to produce a selective increase in dopamine at the terminals of dopaminergic neurons.

One of the most striking features of the fluorescence technique which lends itself as an excellent histochemical tool, lies in its high degree of sensitivity. Within 10µ-thick sections, monoamines have been demonstrated in concentrations of 0.005–0.001% (w/v) or 1–5µg/g of protein (Corrodi and Jonsson, 1967). Anden and co-workers (1966a) utilized the sensitivity of this method in a quantitative study and calculated the average dopamine content of a neuron located in the nigro-neostriatal system of the rat to be \( 1.1 \times 10^6 \) g. The limiting factor to the sensitivity of resolution of adrenergic structures is the background fluorescence. However, one needs to be cautious in evaluating fluorescence results without adequate controls and correlative studies. In a physiological and biochemical study in the cat, for example, the norepinephrine content of the brain stem was found to significantly decrease during sham rage (Reis et al., 1967). However, Reis and Fuxe (1968), in a similar study, found slight if any depletion of the fluorescence in the brain stem during sham rage, and only with blockade of norepinephrine synthesis by [H/4]68 was a significant decrease in the fluorescence of the terminals observed.
IV. Fluorescent Characteristics of the Monoamine Neuron

As demonstrated particularly well in studies on sympathetic chain tissue cultures, (Sano et al., 1967b) the histofluorescence method reveals that monoamines are located throughout the entire neuron. When viewing sectioned material, however, the fluorescence may appear cellular, fibrous, or dotted, corresponding, respectively, to the nerve cell body, processes, or terminals. The characteristics and location of each of these neuronal components will be considered as a prelude to a discussion of mapping of the monoamine pathways. Since most of this work has been performed on the rat, the following presentation refers to this animal unless otherwise noted.

Cell bodies: Generally the fluorescence in monoamine-containing neuronal cell bodies is weaker than in their terminals, indicating a lower amine concentration in the perikarya. Dahlstrom and Fuxe (1965a), in an extensive study, found most of the fluorescent cell bodies located within the lower brain stem and divided them into two types: (1) type A, consisting of twelve groups containing catecholamines and (2) type B, consisting of nine groups, containing serotonin. Generally, type A neurons (catecholamine-containing) are found in a more lateral position in the brain stem except in the cranial part of the mesencephalon. They extend from the medulla to the diencephalon, being most concentrated in the mesencephalon. Type B neurons (serotonin-containing) on the other hand, are localized mainly within the midline raphé nuclei of the lower brain stem, surrounding the pyramidal tract and in the medioventral part of the caudal tegmentum; none have been observed in the spinal cord, diencephalon or telencephalon. Briefly, these groups and their location are summarized as follows:
Medulla Oblongata
A1 - found in the ventrolateral part of the reticular formation; largest group in the medulla.
A2 - small group found mostly within the nucleus commissuralis.
A3 - weakly fluorescent cells found in the nucleus accessorius dorsalis.
A4 - strongly fluorescent cells in the lateral part of the roof of the fourth ventricle.
B1 - medium-sized cells around the medial and ventral portion of the pyramidal tract, extending from the pyramidal decussation to the nucleus facialis.
B2 - small group of cells within the nucleus raphe obscurus.
B3 - a large group surrounding the pyramidal tract at different levels of the nucleus facialis; no distinct border between groups B1 and B3.
B4 - located just under the fourth ventricle, dorsal to the vestibular nuclei and abducens nerve nucleus.

Pons
A5 - small group of cells among the fibers of the rubrospinal tract, mainly at the level of the caudal and middle third of the superior olivary nucleus and medial to the out-going fibers of the facial nucleus.
A6 - group of cells within the locus coeruleus; strongly fluorescent.
A7 - small group of cells at the level of the caudal third of the griseum pontis.
B3 - continuation of the same group from the medulla oblongata.
B5 - found within the nucleus raphe pontis at the level of the motor nucleus of the trigeminal nerve.
B6 - similar to group B4; found in the midline just ventral to the fourth ventricle.

Mesencephalon
The caudal portion of the mesencephalon contains almost exclusively serotonin cells, while the rostral portion contains mostly catecholamine cell bodies.
A8 - large group located within the reticular formation; most of the cells just caudal to the red nucleus.
A9 - large group within the substantia nigra, mostly within the zona compacta.
A10 - largest group of the mesencephalon; cells are mainly localized in the area dorsal to the nucleus interpeduncularis.
B7 - large group in the substantia gisea centralis with most of the cells located within the nucleus dorsalis raphe.
B8 - fairly large group, mainly located within the nucleus medius raphe.
B9 - another large group situated within and around the medial lemniscus; many cells also found dorsal to the lemniscus in the reticular formation.
Diencephalon

All - small group of scattered cells close to the third ventricle.
Al2 - small group of cells within the arcuate and periventricular nuclei.

In the hypothalamus of the guinea pig (Barry and Leonardelli, 1967), fluorescing cell bodies were described which correspond to groups All and Al2 in the rat (Dahlström and Fuxe, 1965a); similar findings were reported in a study of the hypothalamus of the golden hamster (Leonardelli and Herman, 1967). In a study of the brain stem of the cat (Pin et al., 1968a, 1968b), an important group of serotonin-containing cells is described in the large-cell region of the red nucleus which is not found in the rat, and an important group of catecholamine-containing neurons in the medulla oblongata of the rat (group Al) is not observed in the cat. Otherwise, the distribution of fluorescing cell bodies in the cat was found to be similar to that of the rat. However, it is important to note that the description of the monoamine-containing neurons in the brain of the cat is not as detailed as one would like. At this time, one can not exclude the possibility that an in-depth study may reveal other differences than the ones which have been reported in the literature to date.

Fibers and terminals: The axons of monoamine cell bodies may be differentiated into two forms: (1) non-terminal and (2) terminal axons. Non-terminal axons contain low concentrations of monoamines, approximately that of the cell body (100 µg/g) and usually have a smooth outline. Some fiber tracts, however, are characterized by ovoid enlargements with low amine concentration along their length. The functional basis of these enlargements is not known, although they have been related to peristaltic activity of the axon (Dahlström and Fuxe, 1965b).
Terminal axons, on the other hand, characteristically contain high quantities of amines, as evidenced by their strong fluorescence, and appear as varicosities. A varicosity (Hillarp et al., 1966) is an enlargement of an axon which (1) contains a high concentration of the amine and (2) must lie in the area to be innervated. The diameter of the varicosities ranges from 0.3µ to 1.0µ. In the spinal cord, the varicosities have been determined to contain an amine concentration of 10,000 µg/g; the terminals in the neostriatum contain 50-150 times more dopamine than do their cell bodies (Andén et al., 1966a). The number of varicosities per unit length of fiber in the cerebral cortex ranges from 15 to 30/100µ (Fuxe et al., 1968a); the terminal system of one neuron in the nigro-neostriatal pathway, calculated to be 55-77cm in length, contains approximately 500,000 varicosities, or 5-7 varicosities per 7.7µ (Andén et al., 1966a). The varicosities are generally considered to represent those structures which are specialized for the synthesis, storage and release of the amine neurotransmitters, i.e., the pre-synaptic ending (Fuxe, 1965a, 1965b). Each varicosity, however, does not represent a dense-cored vesicle since the vesicles are only about 0.05µ in diameter, but rather, the varicosities appear to be accumulations of the vesicles. Several studies have attempted to correlate the varicosity fluorescence with the fine structure of monoamine endings. One unsuccessful attempt sought to correlate the morphological substrate of fluorescence by observing freeze-dried material treated with 100% formaldehyde with an electron microscope (Eneström and Svalander, 1967).

By the methods indicated earlier, monoamine terminals are differentiated into dopamine-, norepinephrine-, and serotonin-containing structures.
Dopamine endings, very fine in appearance (0.3–0.5 µ thick), are located mostly in circumscribed regions of the telencephalon (the neostriatum, tuberculum olfactorium and nucleus accumbens) as well as in the external layer of the median eminence (Fuxe, 1965b). In the lower brainstem, dopamine terminals are located around the nucleus tractus solitarius and the dorsal motor nucleus of the vagus nerve (Dahlström and Fuxe, 1964a). Norepinephrine terminals are thicker than their dopamine counterparts, vary greatly in size, and have a more widespread distribution. They form a rich plexus in the limbic lobe, around nuclei of the autonomic hypothalamus, around visceral afferent and efferent nuclei of the cranial nerves, and in certain parts of the reticular formation and the raphé complex (Fuxe, 1965b). Localization of serotonin terminals, however, is complicated by their very fine size and the rapid photodecomposition of the yellow fluorescence. In the brainstem, serotonin terminals have been identified within nuclei with catecholamine terminals such as in the motor nuclei of the vagus and trigeminal nerves and the nucleus tractus solitarius (Dahlström and Fuxe, 1964a) and in areas with few catecholamine terminals as in the subfornical organ (Lichtensteiger, 1967). Numerous serotoninergic terminals have been reported throughout the spinal cord (Carlsson et al., 1964; Fuxe, 1965b), especially in the sympathetic intermedio-lateral column of the mouse (Carlsson et al., 1964). In addition, large numbers of serotonin terminals are found in the superficial part of the substantia grisea periventricularis, the pretectal area and in the area just dorsal to the nucleus interpeduncularis (Dahlström and Fuxe, 1964a).

In the cerebellar and cerebral cortices, the monoamine terminals do not
display any distinct degree of stratification or delineation of innervated structures. Catecholamine terminals (probably norepinephrine) appear to make axodendritic contacts in the molecular and granular layers of the cerebellum while serotonin terminals are located mainly in the molecular layer (Arbuthnot, 1966; Hökfelt and Fuxe, 1969). Norepinephrine terminals are found in all areas of the cerebral cortex but appear more frequently in the molecular and external granular layers, possibly forming axodendritic contacts (Fuxe et al., 1968a).

The specificity of monoamine neurons, i.e. the inability of monoaminergic neurons of one type to take up and store monoamines of another type, has been studied by fluorescence microscopy coupled with uptake studies of labeled and unlabeled monoamines and their precursors. However, with a blood-brain barrier mechanism for monoamines (Weil-Malherbe et al., 1961) in all parts of the brain except at the median eminence, area postrema and subfornical organ (Hamberger and Hamberger, 1966; Lichtensteiger, 1967), the amines are unable to penetrate further than the glial basement membrane of the astrocytes surrounding the blood vessels (Samorański and Marks, 1962). Hence the uptake studies need to be performed on slices incubated in vitro (Hamberger and Masuoka, 1965) or in vivo with lesions made in the blood-brain barrier via cold injury or mercuric dichloride (Hamberger and Hamberger, 1966). The amines may be injected into the cerebral vesicles to avoid the barrier but they will only be localized to a zone 200-400 µ thick close to the ventricles (Fuxe and Ungerstedt, 1966; Fuxe et al., 1968b).

These investigations, coupled with pharmacologic studies, indicate a relatively high degree of specificity of the serotoninergic neurons for
serotonin uptake and the adrenergic neurons for catecholamines, suggesting a basic metabolic difference between the two types of monoaminergic neurons (Fuxe et al., 1968b; Hillarp et al., 1966). However, reports indicate that after pretreatment with reserpine and nialamide, serotonin is taken up by catecholamine-containing neurons outside of the blood-brain barrier, although not readily (Lichtensteiger and Langeman, 1968). In addition to being specific for catecholamines, adrenergic nerve uptake is stereospecific for the L-isomers at low amine concentration, but at higher concentrations the uptake does not appear to be dependent upon steric configuration (von Euler and Lishajko, 1964).

V. Mapping of Central Monoamine Pathways

The usual method for mapping monoamine pathways in the central nervous system is to study via fluorescence the antero- and retrograde degenerative changes following lesions of the brain and spinal cord. As Dahlström and Fuxe (1964b) demonstrated, 24 hours after a monoamine-containing axon has been severed, the fluorescence becomes quite intense in the swollen and deformed axon proximal to, but not distal to the lesion. The fact that this fluorescence is depleted by reserpine (Dahlström and Fuxe, 1964b) supports the view that the dense-core vesicles seen with the electron microscope are transported to the nerve ending from the cell body.

As demonstrated in the nigro-neostriatal system (Andén et al., 1965), more specifically, the accumulation of fluorescence proximal to the lesion is very marked during the first week after axotomy, but completely disappears during the third and fourth week. Distal to the lesion, as illustrated by transection of the spinal cord (Dahlström and Fuxe, 1965b), the terminals
first appear more distinct (on the second post-operative day), but by the fourth day, the fluorescence starts to disappear until the twelfth day when no monoamine terminals are evident below the lesion. At the electron microscopic level, Hökfelt and Ungerstedt (1969) found that after destruction of the axon, the nerve endings lose their ability to take up amines and accumulate them at the granular storage sites; this inability is correlated with loss of fluorescence of the terminals. Monoamine perikarya also undergo post-operative changes, for they appear swollen with a marked increased fluorescence intensity soon after axotomy. After the third to fourth week, however, the fluorescence greatly decreases with a corresponding loss of staining ability of the Nissl substance (Andén et al., 1965).

In addition to electrolytic and mechanical lesions in the central nervous system, a chemical substance, 6-hydroxy-dopamine, offers possibilities for mapping by chemically producing selective degeneration of adrenergic pathways (cf. Lavery et al., 1965; Malmfors and Sachs, 1968; Mueller et al., 1969; Porter et al., 1963; Tranzer and Thoenen, 1968). Ungerstedt (1968), in histofluorescence investigations on the effect of 6-hydroxy-dopamine, found that when the drug was injected into a known dopaminergic pathway or nucleus, the fluorescence distal to the injection started to disappear after one day and was completely gone after twelve days, while fluorescence accumulated proximal to the injection. Therefore, as evaluated by histofluorescence, the response of neurons to 6-hydroxy-dopamine is essentially the same as when mechanical or an electrolytic lesion is produced.

Thus degenerative changes, whether produced physically or chemically, occur in monoamine cell bodies, axons, and terminals and these changes may be
directly visualized by fluorescence. By correlating these changes with lesions at known sites, researchers have been able to trace specific monoamine pathways. The following sections summarize the systems which have been mapped out by this method.

**Catecholamine Pathways**

**Bulbospinal:** Nerve cells of a norepinephrine bulbospinal neuronal system (Dahlström and Fuxe, 1965b) appear to be located mainly in the ventrolateral part of the reticular formation of the medulla oblongata (group Al). From these cells, two main pathways descend in the spinal cord: one large system courses in the anterior funiculus and in the most ventral part of the lateral funiculus, terminating on alpha-motorneurons in the ventral horn. The second system, somewhat smaller, descends in the dorsal portion of the lateral funiculus, terminating in the sympathetic cell column and the dorsal horn, especially in the substantia gelatinosa (Andén et al., 1964a; Carlsson et al., 1964; Corrodi et al., 1964). Approximately 50% of the fibers to the sympathetic intermediolateral column cross in the spinal cord via the posterior gray commissure while fibers to the ventral and dorsal horns do not cross at spinal levels but may decussate in the medulla (Dahlström and Fuxe, 1965b; Hillarp et al., 1966).

**Ascending rhombencephalic norepinephrine pathways:** Approximately 70% of the catecholamine terminals in the prosencephalon originate from neurons in the medulla oblongata and pons (Hillarp et al., 1966). Axons arising from groups Al,A5,A6, and A7 ascend through the dorsal part of the reticular formation of the medulla and pons, just medial to the outgoing fibers of the facial nerve, and continue as a bundle through the lateral part of the caudal
mesencephalic reticular formation. This bundle then courses dorsomedially and passes through the diencephalon dorsal to field \( H_1 \) of Forel. At the level of the infrahypophyseal recess, the pathway splits into two parts: (1) a ventromedial division terminates in the medial hypothalamic nuclei, while (2) a ventrolateral portion joins the medial forebrain bundle to terminate in the limbic forebrain structures (amygdaloid cortex, gyrus cinguli, hippocampal formation, septal area) as well as in lateral hypothalamic regions, the preoptic area and parts of the thalamus (Andén et al., 1966b, 1966d, 1967; Hillarp et al., 1966; Loisou, 1969). In addition, fibers suggested to be aspecific afferents (Fuxe et al., 1968a) reach the neocortex from the medial forebrain bundle via the tractus diagonalis (Andén et al., 1966b; Fuxe et al., 1968a). The cerebellar cortex appears to receive fibers from the ascending norepinephrine system via the inferior cerebellar peduncle (Andén et al., 1967; Hökfelt and Fuxe, 1969).

**Nigro-neostriatal system:** The nigro-neostriatal pathway, perhaps the most studied dopamine system of the brain, ascends as an uncrossed tract, arising mainly from the zona compacta of the substantia nigra (group A9). Most of the fibers leaving the cell bodies form a bundle which courses medial and dorsomedial to the ventral part of the crus cerebri. At the level of the posterior part of the median eminence, the fibers turn into the ventro-rostral part of the crus cerebri and run in the retrolenticular portion of the internal capsule where they course rostrally and dorsally to terminate in the nucleus caudatus-putamen (Andén et al., 1964b, 1965, 1966b; Bertler et al., 1964; Fuxe et al., 1964; Hökfelt and Ungerstedt, 1969).

**Ascending mesencephalic dopamine system:** Another ascending dopamine
system arises from group A10 cells in the mesencephalon. Its axons run medial to the nigro-neostriatal system, coursing uncrossed in the medial forebrain bundle and terminate in the tuberculum olfactorium, nucleus accumbens and the dorsolateral part of the nucleus interstitialis stria terminalis (Andén et al., 1966b; Bőrter, 1964).

Tubero-hypophyseal system: Due to its close relationship with the endocrine system, a monoamine hypothalamic-hypophyseal pathway has undergone considerable investigation in not only the rat, but also in other animals such as the pig (Björklund, 1968), mouse (Björklund et al., 1966b), and cat (Fuxe, 1964). This system originates from nerve cells of the arcuate and periventricular nuclei in the diencephalon (group A12); its fibers terminate in the external layers of the median eminence and the infundibulum (mainly on the primary capillary plexus of the hypophyseal portal system) and in the intermediate lobe of the pituitary gland (Björklund, 1968; Björklund et al., 1966b, 1969; Fuxe, 1964; Fuxe et al., 1966a; Odake, 1967; Sano et al., 1967a). This pathway, which is not fully developed until the first postnatal weeks (Björklund et al., 1966b, 1969) appears to be primarily dopaminergic with some norepinephrine activity. Due to the relatively diverse studies on this system, several species and genus differences have been reported. The neurohypophysis of the rat and pig (Björklund, 1968), for example, contains a rich plexus of varicose fibers, while few fluorescent fibers are found in the same structure in the mouse (Björklund et al., 1966b) and cat (Fuxe, 1964). With the finding of a tubero-hypophyseal monoamine pathway, adrenergic mechanisms have been postulated to directly control the intermediate lobe (Odake, 1967) or indirectly regulate the
adenohypophysis via the hypophyseal portal plexus (Fuxe, 1964). A unique feature of this system is the presence of "droplets" of fluorescence found in almost all species investigated. Observed within the median eminence and infundibular stem, the droplets appear to be terminal swellings or end-bulbs on smooth and varicose fibers quite distinct from the varicosities of terminal axons. Due to their relationship to known neurosecretory fibers, the droplets may contain neurosecretory products. Several workers, however, using aldehydefuchsin staining which is specific for neurosecretory neurons, report no correlation between the location of the fluorescent droplets and stained structures (Björkland, 1968; Björkland, et al., 1968b).

**Serotonin Pathways**

**Bulbospinal:** Like their corresponding norepinephrine systems, two descending serotonin pathways originate from neurons in the medulla oblongata (groups B1, B2, and B3). One system descends in the medial part of the anterior funiculus and the anterior part of the lateral funiculus to terminate in the ventral horn. The serotonin endings on the sympathetic intermediolateral column and dorsal horn are terminals of fiber tracts in the lateral funiculus, especially its dorsal part. The serotonin system displays the same pattern of decussation as the descending norepinephrine pathways (Dahlström and Fuxe, 1965b). These bulbospinal systems - both serotonin and norepinephrine - are suggested to be inhibitory stemming from the vaso-depressor region of the medulla (Carlsson et al., 1964).

**Ascending mesencephalic serotonin pathway:** The majority of the serotonin terminals in the prosencephalon originate from nerve cells in the mesencephalon (groups B7, B8, B9). Most of the axons enter the medial fore-
brain bundle to the diencephalon and course uncrossed to the limbic forebrain structures and hypothalamus. The serotonin-containing axons to the neocortex and hippocampal formation assume the same course as do ascending noradrenaline-containing neurons; i.e., from the medial forebrain bundle to the tractus diagonalis and then into the superficial part of the white matter (Andén et al., 1966b, 1967).

The close similarity between these serotonin and norepinephrine pathways correlates well morphologically with the parallelism noted between serotonin and norepinephrine concentrations in the brain determined biochemically as indicated earlier in this review.

Pineal gland: The pineal gland should be briefly considered, for although no known monoamine pathways (besides sympathetic fibers) reach this structure, it has the highest concentration of serotonin of any organ in the rat. Within the pineal gland, serotonin is found by fluorescence microscopy to be localized within the pinealocytes and intrapineal sympathetic nerves (Falck et al., 1966). Using a potassium dichromate fixative specific for monoamines, however, Etcheverry and Zieher (1968) found no reactive sites in the pineal parenchymal cells at the ultrastructural level. On the other hand, in a combined kinetic and histochemical analysis of the serotonin compartments of the rat pineal gland, it was found that 30% of the total pineal serotonin content is stored in the sympathetic nerves (Neff et al., 1969b). Since this store disappears after treatment with desmipramine, an agent which prevents uptake of the amine, the authors postulated that serotonin is synthesized in the pinealocytes and, subsequently, is taken up by the sympathetic axons; the high turnover rate of serotonin in the pinealocytes
supports this idea (Falck et al., 1966; Neff et al., 1969b). This finding also supports the view that amine uptake in nerve terminals is non-specific, at least for serotonin uptake by adrenergic endings.

Thus, a study of the literature reveals several important aspects concerning the distribution, localization, and identification of monoamines in the central nervous system.

1. In those regions which have been biochemically determined to contain the monoamines, the morphological distribution of these substances in all portions of the neuron may be observed by a histochemical fluorescent method at the light microscopic level.

2. This fluorescent technique is highly sensitive and, with the appropriate pharmacologic manipulations, is sufficiently specific to preferentially demonstrate dopamine, norepinephrine, or serotonin.

3. Only a portion of the central nervous system, namely the brain stem and hypothalamus, contains a significant number of monoaminergic cell bodies.

4. By knowing the location of these cell bodies and by means of selective lesions of their axons, specific monoaminergic pathways may be mapped out in the central nervous system.

5. A morphological understanding of the distribution of these significant neurotransmitter substances provides valuable information concerning the functioning of the central nervous system.
VI. The Squirrel Monkey as an Experimental Animal in Neurobiology

In considering an experimental animal for this work, the squirrel monkey (Saimiri sciureus) was chosen because of several factors. An important reason is that of the size of the animal, permitting ease in care and handling as well as the possibility of purchasing a significant number of the primates for a relatively low cost. Technically, the smaller-sized brain allows a greater number of tissue specimens to be processed within a given period of time. Although the brain is small, being approximately the same size and weight as that of the cat (Pinneo, 1968), the ratio of the brain weight to body weight is relatively large. In the cat, for example, this ratio is approximately 0.013 (Hlinkow and Glezer, 1968) while in the squirrel monkey, the ratio is about 0.035 (Pinneo, 1968), or approximately three times as great.

While the small size of the brain facilitates the technical aspects of this problem, the animal appears to possess a significant degree of intelligence as determined by a close observation of the animal while in captivity. More objectively, in a study (Stephan and Andy, 1969) of the comparative neuroanatomy of the primates considering the progressive development of the neocortex (a good indication of neuroanatomical evolutionary advancement), the squirrel monkey is positioned approximately half-way between the lowest-placed simian and man. In terms of learning capabilities, Rumbaugh (1968) concludes that, although the behavioral potential of the squirrel monkey has not been fully evaluated, the perceptual skills which it displays are minimal in non-primates, but are most characteristic of other primates considered more highly evolved than the squirrel monkey.
Specific neurobiological studies on this primate have dealt with neuroanatomical and neurophysiological mapping of regions of its brain. These investigations have included mapping of somatic receiving areas in the cerebral cortex (Benjamin and Welker, 1957); description of the motor cortex in terms of topographical organization, stimulation threshold, and cytoarchitectural criteria (Welker et al., 1957); mapping of sites in the brain which produce different forms of vocalization when stimulated (Jürgens and Ploog, 1970); description of corticospinal projections from the pre- and post-central gyri (Harting and Noback, 1970); anatomical and physiological studies on the auditory system in terms of the auditory cortex (Hind et al., 1958) and the cochlear nerve (Alving and Cowan, 1971); studies on the subcortical and cortical connections of the striate cortex (Spatz et al., 1970); research on the topographical organization of the fasciculus gracilis (Whitsel et al., 1969, 1970); and investigations of neural-sexual integrating mechanisms such as cerebral representation of penile erection (MacLean and Ploog, 1962). These studies are complemented by an extensive series of investigations on the histochemical localization of the enzymes acid phosphatase, acetylcholinesterase, alkaline phosphatase, adenosine triphosphatase, non-specific butyrylcholinesterase, cytochrome oxidase, glucose-6-phosphate dehydrogenase, lactic dehydrogenase, monoamine oxidase, and succinic dehydrogenase in the brain and choroid plexus of the squirrel monkey (Iijima et al., 1968, 1969; Manocha and Bourne, 1966a, 1966b, 1967, 1968; Manocha et al., 1967; Manocha, 1970a, 1970b; Shantha et al., 1967, 1968; Shantha and Manocha, 1968). In addition, two stereotaxic atlases (Gergen and MacLean, 1962; Emmers and Akert, 1963) of the brain of the...
squirrel monkey are available.

Other interesting studies on the squirrel monkey have found that tetrahydrocannabinols produce increased responsiveness of the cerebral cortex to stimulation of the primary somato-sensory cortex (Boyd et al., 1971) and that cobalt powder applied to the surface of the somato-sensory cortex induces acute and short-term epileptogenesis (Grimm et al., 1970).

Recent research dealing with the monoamines in the brain of the squirrel monkey has been reported. In a study of the depletion of these substances caused by chronic manganese dioxide administration (Neff et al., 1969), the authors describe manifestations of extra-pyramidal motor dysfunction which correlate with reduced levels of dopamine and serotonin in the caudate nucleus. In another study of the caudate nucleus (Ordy et al., 1969), long-term treatment with the tranquilizer haloperidol, when coupled with electric shock stress, was found to cause a greater decrease in dopamine levels in the caudate nucleus than with haloperidol alone resulting in a greater incidence and extent of tremors. Behaviorally, amphetamine, which stimulates noradrenergic activity, has been found to cause a dose-related increase in the rate of an operant response, licking from a water tube, in the squirrel monkey (Wuttke, 1970).
VII. Summary of the Review of the Literature

Thus, this survey of the literature has analyzed the fluorescence histochemical method for the intraneuronal localization of monoamines used in the research for this dissertation; this discussion has dealt with the development, methodology, and chemistry of the technique. Investigations using this technique in the study of the brains of lower mammals, mostly the rat, have been discussed in relation to distribution of monoamine-containing cell bodies in the brain stem, description of the location and characteristics of monoamine terminals, and, by degeneration studies, mapping of monoamine pathways in the brain. A brief review of the squirrel monkey as an experimental animal has also been presented in order to provide the reader with a perspective of the increasing use of this animal in neurobiological research.
MATERIALS AND METHODS

In this work, the brain stems of eleven juvenile (400-500 grams) squirrel monkeys were studied. Four of the animals were treated by intraperitoneal route with the monoamine oxidase inhibitor iproniazid (1-isonicotinyl-2-isopropyl hydrazide phosphate, Hoffman La Roche, Inc.; 200mg/kg body weight) four hours before sacrifice in order to enhance the fluorescence of the serotonin reaction product (Dahlström and Fuxe, 1965a). The remaining seven monkeys were untreated. One of the animals was perfused through the aorta with 4% formaldehyde in 1.25% sodium chloride (Emmers and Akert, 1963); the brain was removed and placed in fresh fixative for one week, dehydrated through a graded series of alcohols, and embedded (see Appendix V for a more detailed account of this procedure).

Young animals were preferred in order to limit the amount of lipofuscin in nerve cells which is found in greatest amounts in the phylogenetically older cellular structures in the brain of the squirrel monkey (Creswell, et al., 1964). This intracellular material, which increases in accumulation with age of the animal, fluoresces at a wavelength close to that of the serotonin reaction product and interferes with the observations aimed at the localization of the monoamine. Although no quantitation of fluorescence was planned, initially male animals were specified for this work in order to reduce variability in general and specifically to reduce any variations which might possibly occur in the monoamine levels due to hormonal influences related to the female reproductive cycle. However, not all of the animals
which were obtained were found to be male, one being a female and another a pseudo-hermaphrodite. All animals were sacrificed in the early afternoon to reduce any possible variations in monoamine levels due to circadian rhythmic activity (Reis et al., 1968).

The squirrel monkeys, purchased from Tarpon Zoo, Inc. of Florida, were housed at the animal research facility of this medical center in small primate cages (Acme Research Products) using sawdust bedding. The quarters were kept at a constant temperature (72±2°F), humidity (40%), and light cycle (twelve hours on and twelve hours off, 7:00 to 7:00). The animals were maintained on a standard primate diet (Wayne Monkey Diet, Allied Mills, Inc.; see Appendix I for the composition of the diet), being fed and watered ad libitum. The squirrel monkeys were not kept for a prolonged period of time, being purchased only as needed. However, as a rule, the animals were housed at this research facility for a period long enough to allow observation of their behavior, so that only apparently healthy monkeys would be employed in the study.

While under anesthesia (pentobarbital sodium - Holmes Serum Co., Inc.; 50mg/kg by intraperitoneal route) each of the squirrel monkeys was sacrificed by bilateral thoracotomy and the brain exsanguinated by severing the superior vena cava. The indicated dosage of the pentobarbital produced a profound anesthesia within a short interval of time such that all animals were able to be sacrificed within ten minutes after administration of the barbiturate. The calvarium was cut with an electric saw and, after removing the attached dura mater and severing the cranial nerves and rostral-most portion of the spinal cord, the brain was carefully lifted from the cranial
cavity. In order to minimize diffusion artifacts, the dissection of the brain was performed in a cold room (5°C).

Frontal sections of the brain were cut at an angle approximating that used in the stereotaxic atlases (Gergen and MacLean, 1962; Emmers and Akert, 1963). To achieve this, a paraffin template was made (text-figure 8); the angles of the template were measured directly from the in situ angle of the brain as indicated in the atlas by Emmers and Akert (page 22). The brain was placed upon the template and cut in 2mm.-thick sections with a pathology knife. The vertical path of the knife was guided by a glass plate held at the side of the template.
Text-figure 8. Diagram of the side view of the paraffin template used to cut frontal sections of the brain stem of the squirrel monkey.
The slices of brain were picked up by means of a "paddle" made of an applicator stick and a rectangular piece of glassine paper glued to poster board of the same shape to provide support. The sections were flattened and shaped on the glassine paper to approximate their in situ form. The tissues were identified by a label attached to the other end of the paddle. The paddles with the tissue were first plunged into liquid propane cooled by liquid nitrogen, and then each paddle was placed in a glass tube containing liquid nitrogen for further cooling. The liquid propane was produced by condensing propane gas in a bath of liquid nitrogen via coiled tubing. The liquid formed was collected in a pyrex tube which was kept in a wide-mouthed Dewar flask containing liquid nitrogen. When properly stoppered and stored in a freezer (-74° to -78°C), the liquid propane may be used several times. The propane used in this study (Matheson Gas Company), was of commercial grade; purer grade of propane will solidify at the temperature of liquid nitrogen.

The entire procedure from exsanguination of the animal to freezing of the last brain slice took no longer than 45 minutes and was carried out in the cold room. When all of the brain had been cut and collected, the tubes containing the tissues were transferred to a deep freezer (-79°C) for storage until further processing.

In the next step of the procedure, the tissues were freeze-dried for ten days. The freeze-dryer used in this study was designed and assembled by the candidate, following in principle the design of Mendelow and Hamilton (1950). As illustrated in text-figure 9, the freeze-dryer consisted of a specimen tube to contain the tissues, a refrigeration unit maintaining a low
Text-figure 9. Semi-schematic diagram of the freeze-drying apparatus used in this study.
temperature (-30°C to -32°C), a vacuum trap containing a chemical desiccant (phosphorous pentoxide) to create a water vapor gradient, and a two-stage mechanical pump. This basic design is required for this procedure; the port-type freeze-dryer which is commonly used in the laboratory will not produce satisfactory results. Appendix II at the end of this dissertation describes the freeze-drying apparatus and procedure in greater detail.

After lyophilization, the tissues are quite hygroscopic and must either be stored with a desiccant in a sealed container or immediately undergo the gas phase histochemical reaction. This portion of the procedure essentially follows the methodology described in detail by Falck and Owman (Falck and Owman, 1965).

The tissues were transferred directly from the freeze-dryer with entomologist's forceps to a one-liter wide-mouth glass jar containing ten grams of paraformaldehyde evenly distributed on the bottom of the jar. The water content of the paraformaldehyde was standardized (Hamberger et al., 1965) to a relative humidity of 60% with sulfuric acid (see Appendix III) in a sealed desiccator jar for one week. Standardization of the paraformaldehyde is important, for as discussed in the Review of the Literature, too low a water content will produce relatively weak fluorescence while too much humidity will produce strong fluorescence but with marked diffusion of the monoamines. The tissues were placed upon aluminum foil discs positioned one above the other and connected by a central supporting rod. This tissue holder was supported in the jar upon a rectangular base. High-vacuum grease was applied to the ground-glass mouth of the jar and the corresponding ground-glass lid was clamped into place with a three-pronged clamp. This
arrangement assured an air-tight seal. The specimens were incubated with the paraformaldehyde at 80°C for one hour and fifteen minutes during which time formaldehyde vapors were liberated to react with the monoamines in the tissue.

After the tissues had reacted with paraformaldehyde, they were removed from the incubating jar with entomologist's forceps, placed in degassed paraffin (Paraplast) and infiltrated in vacuo at 60°C for five minutes, with a two-stage mechanical pump supplying the vacuum. The brain specimens were then embedded in paraffin in plastic molds and allowed to cool. Since the monoamine reaction products, especially the beta-carbolines, are prone to photodecomposition, the paraffin blocks were kept in the dark while cooling. After the blocks reached room temperature, they were stored in a box containing phosphorous pentoxide in a refrigerator.

When a paraffin block was to be sectioned, it was removed from the refrigerator and allowed to reach room temperature in the dark. The glass slides used in this study were first cleaned over-night in an acid dichromate cleaning solution, rinsed the next day in running tap water for at least eight hours, briefly rinsed in distilled water, placed in 95% ethyl alcohol for at least two hours, and then wiped dry. This extensive cleaning of the slides assured that no extraneous fluorescence would be produced by particles on the slides. In order to accurately localize the monoamines in the tissue sections within practical limits, the following protocol was observed: a ribbon of 10µ-thick sections was cut on an American Optical rotary microtome from the paraffin block. From the ribbon, the first section was designated for fluorescence observation, the next section for staining with toluidine
blue, and the third section for myelin staining; the next two sections were discarded. This pattern was repeated over and over until the entire tissue had been cut. On the average, five sections were collected per glass slide. Text-figure 10 illustrates the distribution of the sections from a ribbon of tissue.

|   |   |   |   |   |   |   |   |   |   | F | T | L | X | X | F | T | L | X | X | F | T | L | X |
| F = for fluorescence observation |
| T = to be stained with toluidine blue |
| L = to be stained with luxol fast blue |
| X = to be discarded |

Text-figure 10. Diagram of the pattern of distribution of the sections from a ribbon of sectioned material.

Using this system, each section on the slide was an interval of 40µ from the next section on the slide; each slide held four to six sections. Considering cell body diameter, this interval is not deemed great enough to miss any significant groups of monoamine-containing cell bodies. Since little quantitative data are available on the diameters of nerve cell bodies in the brain stem of the squirrel monkey, the following data concerning some brain stem nuclear structures in the cat are cited. The diameters of the cells of the main sensory trigeminal nucleus of the cat, for example, ranges from 10µ to 30µ with occasional cells ranging up to 50µ (Gobel and Dunbar,
1969). The diameters of cell bodies in the mesencephalic nucleus range from 30 µ to 50 µ (Hinrichsen and Larramendi, 1969), the average cell size in the zona compacta of the substantia nigra is 25 µ in diameter (Rinvik and Grofová, 1970), the majority of cell bodies in the ventral basal thalamus are 25-30 µ in diameter (Ralston and Herman, 1969), and the mean diameter of neurons in the inferior olive is 24 µ (Escobar et al., 1968). Thus, only two or three neurons at the most may be missed in the 40 µ distance between each section for fluorescence and, hence, all significant cell groups would be observed.

The slides with tissues to be stained were albumin-coated and the sections floated on the slide using boiled distilled water. They were allowed to dry overnight on a slide warmer (40°C). The sections for fluorescence, however, were floated on paraffin oil using albumin-free warm slides. Paraffin oil rather than water was used to minimize any possible diffusion of the reaction products. These slides were covered with a cover slip and stored in slide boxes in the dark.

As indicated above, two stains were employed in this study - toluidine blue, which stains Nissl substance, for cell bodies, and luxol fast blue for myelin. Since the tissues were previously fixed with formaldehyde vapors during the incubation period, no additional fixation was used before staining. The toluidine blue method of this study employed 0.5% aqueous toluidine blue at pH 6.0 (Dahlström and Fuxe, 1965a); the myelin staining procedure used was a modification of the Klüver-Barrera stain (Klüver and Barrera, 1953), using Darrow red (Powers et al., 1960; Powers and Clark, 1963) instead of cresylecht violet to demonstrate the cell bodies. Since the myelin rather than the cell bodies were of main interest, the sections
were differentiated with this technique until the cell bodies were only lightly stained (see Appendix IV for the staining schedules).

One representative section from each slide, usually the section in the middle, was photographed. The toluidine blue-and luxol fast blue-stained sections that were chosen were always adjacent sections; the location of the section on the slide was identified so that it could be correlated with the corresponding section for fluorescence. Photographs were taken using Kodak High Contrast Copy film with an American Optical cycloptic stereo-microscope mounted with a one-half times reducing lens at the objective with a 40-second exposure time. The negatives were developed with Kodak HC-110 developer and were printed on 5x7 Fotorite paper of #3 (luxol fast blue-stained) and #4 (toluidine blue-stained) contrast.

Analysis of the fluorescing sites involved marking the position of the monoamine-containing cell bodies on photographs of the corresponding stained sections. Photographs of the myelin-stained material were chosen as the basis for localizing the fluorescing cell bodies rather than photographs of the toluidine blue-stained sections. This choice was based upon the fact that under the fluorescence microscope, myelinated pathways may be differentiated from the surrounding gray matter, enabling one to more easily identify the position of the fluorescing cell groups in the myelin-stained material than in photographs of toluidine blue-stained sections. In a few locations, however, such as dorsal to the nucleus reticularis lateralis myelencephali and the nucleus olivaris inferior, the position of the fluorescing cell bodies was best determined by examining and noting their relationship to the nuclear structures. Wherever possible, the identification
of the monoamine-containing cell bodies was also indicated on the photographs of the toluidine blue-stained sections.

In those cases in which positive identification of a particular cell as being monoamine-containing was desired (e.g., in the locus caeruleus), the section was first photographed on the fluorescence microscope, then the paraffin oil removed with xylene, and the sections dipped in celloidin solution, hydrated through a graded series of ethyl alcohol, stained in 0.5% aqueous toluidine blue, differentiated in 95% ethyl alcohol, mounted in glycerol-gelatin, and re-photographed.

The fluorescence was observed using an American Optical series V20 Fluoresstar microscope (50 watt mercury vapor lamp) employing a heat absorbing filter and a Schott BG-12 (3mm thick) exciter filter at the light source and a Schott OG-1 barrier filter. Black and white photographs were taken using Kodak Tri-X Pan film (ASA 400) and color photographs were taken with Kodak High Speed Ekta-chrome film (EHB 135-20) with the ASA rating increased to 320 by special processing (Kodak ESP-1); exposure times for both black and white and color photographs were 10-15 seconds.

To confirm the monoamine-specific nature of the fluorescence, the location of each group identified as monoamine-containing was compared with sections which were heated to 80°C but were not exposed to formaldehyde vapors; those brain tissues not exposed to formaldehyde do not display the specific monoamine fluorescence while autofluorescence is still apparent. In addition, a representative slide showing each group of monoamine-containing cell bodies from each squirrel monkey was treated with 0.1% sodium borohydride (Corrodi et al., 1964; see Appendix VI for the procedure of this
method). The net effect of this treatment is the disappearance of the monoamine-specific fluorescence while the autofluorescence is not affected.

The serotonin-containing cell bodies were differentiated from the catecholamine-containing structures by their color; serotonin-containing cell bodies fluoresce yellow while catecholamine-containing cell bodies fluoresce green. This distinction was easily confirmed by the relative difference of the two forms of fluorescence to photodecomposition. As discussed in the Review of the Literature, the yellow fluorescence is greatly reduced in intensity after ten to fifteen minutes of exposure to ultraviolet light while the green fluorescence requires a more prolonged time of exposure to ultraviolet light (45-90 minutes) before fading is evident. Thus, the neurotransmitter content - i.e., serotonin or catecholamine - of each group of fluorescing cell bodies was further confirmed by exposing the structures to ultraviolet light from the fluorescence microscope for a period of fifteen minutes.

The monoamine-containing cell bodies localized and identified in this study, were described with respect to the following criteria:

Position - The caudorostral extent of each group in the brain stem as well as the relationships of the groups to known nuclei or fiber tracts were characterized. The locations of these groups are identified on photographs of Nissl-stained sections in the atlas of this dissertation.

Size - The greatest diameters of the cells in each group were measured directly from fluorescence photomicrographs of known magnifications. From these values, the mean diameter
for the cells of each group was determined.

**Fluorescence intensity**—As subjectively gauged, the fluorescence intensity for each group was qualitatively determined as being weak, moderate, or strong.

**Nissl staining**—The intensity of staining and the relative granularity of the Nissl substance was characterized for the cells of each group as determined from sections stained with toluidine blue.

**Nuclear staining**—The appearance of the nuclear chromatin and the nucleolus was characterized in terms of intensity of staining with hematoxylin for the cells of each group.

That the structures described in this dissertation are neurons rather than any of the other cellular elements found in large numbers in the central nervous system, i.e., neuroglia, may be appreciated by the typical neuronal shape, larger cell size, and Nissl staining of the fluorescing structures.

In an effort to effectively correlate the fluorescence method with other histochemical techniques to show e.g., monoamine oxidase and acetylcholinesterase activity on adjacent sections of the brain, initial studies by the candidate attempted to utilize the cryostat sectioning techniques described in the Review of the Literature. Although these methods provided satisfactory results on peripheral tissues such as heart, blood vessels, and adrenal medulla, no fluorescence was observed in the substance of the brain. These results agree with those reports in the literature indicating positive results primarily on frozen sections of peripheral organs. This point is further emphasized by the observation of the candidate that although the
monoamine-containing neurons in the brain do not fluoresce, the adrenergic innervation of the blood vessels supplying the brain are clearly demonstrable. These findings have led the candidate to conclude that although the frozen section techniques are valuable for combined histochemical studies of peripheral organs, they may not be satisfactorily applied to the central nervous system.

The location of the groups of monoamine-containing cell bodies described in this study is indicated in the Atlas which is part of this dissertation. This Atlas will be referred to in the text as a complement to the description of the location of each fluorescing group. The nomenclature adopted for most of the brain stem structures in the text as well as in the Atlas is based upon the terminology used in the atlas of the brain stem of the squirrel monkey by Emmers and Akert (1963), which in turn is based upon Nomina Anatomica (Kopsch, 1957). The raphé structures, however, will follow the terminology of Taber et al. (1960). Where they are similar in appearance, figures in the Atlas of this dissertation will be correlated with figures from the atlas by Emmers and Akert. The catecholamine-containing groups of cell bodies will be grouped according to their location and will be designated by the letter "C" while the groups of yellow fluorescing serotonin-containing cell bodies will be designated by the letter "S". In order to facilitate comparison between the groups described in the rat (Dahlström and Fuxe, 1965) and those found in the present study, the numbering of the groups will follow the same numbering system established by Dahlström and Fuxe for the rat.
RESULTS

I. Determination of the Specificity of the Observed Fluorescence

The specific fluorescence emitted by the monoamine reaction products was distinguished from autofluorescence of the tissue on the basis of three criteria: 1) careful examination of the fluorescence; 2) comparison of the fluorescing sites in tissues which were treated with formaldehyde vapors with those which were not treated with formaldehyde; and 3) treatment of the slides which display monoamine-specific fluorescence with sodium borohydride.

With careful examination of the fluorescence at relatively high magnification (200 times or greater), lipofuscin appeared as small, distinct, orange or brown granules (Fig. 24) while the monoamine-specific fluorescence was characterized by a more "hazy" appearance which completely filled the cytoplasmic portion of the cell (Fig. 25). In considering the second criterion indicated above, the monoamine-specific fluorescence seen in the half-sections of the brain stem treated with hot formaldehyde vapors was not seen in corresponding half-sections which were not exposed to the vapors of paraformaldehyde.

Sodium borohydride treatment of the sections, on which the third criterion for monoamine-specific fluorescence is based, abolished the reaction product fluorescence in all groups observed, but did not affect the autofluorescence (Fig. 26).

The specificity of the particular form of monoamine-produced fluorescence, i.e., due to either catecholamine or serotonin reaction products,
was identified by the particular color; sites which contained catecholamines fluoresced green (Figs. 27 and 27), while sites containing serotonin appeared yellow (Fig. 29). The specificity of the fluorescence was confirmed by examining the fluorescing sites before and after exposure of the tissue on the slide to fifteen minutes of ultraviolet light from the fluorescence microscope. As appreciated by comparing before and after photographs of the monoamine fluorescence, the fluorescence of the catecholamine reaction product is only slightly or moderately diminished after fifteen minutes of exposure to ultraviolet light (Figs. 30 and 32), while serotonin-specific fluorescence, on the other hand, is markedly reduced after this time (Figs. 31 and 32).

Thus, it may be assumed that: 1) the fluorescence of cells reported in this study is due to the monoamine reaction products rather than to auto-fluorescence of the tissue and that 2) sites which contain catecholamines are differentiated from those sites containing serotonin on the basis of their respective green and yellow colors, as well as on the basis of their relative sensitivity to photodecomposition when exposed to ultraviolet light.

The locations of the monoamine-containing cell bodies reported in this study are illustrated in Atlas-figs. 6-27; a key depicting the levels of the Atlas-figures with respect to the entire brain stem is seen in Atlas-fig. 5. Macroscopic views of the whole brain and brain stem of the squirrel monkey are found in Atlas-figs. 1-4.
II. Monoamine-containing Cell Bodies in the Brain Stem of the Squirrel Monkey

A. Catecholamine-containing Groups

*Group Cl (Atlas-figs. 6-13)* Group Cl, consisting of weakly to moderately fluorescing cells, first appeared at the level of the decussatio pyramidum in the caudal portion of the medulla oblongata. The cell bodies were found in the lateral aspect of the brain stem, ventral to the nucleus tractus spinalis nervi trigemini. At more rostral levels, the cell bodies maintained this position ventral to the nucleus. With the development of the nucleus reticularis lateralis myelencephali, the fluorescing cells assumed a position immediately dorsal to this nucleus (Figs. 33 and 34). In its caudal aspect, this group was composed of few cells (four or five per section) which were closely grouped together, while at the level of the caudal portion of the nucleus olivaris inferior, the number of cells increased as they formed a horizontal band which spread toward the midline (Fig. 34). Rostrally, with the decrease in the number of cells of the nucleus reticularis lateralis myelencephali, group Cl also decreased in number of cells and appeared to move ventrally while maintaining its position dorsal to the nucleus reticularis lateralis myelencephali. With the disappearance of the nucleus reticularis lateralis myelencephali, the few fluorescing cell bodies of group Cl were found lateral and dorsolateral to the nucleus olivaris inferior until the development of the nucleus nervus facialis when all of the cells of group Cl disappeared.

Most of the cell bodies of this group were round to oval in shape with some being spindle-shaped; they were all multipolar. The mean diameter of
the cells is 25 µ, with a range of 18 µ to 31 µ. The Nissl substance of these cells stained less intensely and with finer granules than did the more heavily stained Nissl material of the cells of the nucleus reticularis lateralis myelencephali. The round, pale, relatively large nucleus of the cells of group C1 contained a prominent nucleolus and dispersed chromatin.

Group C2 (Atlas-figs. 7-10): The few weakly to moderately fluorescent cells which characterized this group were first found at a level immediately rostral to the decussatio pyramidum, where they were located in a position dorsolateral to the canalis centralis. More rostrally, most of the cell bodies of this group were located dorsal to the nucleus nervi hypoglossi and lateral to the nucleus dorsalis nervi vagi (Fig. 36). At the rostral extent of this group, these cell bodies were found just ventral to the floor of the ventriculus quartus (Fig. 35). Cells of this group were not found rostral to the nucleus nervi hypoglossi. As indicated above, this group was small in number; usually only two or three cells could be seen per section. Most of the cells were found just ventral to the floor of the ventriculus quartus at the level of the nucleus dorsalis nervi vagi.

The cell bodies of group C2 were round to oval in shape and were multipolar. They had a mean diameter of 24 µ with diameters ranging from 22 µ to 27 µ. Due to the small number of cells in this group, it was not possible to correlate the fluorescent cell bodies with the stained sections to determine their cytological characteristics.

Group C3: In the rat, a group (A3) of a few small, very weakly to weakly fluorescent cells was described in the nucleus olivaris accessorius dorsalis (Dahlström and Fuxe, 1965). Although this group was not identified
in the squirrel monkey, a group C3 will be designated in the sequence of groups so that the respective group numbers of the rat and the squirrel monkey will correspond with one another.

**Group C4:** The strongly green fluorescing cell bodies of group C4 were found in the lateral portion of the ventral aspect of the roof of the ventricle quartus. In a transverse section of the cerebellum, this group appeared to be oriented in a horizontal plane ventral to the cerebellar nuclei. The cell bodies of this group were rather closely compacted (Fig. 37) but did not extend for an appreciable distance in a caudal-rostral direction in the cerebellum. At rostral levels, the cells of this group turn ventrally, course down the medial aspect of the pedunculus cerebellaris inferior, and appear to merge with the cells of group C6 at the caudal pole of the nucleus nervus facialis.

Most of the cell bodies of this group were round to oval in shape (Figs. 37 and 38), ranging 30 µ to 36 µ in diameter and having a mean diameter of 34 µ; some of the cells were spindle-shaped and all were multipolar. The medium granularity of the Nissl substance of the cell bodies stained moderately with toluidine blue and the cells displayed a pale, round to oval nucleus with a prominent nucleolus.

**Group C5 (Atlas-figs. 14-18):** Group C5 first appeared as an aggregate of weakly and moderately fluorescing cells dorsolateral to the rostral-most portion of the nucleus olivaris inferior (Fig. 39) immediately deep to the sulcus lateralis anterior. The cells in the caudal region of this group were located ventral to those of the rostral part of group C1. These two groups could be differentiated from each other on the basis of the weaker
fluorescence of group C5 and the fact that more numerous strongly fluorescing terminals were associated with the cells of C5 than with C1. These terminals appeared to be making axosomatic and possibly axodendritic contacts (Figs. 39, 40, 42, 43). With the disappearance of group C1, group C5 became located more medially between the nucleus olivaris inferior and the nucleus nervus facialis. More rostrally, with the development of the nucleus olivaris superior, the cells of C5 increased in number and became localized around the periphery of the nucleus olivaris superior (Figs. 41, 42, 43). In the rostral portion of this group, the cells formed a narrow band which extended dorsally, approaching the ventral part of the locus caeruleus (Fig. 44). The distribution of the cells of group C5 was limited to a triangle formed medially by the fibers of the nervus abducens, laterally by the nervus facialis, and ventrally by the ventrolateral surface of the brain stem. Group C5 ended just rostral to the nucleus olivaris superior.

The multipolar cell bodies of group C5 were round to oval in shape and had a mean diameter of 26 µ with cell diameters ranging from 20 µ to 30 µ. The medium granular Nissl material was stained moderately in intensity. The nuclear chromatin stained lightly and the nucleolus was moderately stained.

Group C6 (Atlas-figs. 15-18): The cells of group C6 are continuous with those of group C4 as these cells extend from the cerebellum to the pontine tegmentum (Figs. 1, 45). As these cells migrated ventrolaterally, the fiber system of the cells also appeared to be directed in a ventrolateral as well as in a dorsomedial direction (Fig. 45). With the beginning of the formation of the tractus mesencephalicus n. trigemini, the cell bodies continued increasing in number and became directed toward this tract (Figs. 2, 3). At the
level where the tract lies medial and slightly ventral to the brachium conjunctivum, the characteristic clustering of the cells of the locus caeruleus, which comprises group C6, attained their greatest density (Figs. 3-16, 47). Most of the cells were found medial to the tractus mesencephalicius n. trigeminalis (Figs. 47 and 48), with numerous cell bodies also distributed ventral and lateral to the tract; some cells were also found within the tract. In the ventral region, the fluorescing cells became disaggregated, decreasing in number (Fig. 47) but in the dorsal portion of C6 the cells remained quite closely compacted along the medial aspect of the tractus mesencephalicius n. trigeminalis, with the greatest density of cells being close to the floor of the ventriculus quartus. As the tract coursed to its dorso-medial position (Figs. 16-23), the cell bodies, although decreasing in number, retained their closely-packed appearance. At the level of the decussatio nervorum trochlearium (Figs. 17-19), the fluorescing cells were reduced in number, clustering along the ventral surface of the decussation and dorso-medial to the more dorsal portion of the brachium conjunctivum (Fig. 51). Rostral to the decussation, a few scattered fluorescing cells of C6 were found about and within the fibers of the nervus trochlearis (Figs. 20-23, 46); this group disappeared just rostral to the decussatio nervorum trochlearium.

The fluorescing cell bodies of group C6 appeared to be of at least two types. One form was round to oval-shaped and was primarily located in the dorsal, more compacted portion of the locus caeruleus (Fig. 47). The mean diameter of these cells was 31 \( \mu \) with a range of 27 \( \mu \) to 35 \( \mu \) and a few measured up to 40 \( \mu \) in diameter. The other form of cell was spindle-shaped,
smaller, and was found mostly in the ventral portion of the locus caeruleus (Fig. 47). Both forms of these cells were characterized by moderately staining, finely to moderately granular Nissl substance and were multipolar. The round nucleus, eccentrically located in many of the cells, contained dispersed chromatin and a prominent nucleolus. The cells of the locus caeruleus could be differentiated from the nonfluorescing cells of the nucleus tr. mesenceph. n. trigemini, which were larger, unipolar, and were characterized by darker staining, coarser Nissl substance (Fig. 50).

As indicated in Figure 49, practically all of the cells located immediately medial to the tractus mesencephalicus n. trigemini were found to be catecholamine-containing. All of the sections of the locus caeruleus which were examined contained no or very few nonfluorescing cells. The nonfluorescing cells were observed mostly in the dorsal region of the tractus mesencephalicus n. trigemini between the tract and the locus caeruleus (Fig. 49D) at the level where the tract lies medial and ventral to the brachium conjunctivum (Figs. 3-12). While the majority of these nonfluorescing cells appeared larger and to stain more heavily with toluidine blue than did the fluorescing cells, in a few cases, nonfluorescing cells were indistinguishable in the stained preparations from the surrounding cells which were found to fluoresce.

Group C7 (Atlas-figs. 16-18): Located ventral to the ventral tip of the brachium conjunctivum was a small group of green fluorescing cells in the region of the pons rostral to the point where the tractus mesencephalicus n. trigemini has coursed dorsal to the brachium conjunctivum (Figs. 18-23). These moderately to strongly fluorescing cells are at first grouped in a
cluster (Fig. 52) and then, more rostrally, they extended in a ventral direction medial to the lemniscus lateralis (Fig. 53). The cells in the rostral aspect of this group appeared to be continuous with the cells of group C6 (Figs. 17, 51).

The cell bodies of C7 were round having a mean diameter of 34 μ with a range of diameters 27 μ to 36 μ and were multipolar. The cytological staining characteristics of the cells of this group were similar to those of group C6.

**Group C8** (Atlas-figs. 22-26): Group C8 began to form caudal to the nucleus ruber, just lateral to the brachium conjunctivum at the level of the rostral portion of the decussatio brachii conjunctivi (Fig. 54). This group appeared to be oriented ventromedially to dorsolaterally, extending from the lateral portion of the formatio reticularis tegmenti mesencephali to intermingling more ventromedially with the green fluorescing cells of the pars compacta of the substantia nigra (Group C9). The scattered cell bodies of C8 formed clusters within the pars diffusa of the substantia nigra (Fig. 56). Proceeding rostrally, the cells of this group maintained their position dorsolateral to the pars compacta and started to decrease in number just rostral to the nucleus ruber. With the formation of the corporis mamillaris group C8 was no longer observed.

The round, multipolar cell bodies of this group ranged in size from 22 μ to 31 μ in diameter, with a mean diameter of 26 μ. The perikarya of many of the cells were characterized by a strongly fluorescing region encircling the nucleus while the rest of the cytoplasmic fluorescence was observed to be of moderate intensity (Fig. 55). The Nissl substance of some
of the cells stained moderately with fine to medium granules, while numerous
other cells were characterized by relatively coarser masses of darker-staining Nissl material in the cytoplasm. The nuclei appeared pale with nuclear staining; they were round, centrally placed, and displayed heavily-staining nucleoli.

Group C9 (Atlas-figs. 23-26): The pars compacta of the substantia nigra was identified as the catecholamine-containing group C9. The pars compacta, which appeared at the level of the nucleus ruber, was located dorsomedial, medial, and ventromedial to the non-fluorescing pars diffusa (Fig. 57). At a more rostral level, the green fluorescing cells of C9 approached the midline, mingling with the exiting fibers of the nervus oculomotorius, and were limited medially by the nucleus interpeduncularis. Laterally, group C9 merged with group C8. Rostrally, this group, like C8, disappeared with the formation of the nucleus corporis mamillaris.

Cytologically, the cellular characteristics of group C9 was identical to those of group C8 in terms of fluorescence intensity, including the strong perinuclear zone (Fig. 59), Nissl staining characteristics, multipolarity, size, and nuclear and nucleolar appearance.

Group C10 (Atlas-figs. 23-25): The cells of group C10 were found in the midline, being limited laterally by the exiting fibers of the nervus oculomotorius and ventrally by the nucleus interpeduncularis (Figs. 60 and 61). The moderately fluorescing cells of this group were characterized by their association with strongly fluorescing terminals which appeared to form axosomatic and possibly axodendritic contacts (Fig. 62). Ventrally, this group was closely associated with the cells of group C9 (Fig. 63).
The multipolar cell bodies of group C10 were round, had a mean diameter of 25 µ with a range of diameters from 21 µ to 30 µ. The finely to medium granular Nissl substance stained lighter than did the Nissl material of the cells in groups C8 and C9. The pale, round nucleus also seemed to have a less intensely stained nucleolus than was found in the cells of groups C8 and C9.

**General Observations on the Catecholamine-containing Groups**

**Position:** All catecholamine-containing groups of cell bodies, except for the midline group C10, were found in the more lateral aspect of the brain stem. Some groups were identified as specific nuclei such as C6 as the locus caeruleus and C9 as the pars compacta of the substantia nigra. Other groups, however, could only be described as being associated with certain nuclear structures such as C1 dorsolateral to the nucleus reticularis lateralis myelencephali, C5 around the nucleus olivaris superior, and C10 dorsal to the nucleus interpenduncularis. The positions of these groups as well as their relative density of cells are represented in Figure 64.

**Cytology:** The mean cell diameters of most of the groups ranged from 24 µ to 26 µ with the exception of groups C4, C6, and C7 which were 31-34 µ in diameter. The fluorescence intensity of the cells was gauged as being weak (C2) to strong (C6). Cells of some groups (e.g., C8 and C9) were further characterized by a strongly fluorescent perinuclear region. In many of the cells, the nucleus contained a fluorescing "dot" which was observed only in cells characterized as monoamine-containing. Further, the nuclear dot was only observed in tissues treated with paraformaldehyde vapors and the fluorescence of the dot was greatly reduced or disappeared completely (as was
the cytoplasmic fluorescence) following treatment of the section with sodium boroxydride (Fig. 26). Generally, the Nissl granules of the catecholamine-containing cells were of medium granularity and stained moderately with toluidine blue. In all cells, the nuclear chromatin stained lightly while the nucleolus was quite prominent. These observations are summarized in tabular form (Fig. 65).
PHOTOMICROGRAPHS OF CATECHOLAMINE-CONTAINING CELL BODIES IN THE BRAIN STEM OF THE SQUIRREL MONKEY

(Saimiri sciureus)
PLATE I
Explanation of Figures

Figs. 1-23 Caudal to rostral sequence of diagrammatic frontal views of the pontine tegmentum of the brain stem illustrating the position of the locus caeruleus, which is represented as dots. Each figure indicates an interval of approximately 0.1 mm from the previous figure.

A - Aqueductus cerebri
B - Brachium conjunctivum
C - Nucleus colliculus inferior
FLM - Fasciculus longitudinalis medialis
T - Tractus mesencephalicus n. trigemini
V - Ventriculus quartus
IV - N. trochlearis
VII - N. facialis
PLATE II
Explanation of Figures

Fig. 24. Lipofuscin autofluorescence seen in freeze-dried material not exposed to hot vapors of formaldehyde. Note the granular appearance of the fluorescing material in the cells and compare with that seen in Fig. 25. X224.

Fig. 25. Specific monoamine fluorescence observed in tissue freeze-dried and exposed to hot vapors of formaldehyde (group C1). X224.
Fig. 26. Two views of the catecholamine-containing group C1 before (26A) and after (26B) treatment of the section with 0.1% sodium borohydride. Note the disappearance of the monoamine-specific fluorescence of the cells and of the neighboring terminals; the loss of fluorescence in three particular cells in Fig. 26B is indicated (arrows). The autofluorescence of the cells of the more ventral nucleus reticularis lateralis myelencephali (RL), on the other hand, is not reduced in intensity. X112.
PLATE IV
Explanation of Figures

Fig. 27. Color photomicrograph of the green fluorescing catecholamine-containing neurons in the pars compacta of the substantia nigra. X92.

Fig. 28. Color photomicrograph of the green fluorescing catecholamine-containing neurons in the locus caeruleus. X92.

Fig. 29. Color photomicrograph of the yellow fluorescing serotonin-containing neurons of the raphé. X92.
Explanation of Figure

Fig. 30. Two views of the catecholamine-containing cells of group C8 before (Fig. 30A) and after (Fig. 30B) exposure of the section to ultraviolet light for fifteen minutes. As seen in these two photographs, the fluorescence intensity is not significantly decreased as compared with Figure 31. Both Figs. 30A and 30B were photographed and printed under identical conditions. X112.
Fig. 31. Two views of the serotonin-containing raphé neurons before (Fig. 31A) and after (Fig. 31B) exposure of the section to ultraviolet light for fifteen minutes. Note that the fluorescence of the cell bodies as well as the background fluorescence are markedly reduced in intensity while the autofluorescence is not significantly affected. Compare with Fig. 30. Both Figs. 31A and 31B were photographed and printed under identical conditions. XIl2.
Fig. 32. Two views of the pineal gland of the squirrel monkey before (Fig. 32A) and after (Fig. 32B) exposure of the section to ultraviolet light for fifteen minutes. In this structure, the difference in sensitivity of the catecholamine- and serotonin-produced fluorescence is demonstrable. The fluorescing serotonin-containing pinealocytes which are distinctly visualized in the dorsal aspect of the gland, show markedly reduced fluorescence after ultraviolet irradiation. On the other hand, the catecholamine-containing sympathetic fibers innervating the gland are not noticeably changed in fluorescence intensity. Both Figs. 32A and 32B were photographed and printed under identical conditions. X112.
PLATE VIII
Explanation of Figures

Fig. 33. The strongly fluorescing catecholamine-containing cells of group Cl located immediately dorsal to the nucleus reticularis lateralis myelencephali (RL). X112.

Fig. 34. Low-power view of the cells of group Cl at a level rostral to Fig. 33. Note the horizontal spreading of the cells toward the midline, past the fibrae arcuatae internae (AI). The dorsal position of Cl with respect to the nucleus reticularis lateralis myelencephali (RL) is also indicated. X56.
Explanation of Figures

Fig. 35. View of the cells of group C2 located ventral to the floor of the ventriculus quartus (IV). X128.

Fig. 36. Localization of the cells of group C2 dorsal and lateral to the nucleus dorsalis nervi vagi (NX) and the nucleus nervi hypoglossi (NXII). Note the fluorescing terminals in the nucleus dorsalis nervi vagi, but not in the nucleus nervi hypoglossi. X128.
PLATE X
Explanation of Figures

Fig. 37. Strongly fluorescing closely compacted cells of group C4 located in the ventrolateral part of the cerebellum in the roof of the ventriculus quartus (IV). A cerebellar folium is indicated at the left of the photomicrograph (arrow). X128.

Fig. 38. Two cell bodies isolated from the rest of group C4. Note the extended fluorescing process of one of the cells (arrow) and the bifurcated processes of both cells. X255.
Fig. 39. The caudal portion of group C5 located dorsolateral to the nucleus olivaris inferior (OI). Note the strongly fluorescing terminals apparently making contact with some of the cells (arrows). X112.

Fig. 40. Fluorescing cell bodies of group C5. Note the axosomatic contacts made by fluorescing terminals on one of the cells (arrow). X224.
Fig. 41. Moderately fluorescing cells of group C5 located in the periphery of the nucleus olivaris superior (OS) as well as more dorsally. X128.

Fig. 42. Weakly and moderately fluorescing cells of group C5 dorsolateral to the nucleus olivaris superior (OS) receiving contacts by fluorescing terminals. One of the cells appears to be making contacts along one of its processes (arrow). X255.
Explanation of Figures

Fig. 43. Weakly and moderately fluorescing cells located dorsomedial and medial to the nucleus olivari superior (OS) belonging to group C5. Note the fluorescing terminals on one of the cell bodies (arrow). X128.

Fig. 44. Low-power view of the cells in the dorsal-most aspect of group C5 (arrows) which are extending toward the more strongly fluorescing cells of the locus caeruleus (group C6) (LC). X64.
Fig. 45. The caudal portion of the strongly fluorescing cells of group C6 which appears to be continuous with group C4. This group of cell bodies corresponds to the locus caeruleus. The closely-grouped cells are located dorsolateral to the ventriculus quartus (IV) and are oriented in a ventrolateral direction. X112.

Fig. 46. A few cells of group C6 located rostral to the decussatio nervi trochlearis. Note the close association of the fluorescing cells with the fibers of the nervi trochlearis (nIV). X224.
Fig. 47. View of the strongly fluorescing cells of the locus caeruleus (group C6) in the region of their greatest density. In the dorsal portion of the group, the large fluorescing cells are closely packed and are located medial to the tractus mesencephalicus n. trigemini (TMv). More ventrally, the cells appear to disaggregate, are smaller and more elongated. At several places, fibers from those cells can be seen traversing the tractus mesencephalicus n. trigemini. X140.
Fig. 48. Three views of the region in the pontine tegmentum where group C6 is located at the level of greatest density of its cell bodies.

Fig. 48A. Photomicrograph of a toluidine blue-stained section demonstrating the close association of C6 to the tractus mesencephalicus n. trigemini (TMv).

Fig. 48B. Photomicrograph of a section adjacent to Fig. 48A. The fluorescing cell bodies are seen located medial to the tractus mesencephalicus n. trigemini (TMv); the tract lies ventromedial to the brachium conjunctivum (BC). Note the large closely-packed cells in the dorsal portion of the group (arrow) compared with the loosely packed, smaller cells ventrolateral to the tractus mesencephalicus n. trigemini.

Fig. 48C. Photomicrograph of a section adjacent to that seen in Fig. 48A. This section, stained for myelin with luxol fast blue, clearly demonstrates both the tractus mesencephalicus n. trigemini (TMv) and the brachium conjunctivum (BC).
PLATE XVII
Explanation of Figure

Fig. 49. Two different views of the locus caeruleus (group C6) located medial and dorsal to the tractus mesencephalicus n. trigemini (TMv) before (Figs. 49A and 49C) and after (Figs. 49B and 49D) staining of the sections with toluidine blue. The cell bodies in the region of the locus caeruleus which have been stained in Fig. 49B are observed to fluoresce in Fig. 49A. In Fig. 49B, however, three cell bodies (arrows) dorsal to the tractus mesencephalicus n. trigemini and lateral to the locus caeruleus are stained but are not observed to fluoresce in Fig. 49C. X42.
Explanation of Figure

Fig. 50. Photomicrographs of the cell bodies of the locus caeruleus (Fig. 50A) (group C6) and of the nucleus tr. mesenceph. n. trigemini (Fig. 50B) stained with toluidine blue from sections obtained from the squirrel monkey perfused with 4% formaldehyde. Note that the cells of the nucleus tr. mesenceph. trigemini are larger and stain more heavily with coarser Nissl granules than do the cells of the locus caeruleus. X378.
PLATE XIX
Explanation of Figure

Fig. 51. Composite of photomicrographs illustrating the relationship of both groups C6 and C7 to the brachium conjunctivum (BC). The rostral aspect of group C6, as seen in the figure, is rather loosely packed and positioned medial and dorsal to the brachium conjunctivum. Group C7 first appears as a few cells ventral to the brachium conjunctivum. The presence of fluorescing cells midway between these two groups suggests a degree of continuity of the groups with each other.
PLATE XX

Explanation of Figures

Fig. 52. Fluorescing cells of group C7 located ventral to the brachium conjunctivum (BC). Cells of the rostral portion of group C6 are also seen (arrow). X64.

Fig. 53. Cells of group C7 observed ventral to the brachium conjunctivum (BC) oriented in a ventral direction medial to the lemniscus lateralis (LL). X64.
Explanation of Figures

Fig. 54. The caudal portion of group CS which appears lateral to the rostral-most portion of the brachium conjunctivum (BC). The arrow points to a fluorescing artifact. X128.

Fig. 55. Moderately fluorescing cells of group CS. Several of the cell bodies are characterized by a more strongly fluorescing perinuclear region. X255.
Fig. 56. Cells of group C8 which form clusters of fluorescing cell bodies in the non-fluorescing pars diffusa of the substantia nigra (SNd). X128.
Fig. 57. The moderately fluorescing cells of group C9 belonging to the zona compacta of the substantia nigra are positioned dorsomedial, medial and ventromedial to the non-fluorescing pars diffusa (SNd). X128.

Fig. 58. Cell bodies of group C9 located ventral to the pars diffusa of the substantia nigra and dorsal to the pedunculus cerebri (PC). X128.
Fig. 59. Cell bodies of group C9 belonging to the zona compacta of the substantia nigra. Note the fluorescing perinuclear region which is stronger in intensity than the rest of the cytoplasm in some of the cells. X224.
Fig. 60. Low-power view of the cells of group ClO (arrows). This group is closely associated with numerous fluorescing terminals. It is bounded laterally by the exiting fibers of the nervus oculomotorius (nIII) and ventrally by the nucleus interpeduncularis (IP). X56.
Fig. 61. Cell bodies of group C10 which are located medial to the exiting fibers of the nervus oculomotorius (nIII). X128.

Fig. 62. Higher magnification of the cells of group C10. Note the somatic and possibly dendritic contacts made by fluorescing terminals on these cells (arrows). X255.
PLATE XXVII
Explanation of Figure

Fig. 63. Photomicrograph of portions of both groups C9 and C10 which are both located dorsal to the pedunculus cerebri (PC). Group C10 is found more medially than C9 in the midline and is associated with numerous fluorescing terminals. X128.
Fig. 64. Diagram of the brain stem of the squirrel monkey indicating the approximate caudostral and dorsoventral extents of the catecholamine-containing groups of cell bodies (dots). The density of the dots represents the relative density of the cell bodies in each group.

(OL - nucleus olivaris inferior, OS - nucleus olivaris superior)

Fig. 65. Summary of the catecholamine-containing groups in the brain stem of the squirrel monkey indicating the position of the groups as well as the fluorescence and cytological characteristics of the cell bodies of each group. The following grading system refers to fluorescence intensity, Nissl staining, chromatin staining, and nucleolus staining:

* = absent or negligible
** = weak
*** = moderate
**** = strong

The following grading system refers to the characteristics of the Nissl granules:

* = indistinguishable
** = fine
*** = moderate
**** = coarse
B. Serotonin-containing Groups

Group S1 (Atlas-figs. 9-18): Group S1 was limited primarily to the nucleus raphe pallidus situated in the ventral portion of the brain stem midline. The group was first found at a level just caudal to the nucleus olivaris inferior. The cells which formed this group in the raphe were located medial to the tractus pyramidalis and did not extend any further dorsally than the dorsal portion of the nucleus olivaris inferior (Fig. 66). At more rostral levels, the cells of S1 were observed to merge dorsally with those of group S3 found in the nucleus raphe magnus (Figs. 70 and 71). At the level of the rostral half of the nucleus olivaris inferior, the cells of group S1 became closely associated with numerous strongly fluorescing fibers which appeared from the most ventral region of the midline and spread dorsally and laterally like a bush (Figs. 67,70,71). Rostrally, these fibers were found in the corpus trapezoides. With the full development of the nucleus olivaris superior, the fibers disappeared and, shortly thereafter, the cells of group S1 were no longer observed. The rostral extent of S1, however, was ill-defined. Although group S1 was found for a considerable distance throughout the brain stem, only a few cells were observed per section in the nucleus raphe pallidus. The number of cells appeared to increase proceeding rostrally, in addition to the cells found in the nucleus raphe pallidus, cells belonging to S1 also extended laterally and ventrally, following the dorsal border of the nucleus olivaris inferior. These cells were found among the fibers of the nervus hypoglossus as this nerve passed through the ventrolateral portion of the nucleus olivaris inferior (Fig. 68). At the level of the formation of the catecholamine-containing group C5, the lateral-most cells of S1 were found
between the cells of group C5 and the nucleus olivaris inferior. At the most rostral portion of the nucleus olivaris inferior, these lateral cells of S1 disappeared.

Group S1 cells were round to oval in shape, multipolar, and had a mean diameter of 22 µ with a range of 14 µ to 25 µ. The finely to moderately granular Nissl substance of these cells stained with medium intensity while in some cells, dark staining clumps of Nissl material was observed in the periphery of the cytoplasm. Generally, S1 cells were observed to have quite sparse cytoplasm. The cells had pale staining nuclei with heavily stained nucleoli.

Group S2 (Atlas-fig. 7-13): Group S2 was found to extend more caudally in the brain stem than did the cells of S1. S2 was limited to the nucleus raphe obscurus (Fig. 66), first appearing along the lateral edges of the decussatio pyramideum in the medulla oblongata. This group was characterized by two narrow symmetrical paramedian rows of fluorescing cell bodies (Figs. 66 and 69). At its greatest development, group S2 was found to extend throughout most of the raphe in a ventrodorsal direction from the level of the dorsal portion of the nucleus olivaris inferior to the floor of the ventriculus quartus (Fig. 66). More rostrally, the cells of group S2 were limited to the dorsal portion of the raphe while ventrally, the group became continuous with the cells of group S3 located within the nucleus raphe magnus. Group S2 disappeared with the development of the nucleus n. facialis.

Cytologically, the cells of group S2 were similar to those of group S1. They were round to oval, measuring from 17 µ to 28 µ in diameter with a mean diameter of 21 µ. The cells also had little cytoplasm with fine to
medium granular Nissl substance which stained lightly to moderately with toluidine blue. In many of the cells, a small peripheral region of the cytoplasm was observed to contain darkly stained coarse Nissl granules. The nuclei of the cells of this group were pale and displayed prominent nucleoli.

**Group S3** (Atlas-figs. 12-18): The cells of group S3 were found to be located both in the midline and to extend laterally. Within the raphe system, group S3 was found within the nucleus raphe magnus, first observed at a level just rostral to the exiting fibers of the nervus hypoglossus. The cells of S3 first appeared at approximately that level at which the cells of the catecholamine-containing group C1 were disappearing. Dorsally, S3 merged with group S2 in the nucleus raphe pallidus. The two groups could be differentiated, however, by the fact that unlike S2, cells of group S3 were randomly distributed throughout the midline and spread much farther in a lateral direction (Figs. 70 and 71). More rostrally, the cells of group S1 and S3 became continuous such that the two groups could not be distinguished except for the larger cell size of S3 (Fig. 71). Cells from group S3 were found to also extend laterally within the corpus trapezoideum (Figs. 71 and 72). Both the midline and lateral distribution of the cells of group S3 terminated at the rostral pole of the nucleus olivaris superior.

With a mean diameter of 26 µ and a range of 21 µ to 30 µ, the cells of group S3 were found to be larger than those of groups S1 and S2. The moderate to coarsely granular Nissl material stained moderately to heavily with toluidine blue. The nuclei were pale and the nucleoli stained darkly. These multipolar cells were round to oval in shape with some being spindle-shaped,
particularly those in the more lateral regions of this group.

**Group S4:** In the rat (Dahlström and Fuxe, 1965a), a few cells designated as group S4 were described as being found "just under the fourth ventricle, dorsal to the vestibular nuclei and nuc. nervi abducens." While a comparable group was not found in the squirrel monkey, as with group C3, a group S4 is being indicated to maintain a corresponding sequence of numbers between the rat and squirrel monkey.

**Group S5** (Atlas-fig. 14): At a level just caudal to the full development of the locus caeruleus, a few cells belonging to group S5 were found in the caudal portion of the nucleus raphe pontis. Some of the cells appeared to be clustered into small groups and their processes appeared to be directed ventrally and dorsally (Fig. 73). Ventrally, these cells mingled with those of group S3 in the nucleus raphe magnus. Group S5 cells were no longer observed with the appearance of groups S6 and S8.

The cell diameters for these round to oval multipolar neurons ranged 18-30 µ with a mean diameter of 25 µ. Their moderately granular Nissl substance stained medium in intensity with toluidine blue while the nuclei of these cells were pale with prominent nucleoli.

**Group S6** (Atlas-figs. 15-18): Rostral to group S5 at the level of the locus caeruleus, a group of cells belonging to group S6 was observed as being positioned just ventral to the rostral portion of the ventriculus quartus (Fig. 74). The cells were located immediately dorsal to the fasciculus longitudinalis medialis, forming a "cap" on the fasciculus. Cell bodies of this group were also found within the fasciculus, their processes often found coursing through the pathway (Fig. 75).
The majority of these round, multipolar cells had moderately stained Nissl material of medium granularity. Some of the cells were further characterized by a rim of darker staining Nissl substance around a large portion of the periphery of the cytoplasm. The cells of S6 had lightly staining nuclei and darkly staining nucleoli. They had a mean diameter of 28 µ with a range of 21 µ to 35 µ.

Group S7 (Atlas-figs. 17-21): The cell bodies of group S7 were located within the nucleus raphe dorsalis which is identified as the nucleus dorsalis tegmenti (Gudden) in the atlas of the squirrel monkey by Emmers and Akert (1963). This group was first observed dorsal to the nucleus n. trochlearis at the level of the rostral portion of the locus caeruleus. At the caudal portion of this densely-packed group, the cells formed two symmetrical clusters on either side of the midline ventral to the aquaeductus Sylvii and medial to the fasciculus longitudinalis medialis (Fig. 76). More rostrally, S7 extended laterally, dorsal to the nucleus n. trochlearis (Figs. 76 and 80) toward the few remaining cells of the rostral-most portion of the locus caeruleus. The cells of S7 merged ventrally with those of group S8. The two groups could be distinguished, however, by the seemingly less intense fluorescence of S7. In addition, S7 appeared to be associated with a greater amount of background fluorescence (Fig. 80) which may possibly be due to numerous small fluorescing fibers (this possibility is suggested in Fig. 76). Rostrally, group S7 decreased in size and disappeared with the formation of the nucleus n. oculomotorius.

The round to oval and elongated cells of this group were multipolar, stained medium in intensity with toluidine blue, and had moderately granular
Nissl substance. The nuclei were pale with prominent nucleoli. The cell diameters ranged from 19-28 µ with a mean diameter of 24 µ.

Group S8 (Atlas-figs. 16-22): The cells of the large group S8 were found primarily within the nucleus centralis superior, being first observed at the level of the nucleus motorius n. trigemini. At caudal levels, cells of group S8 formed a bilateral paramedian row which extended from the corpus trapezoidum dorsally to the fasciculus longitudinalis medialis and, in the midline, mingled with those of S7(Fig. 75). At more rostral levels, the main portion of S8 was separated by the decussatio brachii conjunctivi with some cells found along the lateral edges of the decussation as well as within the decussation (Fig. 80). Cells of S8 were also found to be scattered laterally, ventral to the nucleus n. trochlearis. Processes from some of these cells which were found more dorsolaterally, were observed to course dorsally, around the nucleus n. trochlearis toward the nucleus raphe dorsalis (Fig. 78).

Cells of group S8 often appeared to be clustered in small groups, some appearing to be fused (Fig. 77). With the formation of the nucleus interpeduncularis, group S8 has decreased in size and soon disappears.

The cell bodies of S8 were of two types: larger cells found in bilateral paramedian columns along the midline and smaller cells in a midline row (Figs. 79 and 80). The larger paramedian cells were found to have a mean diameter of 27 µ with a range of 23µ to 34 µ. The staining characteristics and appearance of these cells were similar to those of group S6. The smaller midline cells were more elongated and were found more in the rostral portion of group S8. The cells had a mean diameter of 20 µ, a small amount of cytoplasm that stained moderately with toluidine blue, pale nuclei, and darkly staining nucleoli.
Group S9 (Atlas-figs. 19-21): The cell bodies belonging to group S9 were found spreading laterally from the ventral portion of group S8, starting just rostral to the nucleus olivaris superior (Figs. 80, 81, 82). These cells were observed to be dorsal to the lemniscus medialis and corpus trapezoideum (Fig. 81) extending as a horizontal band to the ventral portion of the formatio reticularis pontis caudalis and formatio reticularis pontis oralis.

These cells of S9 had a mean diameter of 27 µ with a range of 20 µ to 32 µ. Their cytological staining characteristics and appearances resembled those of group S6 and the larger cells of S8.

General Observations on the Serotonin-containing Groups

Position: Unlike the catecholamine-containing neurons, the serotonin-containing cell bodies were found within specific nuclear structures, i.e. those of the raphe system. Correspondingly, these cells were found mostly in the region of the midline, although portions of some groups (S1, S3, S8) and the majority of another group (S9) extended toward the lateral aspect of the brain stem. The relative positions of these groups in the brain stem are diagrammatically depicted in Figure 83.

Cytology: The mean cell diameters of these groups of serotonin-containing cell bodies ranged from 20 µ (small cells of S8) to 28 µ (S6). The fluorescence intensity of these cells was not evaluated since, after iproniazid treatment, they all fluoresced with approximately the same degree of intensity. However, it was particularly noted that in tissues from untreated animals, groups S6, S8, and S9 fluoresced much more strongly than did the cells in the other groups which were barely visible. As with the
catecholamine-containing cells, the Nissl substance was usually of moderate granularity and stained moderately in intensity, although the cells in groups S1 and S2 appeared to stain lighter than the cells in the other groups. Additionally, cells in almost all of the serotonin-containing groups were characterized by a darker staining mass of Nissl material that was located in a rim or a clump in the periphery of the cytoplasm. Like the catecholamine-containing groups, all of the nuclei of the serotonin-containing cells stained lightly with a darkly staining nucleolus. An interesting feature of some of the cells, particularly those in groups S1 and S2, involved an apparent disparity between the appearance of the cells after Nissl staining and their appearance under the fluorescence microscope. With toluidine blue staining, the cells appeared to have little cytoplasm. However, these cells appeared to display a greater area of fluorescence than one would expect assuming that the fluorescence reaction product was limited to the cytoplasm. Further, like the catecholamine-containing cells, the nuclei of many of the serotonin-containing neurons were found to have a "dot" of formaldehyde-induced fluorescence.
PHOTOMICROGRAPHS OF SEROTONIN-CONTAINING CELL BODIES
IN THE BRAIN STEM OF THE SQUIRREL MONKEY
(Saimiri sciureus)
Fig. 66. Montage of low power photomicrographs of the midline raphe region in the medulla oblongata at a level approximating Atlas-Fig. 11. In this view, two serotonin-containing groups, S1 and S2, are evident. Cells of group S1 are found within the nucleus raphe pallidus (RPa) medial to the tractus pyramidalis (Py). This group is located ventral and medial to the nucleus olivaris inferior (OI). An artery (A) is indicated on the ventral surface of the brain stem. Dorsal to S1 are the cells of group S2 within the nucleus raphe obscurus (RO) characteristically arranged in two symmetrical narrow paramedian rows. The curve in this group is artifactual caused by distortion in the tissue. Group S2 reaches close to the floor of the ventriculus quartus (IV). X43.
Explanation of Figures

Fig. 67. The rostral portion of group Sl within the nucleus raphe pallidus is observed as being closely associated with numerous fluorescing fibers. This group is located medial to the tractus pyramidalis (Py) in the ventral part of the brain stem. An artery (A), probably the basilar artery is also seen. Located on the ventral surface of the brain stem, this vessel displays the characteristic features of arteries which are seen in these preparations, i.e., an inner auto-fluorescent layer of elastic tissue and an outer band of fluorescing dots and fibers which are the norepinephrine-containing sympathetic nerves to the muscular layer. X104.

Fig. 68. Cells of group Sl located among the exiting fibers of the nervus hypoglossus as the nerve passes ventrolateral to the nucleus olivaris inferior. X104.

Fig. 69. Cells of group S2 located within the nucleus raphe obscurus. X104.
PLATE XXXI
Explanation of Figures

Fig. 70. Composite of low power photomicrographs of the ventral half of the midline raphe region at the level of the corpus trapezoideum. The rostral part of group S1 in the nucleus raphe pallidus (RPa) located medial to the tractus pyramidalis (Py) as well as group S3 in the nucleus raphe magnus (RM) are evident. Cells belonging to group S3 are also seen to extend laterally among fluorescing horizontal fibers. X43.

Fig. 71. Higher magnification of the cells of groups S1 and S3 at the level of the corpus trapezoideum (CT). Note the fluorescing fibers associated with the cells in this rostral portion of group S1 and further that group S1 merges dorsally with the cells of group S3. A few cells of S3 may be seen spreading laterally. The curve in the ventral midline is due to distortion of the tissue. (RM = nucleus raphe magnus; RPa = nucleus raphe pallidus; Py = tractus pyramidalis). X104.

Fig. 72. High power view of some of the cells of group S3 found in the more lateral region of the corpus trapezoideum. X207.
PLATE XXXII
Explanation of Figures

Fig. 73. A few cells of group S5 located within the nucleus raphe pontis. X104.

Fig. 74. The caudal portion of group S6 located just ventral to the ventriculus quartus. X104.
Explanation of Figures

Fig. 75. Low power view of the region of the fasciculus longitudinalis medialis (LM) at the level of the nucleus trochlearis. Cells of group S7 are seen within the nucleus dorsalis raphe located medial to the fasciculus; the cells of this group extend dorsally. Cells of group S8 located within the nucleus centralis superior are found ventral to the fasciculus. A few cells, probably representing the rostral part of C6, are seen within and immediately dorsal to the fasciculus longitudinalis medialis. X65.

Fig. 76. Cell bodies belonging to group S7 located in the nucleus dorsalis raphe, medial to the fibers of the nervus trochlearis (niv). X104.
PLATE XXXIV
Explanation of Figures

Fig. 77. View of some of the large cells of group SB located within the nucleus centralis superior. Note that some of the cells are in close proximity to each other and, in one case, they appear to be fused.

Fig. 78. Cells of group SB located lateral to the nucleus centralis superior, ventral to the nucleus trochlearis (Niv). These cells appear to be sending long fluorescing processes which are directed dorsally and laterally around the nucleus trochlearis to the region of group S7. X104.

Fig. 79. Two types of cells are found in group SB in the nucleus centralis superior; these include a median-placed row of smaller cells and para-median larger cell bodies. X104.
Explanation of Figure

Fig. 80. Montage of low power photomicrographs of a transverse section through the mesencephalon at the level of the decussatio brachii conjunctivi (DBC). In this composite, several serotonin-containing groups located in the midline raphe are observed. Group S7 is found dorsal and medial to the fasciculus longitudinalis medialis (FLM) and the nucleus trochlearis (Niv) within the nucleus dorsalis raphe (DR). Cells of group S8 are ventral to the fasciculus, on the periphery and within the decussation; a few cells also spread laterally. Ventral to the decussation, group S8 cells are observed in greater numbers within the nucleus centralis superior (CS); both median small cell bodies and paramedian large cells are present. Ventral to group S8 are the laterally extending cells of group S9. X45.
Fig. 81. Low power view of group S9 which spreads laterally from the ventral portion of group S8. The cells of group S9 are found dorsal to the lemniscus medialis (LM). The dashed line represents the midline. X43.

Fig. 82. Higher magnification of the cells of group S9 seen at the left; cells of group S8 are on the right side of the photomicrograph. X104.
Fig. 83. Diagram of the brain stem of the squirrel monkey indicating the approximate position of the serotonin-containing groups of cell bodies (S1-S9) in caudorostral and ventrodorsal planes.

CoI  Colliculus inferior
CoS  Colliculus superior
CS  Nucleus centralis superior
DR  Nucleus dorsalis raphe
RM  Nucleus raphe magnus
RO  Nucleus raphe obscurus
RPa  Nucleus raphe pallidus
RPo  Nucleus raphe pontis
AN ATLAS LOCALIZING MONOAMINE-CONTAINING CELL GROUPS IN
HISTOLOGICAL SECTIONS OF THE BRAIN STEM OF THE
SQUIRREL MONKEY (Saimiri sciureus)
Introduction to the Atlas

The purpose of this Atlas is to provide the reader with a pictorial presentation of the distribution of the catecholamine- and serotonin-containing cell bodies in the brain stem of the squirrel monkey as reported in this study. The Atlas is composed of two parts: the first portion (Atlas-figs. 1-4) presents various macroscopic views of the fixed whole brain as well as brain stem; in the second part (Atlas-figs. 6-27) the positions of the various monoamine-containing cell groups are indicated on frontal histological sections of the brain stem. Each figure in this portion of the Atlas consists of a toluidine blue-stained section for Nissl substance and a next adjacent section stained with luxol fast blue for myelin. On the Nissl stained sections, the positions of the catecholamine-containing groups are represented as crosses and the locations of the serotonin-containing groups are indicated with triangles. Atlas-figs. 6-14 have been magnified seventeen times while Atlas-figs. 15-27 have been magnified twelve times. A key is presented in Atlas-fig. 5 to indicate the approximate levels from which these sections were taken. Whenever a particular section resembles a section in the stereotaxic atlas of Emmers and Akert (1963), the co-ordinates of that atlas are indicated on the Atlas figure of this dissertation. The terminology of the Atlas follows that of Emmers and Akert (1963) except the raphe nuclei for which the nomenclature of Taber et al. (1960) is used.
Lateral view of the whole brain. The scale is in centimeters.
Occipital view of the whole brain. The scale is in centimeters.
Ventral view of the whole brain. The scale is in centimeters.
Dorsal view of the isolated brain stem. The scale is in centimeters.
Explanation of Atlas-Fig. 5

In the upper drawing of the brain stem of the squirrel monkey, a key of the levels corresponding to the histological sections used in this atlas is presented. The numbers (6-27) at the top of the vertical frontal section lines, which are approximately 1.0 mm apart, refer to the atlas figure numbers. In the lower diagram, a few superimposed structures are labeled on the drawing for reference purposes.

- CI: Colliculus inferior
- CM: Corpus mamillaris
- CO: Optic decussation
- CS: Colliculus superior
- mO: Nucleus n. facialis
- IO: Nucleus olivaris inferior
- OS: Nucleus olivaris superior
- SN: Substantia nigra
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<tr>
<td>BP</td>
<td>Brachium pontis</td>
</tr>
<tr>
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</tr>
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<td>CoS</td>
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</tr>
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<td>CT</td>
<td>Corpus trapezoidium</td>
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<td>Decussatio brachii conjunctivi</td>
</tr>
<tr>
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<td>Decussatio pyramidum</td>
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<tr>
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</tr>
<tr>
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Atlas-Fig. 6a
Atlas-Fig. 6b
Atlas-Fig. 7b
Atlas-Fig. 8 resembles the sections in co-ordinates P5.0 and P5.5 of Emmers and Akert.
Atlas-Fig. 9a

Atlas-Fig. 9b

Atlas-Fig. 9 resembles the sections in co-ordinates P4.0 and P4.5 of Emmers and Akert.
Atlas-Fig. 11a

Atlas-Fig. 11b

Atlas-Fig. 11 resembles the sections in co-ordinates P3.0 and P3.5 of Emmers and Akert
Atlas-Fig. 13a

Atlas-Fig. 13b

Atlas-Fig. 13 resembles the sections in co-ordinates P2.0 and P2.5 of Emmers and Akert
Atlas-Fig. 14 resembles the sections in co-ordinates P1.0 and P1.5 of Emmer and Akert.
Atlas-Fig. 15a

Atlas-Fig. 15b

Atlas-Fig. 15 resembles the sections in co-ordinates P0.5 of Emmer and Akert.
Atlas-Fig. 16 resembles the sections in co-ordinates APO.0 of Emmer and Akert
Atlas-Fig. 17 resembles the sections in co-ordinates A0.5 of Emmers and Akert.
Atlas-Fig. 18 resembles the sections in co-ordinates A1.0 of Emmers and Akert.
Atlas-Fig. 19 resembles the sections in co-ordinates Al.5 of Emmers and Akert.
Atlas-Fig. 20 resembles the sections in co-ordinates A2.0 of Emmer and Akert.
Atlas-Fig. 21 resembles the sections in co-ordinates A2.5 of Emers and Akert.
Atlas-Fig. 22 resembles the sections in co-ordinates A3.0 of Enners and Akert.
Atlas-Fig. 2Ja resembles the sections in co-ordinates A3.5 of Emmers and Akert.
DISCUSSION

I. Consolidation of Several Monoamine-containing Groups

From observations made in this study, several changes in the designation of particular monoamine-containing groups are suggested. Of the catecholamine-containing groups, it would seem that groups C4, C6, and C7 could be classified together as belonging to the locus caeruleus. This suggestion is based upon the fact that all three groups have similar fluorescence intensity, size, Nissl staining characteristics, and are found in relatively close sequential proximity with each other in a caudal to a rostral direction.

Likewise, the cells of these comparable groups in the rat (A4, A6, A7) as reported by Dahlström and Fuxe (1965a), were described to have similar shapes, sizes, staining characteristics, and fluorescence intensity which was characterized by a strongly fluorescent perinuclear zone. That group A4 is the caudal-most portion of the locus caeruleus in the rat was confirmed quite recently (Olson and Fuxe, 1971) in a study of the efferent pathways of this structure by the fluorescence histochemical method for monoamines. Group C6, of course, represents the main portion of the locus caeruleus which, medial to the tractus mesencephalicus n. trigemini at the level where the tract lies ventral and medial to the brachium conjunctivum, the nucleus becomes a closely-packed group of cells. The third group which might be considered with the locus caeruleus, group C7, is located at the ventral tip of the brachium conjunctivum. In consecutive sections from this region, one receives the impression that group C7 is actually a portion of C6 which, in
the rostral portion of the locus caeruleus, becomes positioned ventral to the brachium conjunctivum. However, in the study of the efferent pathways of the locus caeruleus in the rat by Olson and Fuxe (1971), it was revealed that while the cell bodies of groups A4 and A6 displayed the characteristic fluorescence retrograde changes which accompany degeneration of a monoamine-containing neuron, such changes were not reported in the cells of group A7. Similarly, in a stereotaxic study of the monoamine pathways in the brain of the rat (Ungerstedt, 1971a), the cells of group A7 were described as contributing to a ventral norepinephrine ascending pathway while axons from noradrenergic cells of the locus caeruleus were observed to form a dorsal ascending pathway. Thus, although the morphological features of the cells of group A7 in the rat are similar to those of groups A4 and A6, experimental evidence from other workers does not suggest that the fibers from the cells of group A7 follow the same route as those fibers from the cells of A4 and A6. It would be of considerable interest to map these pathways, as well as all of the other monoaminergic pathways, in the brain of the squirrel monkey and compare these results with those reported for the rat.

A second region of catecholamine-containing cells which lends itself to consolidation of groups is that of the substantia nigra. In their classification, Dahlström and Fuxe (1965a) distinguish two different groups of cells in the region of the substantia nigra of the rat: group A9, which is identified as the zona compacta of the substantia nigra, and group A8, which forms dorsolateral and slightly caudal to group A9. As indicated in the results reported by these authors, both groups appear to merge and are indistinguishable on the bases of both fluorescence and cytological characteristics.
These same observations were made in the squirrel monkey in the present study, the results of which suggest that both groups C8 and C9 are portions of the catecholamine-containing pars compacta of the substantia nigra. This possibility is supported in the rat, at least, by a recent report on the cytoarchitecture of the substantia nigra in the rat based upon conventional histological methods (Hanaway et al., 1970), in which the authors describe the area of the cells of group A8 as being the caudal part of the zona compacta. In addition, as reported by Ungerstedt (1971a), destruction of the corpus striatum in the rat leads to retrograde degenerative changes not only in the cell bodies of group A9, but also in the cells of group A8. An understanding of the distribution of the catecholamine-containing cells of the substantia nigra, especially in a primate as presented in this study, may provide additional morphological information on which to base investigations of disorders of the extrapyramidal system such as seen in Parkinsonism.

Of the serotonin-containing groups, due to their similar cytological and fluorescence characteristics, it would seem that groups S6, S8, and S9 could also be classified as one group. In the rat, Dahlström and Fuxe (1965a) also describe these three groups (B6, B8, B9) as having similar cytological characteristics. That group S6 may be a portion of the nucleus centralis superior (S8) is supported by Gerhard and Olszewski (1969) who describe the nucleus centralis superior as being divided into a dorsal portion which is situated dorsal to the fasciculus longitudinalis medialis, and a medial portion which is located ventral to the fasciculus. Group S6 would then correspond to the dorsal portion of the nucleus and group S8 would be found in the main medial portion of the nucleus centralis superior.
II. Correlation of the Formaldehyde-induced Fluorescence with Cytological Features

It would be of particular interest to be able to distinguish certain cytological features as seen in classically stained tissues which are unique to monoamine-containing cell bodies. This ability would be of immediate practical benefit by enabling one to identify such neurons on toluidine blue or hematoxylin stained sections rather than to require the involved fluorescence histochemical technique. Such a correlation would also be of significant value to the understanding of the relationship between the features of neuronal cytology as seen on stained sections as compared with the fluorescence characteristics, i.e. the monoamine-containing nature, of the cells. This point is emphasized by the finding that three to four weeks after the axon of a monoamine-containing neuron has been cut, the fluorescence intensity of the cell body greatly decreases with a corresponding loss of staining ability of the Nissl substance (Andén et al., 1965).

In analyzing the cytological features of the monoamine-containing cell bodies as seen in toluidine blue and hematoxylin stained tissue, most of these cells appear to stain moderately with toluidine blue, displaying Nissl substance of medium granularity. Further, as seen on hematoxylin-stained sections, all of the monoamine-containing cells contain a pale nucleus with a prominent nucleolus. However, although these features appear to be characteristic of monoamine-containing neurons, they are not unique to these cells. Other cell bodies which do not exhibit the monoamine-specific fluorescence, such as those found in the nucleus olivaris inferior, resemble the monoamine-containing cells in terms of cytoplasmic and nuclear staining characteristics.
While no apparent correlation could be detected between the fluorescence characteristics of monoamine-containing cells and the intensity of staining and granularity of their Nissl substance, a quantitative study employing microfluorimetric analysis of the fluorescence combined with a microspectrophotometric evaluation of the staining intensity of the Nissl substance may yield more fruitful results. This particular study would be especially interesting in view of the above-mentioned results of Andén and co-workers (1965).

Several interesting facets of neuronal cytology which would also appear to warrant further examination were revealed in this study. One aspect involves the fluorescing "dot" which has been observed in the nuclei of both catecholamine- and serotonin-containing cell bodies and is also seen in illustrations of cell bodies published by other workers (Dahlström and Fuxe, 1965a). Although it is quite evident in many of the cells, to the author's knowledge, no account has described the dot nor the basis of its fluorescence. Since it is present only in paraformaldehyde-vapor treated material and the fluorescence disappears after sodium borohydride treatment, one could suggest that the fluorescence is due to a monoamine reaction product. However, it is difficult to accept the idea that monoamines would be found within the nucleus in sufficient concentration to produce the resulting fluorescence. On the other hand, the similar size and location of the dot suggests that the fluorescence might correspond to the nucleolus. However, since the nucleolus is basically composed of ribonucleic acid and the formaldehyde-induced reaction is quite specific for monoamines, this possibility is also improbable. Another area for further investigation involves the apparent discrepancy
observed between the amount of cytoplasm showing in Nissl stained sections and as seen under the fluorescence microscope in some of the serotonin-containing cells (particularly those from groups S1 and S2). Also, it would be of interest to investigate whether or not the rim or mass of darkly staining Nissl material in the periphery of the fluorescing raphe cells is of significance to the serotonin-containing nature of these cells.

III. Comparison of the Monoamine-containing Groups of Cell Bodies in the Brain Stem of the Squirrel Monkey with those of Lower Mammals

Although minor species-related differences were found, a close basic similarity was observed among the monoamine-containing structures in the brain stem of the squirrel monkey as described in this study and those of the rat as reported by Dahlström and Fuxe (1965a). This interspecies similarity was underscored by the fact that the same numbering system for the monoamine-containing groups as established in the rat, in most cases, could be applied to the squirrel monkey. The results of this study revealed that almost all of the monoamine-containing groups of cells described in the rat by Dahlström and Fuxe (1965a) could be identified in the squirrel monkey brain stem. Further, no additional groups of cell bodies were found in the squirrel monkey which were not described in the rat. In addition, although some minor differences were noted between the rat and squirrel monkey with respect to position and appearance of the groups, the general characteristics of the groups of monoamine-containing cell bodies in these two animals were quite similar.
The similarity of these groups in the rat and squirrel monkey may be discussed in view of the fundamental operations of the brain stem in all mammalian species as well as the similarity of the organization of the brain stem from a phylogenetic point of view. This region contains structures essential for the life of the animal such as the vital centers of the autonomic nervous system as well as for the preservation of the animal such as the motor and sensory nuclei of the cranial nerves. In addition, ascending and descending pathways linking the most primitive portion of the central nervous system, the spinal cord, with the rest of the central nervous system, pass through and are influenced by the brain stem. Phylogenetically, the brain stem is a relatively old structure when compared with the higher centers of the brain. As such, from a comparative neuroanatomical point of view, this region is organized according to the same basic plan throughout the mammalian evolutionary scale. This point is appreciated in a study of comparative neurobiology such as presented by Papez (1967) which indicates that while the position and size of any one structure may vary among the mammalian species, it serves similar functions throughout the scale of phylogenetic development. For example, in all mammals, the medial lemniscus conveys proprioceptive impulses, the vestibular nuclei are necessary for the maintenance of balance and equilibrium, and the pyramidal tracts carry information to the spinal cord concerning voluntary movements. In the field of comparative neuroanatomy, a comprehensive study of the nervous system of vertebrates (Ariëns Kappers et al., 1936) reveals that although the morphological features of structures in the brain stem differ in comparing mammalian with non-mammalian species, a certain uniformity exists within the brain stem.
of all mammals. Further, Le Gros Clark (1960) indicates that in the trends of primate neuroanatomical evolution, the tectum of the midbrain is the only portion of the brain stem which appears to have undergone any significant changes. Thus, it is not inconceivable, and perhaps should even be expected, that the monoamine-containing cell groups, like the rest of the structures of the brain stem would not significantly differ between the rat and the squirrel monkey.

The differences which were observed between the rat and squirrel monkey were based mainly upon location, position, and appearances of the groups. Of the catecholamine-containing groups, group Al in the rat was described as being located lateral, ventral, and medial to the nucleus reticularis lateralis (Dahlström and Fuxe, 1965a) while in the squirrel monkey, the fluorescing cells of group Cl were found primarily dorsal to this nucleus. While the general cellular characteristics of the larger cells of the locus caeruleus (group C6) as well as their closely-packed arrangement, as reported in this study, were similar in the squirrel monkey to those of the rat, two features of the locus caeruleus found in the squirrel monkey were not described in the rat. One point was the striking proximity of the fluorescing cells to the tractus mesencephalicus n. trigemini. In the squirrel monkey, these fluorescing cells were found to be closely associated with the tract from its most caudal levels to the levels at which, moving in a rostral direction, it takes a position lateral to the aquaeductus Sylvii. Another feature in the squirrel monkey which was not described in the rat was a preferential distribution of the larger cell bodies in the more dorsal portion of the locus caeruleus and of the smaller, spindle-shaped cells in the ventral portion of the nucleus.
In another difference between the rat and squirrel monkey with respect to the catecholamine-containing groups, numerous fluorescent terminals were observed on the cell bodies of group C10 in the squirrel monkey, while such contacts were not described for the rat in the comparable group A10 (Dahlström and Fuxe, 1965a) nor were any terminals observed on the cell bodies in the illustrations of this region in the rat.

As far as the serotonin-containing groups are concerned, the cells of group S6 were found to be closely associated with the fasciculus longitudinalis, being located within and immediately dorsal to the fasciculus. The interesting distribution of serotonin-containing cell bodies within the fasciculus longitudinalis medialis was not described in the rat. Considering another serotonin-containing group, the appearance of S8 differed from that of group B8 in the rat. In the squirrel monkey, this group was composed of two columns of paramedian cells and a midline row of smaller cell bodies located within the nucleus centralis superior; the smaller cells were particularly evident at the level of the decussatio brachii conjunctivii. In the rat, however, the cell bodies of group B8 were illustrated as being randomly distributed within the midline region. Such a distribution of cell bodies within the nucleus centralis superior of the squirrel monkey was not indicated by Taber et al. (1960) in the cat using standard histological techniques. In fact, this nucleus in the cat was described by these workers as having larger cell bodies in the midline with smaller cell bodies more in the periphery, quite the opposite to what was found in the squirrel monkey. While a paramedian paired nature of this nucleus was described in man (Braak, 1970) using aldehydefuchsin-stained material, a midline distribution of smaller
cell bodies was not indicated. In another difference between groups S8 and B8, numerous fluorescing varicose fibers were observed in association with the cells of group S8 in the squirrel monkey while such fibers were not described in B8 of the rat. Finally, group S9 in the squirrel monkey did not appear to be as extensive in distribution as group B9 described in the rat.

Two minor monoamine-containing groups found in the rat were not observed in the squirrel monkey. These groups include a catecholamine-containing group A3 described as a small weakly fluorescing group (Dahlström and Fuxe, 1965a) and a serotonin-containing group B4. That both of these groups were not observed in the squirrel monkey may indicate minor species differences. The significance of group A3 may not be very great, for this group was not mentioned in a recent study of stereotaxic mapping of monoamine pathways in the rat (Ungerstedt, 1971) as well as a series of studies using 6-hydroxydopamine in investigations of the monoamine-containing neurons in the brain of the rat (Ungerstedt, 1971b, 1971c).

In the only other relatively systematic study of a mammalian species, (Pin et al., 1968), most of the monoamine-containing groups described in the cat were found to be similar to those described in the squirrel monkey as reported in this dissertation. However, two rather significant differences were found between the cat and the squirrel monkey (as well as between the cat and the rat). These differences involved a group of serotonin-containing cells in the large-celled region of the red nucleus in the cat which was not found in the rat or the squirrel monkey. In addition, an important group of catecholamine-containing cells found in the medulla oblongata of the rat (group A1) and the squirrel monkey (group C1) was not observed in the cat.
The lack of significant interspecies differences between the rat and the squirrel monkey while two rather striking differences were noted between the cat and the squirrel monkey is difficult to explain at this time. Certainly more such comparative studies of the central monoaminergic systems found throughout the entire vertebrate phylogenetic scale will be of considerable value.

IV. Connections and Functional Significance of the Monoamine-containing Groups

Of particular interest to this study would be an understanding of the role that these groups of monoamine-containing cell bodies play in the functioning of the central nervous system of the squirrel monkey. However, since this investigation is the first to deal with the fluorescence histochemistry of the brain stem of a primate, discussion of the interaction of the monoaminergic groups with the rest of the nervous system will be limited to correlations with the available pertinent physiological, chemical, and enzyme histochemical data of the squirrel monkey which have appeared in the literature. In addition, since work on the monoamine-containing groups of cells reported in the literature has been restricted to the lower mammals, discussion of the functional significance and connections of these groups will emphasize these animals, particularly the rat. However, it is hoped that an understanding of the relationships of the monoaminergic groups in the lower mammals will provide an insight into the comparable groups found in the brain stem of the squirrel monkey.
Catecholamine-containing groups: As indicated in the Review of the Literature, the two types of cells containing catecholamines, dopamine and norepinephrine, may be differentiated pharmacologically such as after administration of alpha-methyl-dopa followed by reserpine. In the rat, as also indicated in the Review of the Literature, the catecholaminergic pathways have been described by several workers. These pathways were confirmed and expanded upon recently by Ungerstedt (1971a) using stereotaxic procedures. First of all, this worker described a descending noradrenergic system originating from group A1 and possibly A2 which terminated upon cells of the ventral horn, dorsal horn and intermediolateral cell column of the spinal cord. Secondly, an ascending noradrenergic pathway described as originating from groups A1, A2, A5, and A7 formed a ventral bundle which terminated on structures in the lower brain stem, mesencephalon, and diencephalon. With respect to the corresponding group Cl in the squirrel monkey, it is of interest that stimulation of the region of this group has been reported to elicit chirping, a specific type of vocalization, in the squirrel monkey (Jürgens and Ploog, 1970). Along the same lines, it has been reported that administration of phenoxybenzamine, an adrenergic blocking agent, inhibited vocalization in the cat (Kanai and Wang, 1962). Certainly the catecholaminergic involvement of vocalization, especially with respect to group Cl, would be of interest to examine.

In the rat, just as a ventral noradrenergic bundle was described as originating from groups A1, A2, A5, and A7, so was a dorsal bundle of noradrenergic fibers described as originating from the locus caeruleus (Ungerstedt, 1971a). It is felt that a significant result of the present study is
the positive identification of certain cell groups as belonging to the catecholamine-containing locus caeruleus, rather than to other nuclei such as the nucleus tr. mesenceph. n. trigemini. The cluster of closely-packed cell bodies located medial to the tractus mesencephalicus n. trigemini in the pons of the squirrel monkey, which is interpreted in the literature (Emmers and Akert, 1963; Manocha and Bourne, 1966c; Manocha and Shantha, 1969) as the caudal portion of the nucleus tr. mesenceph. n. trigemini is actually composed almost entirely of green fluorescing cells, which most probably belong to the locus caeruleus. As a matter of fact, the results of the histochemical work of Manocha and Bourne (1966) on the distribution of monoamine oxidase in the brain stem of the squirrel monkey are in good agreement with the results of the present study, since they show a rather high concentration of monoamine oxidase in the area of the catecholamine-containing cell bodies of the locus caeruleus (although, in accordance with the available atlases, this area is labeled as nucleus tr. mesenceph. n. trigemini). On the basis of general consideration, one would expect a positive reaction for monoamine oxidase where monoamine-containing structures are located; in particular, Hashimoto et al., (1962), describe the locus caeruleus as containing large amounts of monoamine oxidase in the rat. Further, in the brain of the developing rat, the fluorescence histochemical reaction product, which is first observed on the fourteenth day of fetal life, displays a close parallelism with the monoamine oxidase activity (Maeda and Dresse, 1969). Other authors (Gerhard and Olzewski, 1969), however, label as locus caeruleus the structure identified as such in this study of the histochemical fluorescence of its cells.
As described in the literature (Russel, 1955), the cells of the nucleus
tr. mesenceph. n. trigemini may be intermingled with the cells of the locus
cæruleus. Indeed, comparison of photomicrographs of sections showing
fluorescing cell bodies of the locus caeruleus with photomicrographs of the
same sections stained with toluidine blue reveal that a few nonfluorescing
cell bodies are found among the fluorescing cells particularly in the dorso-
lateral aspect of the locus caeruleus. Whether or not these nonfluorescing
cells belong to the nucleus tr. mesenceph. n. trigemini is difficult to
establish, however, in view of the disruption of the Nissl staining pattern
due to freeze artifacts. Some of the nonfluorescing cells appear to have
the characteristics of the cells of the nucleus tr. mesenceph. n. trigemini
by being larger, not having any apparent processes (indicating a unipolar
cell), and staining more heavily with toluidine blue than the fluorescing
cells of the locus caeruleus. However, in a few cases, nonfluorescing cells
were indistinguishable in the stained preparations in every way from the
surrounding cells which were found to fluoresce. In sections which were
examined from the animal perfused with 4% formaldehyde, where the cytological
details are better preserved, no cells of the nucleus tr. mesenceph. n.
trigemini were observed within the main portion of the locus caeruleus located
medial to the tractus mesencephalicus n. trigemini, while only in the dorsal
most portion of the tract could a few cells of the nucleus tr. mesenceph. n.
trigemini be found with the cells of the locus caeruleus mostly on the lateral
aspect of it, between this nucleus and the tractus mesencephalicus n. tri-
gemini.
In the rat, the noradrenergic innervation of the cerebral and cerebellar cortices has been attributed mainly to axonal terminals originating from the locus caeruleus with the suggestion that a single cell of the locus caeruleus capable of innervating all cortices of the brain (Olson and Fuxe, 1971).

With respect to the cerebellum, recent physiological studies (Siggins, et al., 1971) have demonstrated that stimulation of the locus caeruleus results in prolonged inhibition of the Purkinje cells after a long latent period. The colliculi, the geniculate bodies, parts of the thalamus, the vagal area, the raphe, and probably the nuclei of the accessory nerve also receive noradrenergic innervation from the locus caeruleus (Olson and Fuxe, 1971). Moreover, according to Loizou (1969) the hypothalamus receives nerve terminals originating from cell bodies located in the locus caeruleus. A similar ubiquitous innervation by the cells of the locus caeruleus to those areas of the brain as well as to the hippocampus was reported by Ungerstedt (1971a). He further observed that such a collateral contribution to all cortices from one single neuron appears to be unique to the locus caeruleus. From a functional point of view, this diffuse monosynaptic noradrenergic system having its cell bodies (or most of its cell bodies) in the locus caeruleus is important for cortical arousal (Fuxe and Hansson, 1967; Jones et al., 1969; Olson and Fuxe, 1971) as well as for the induction of paradoxical sleep (Jouvet, 1969). With respect to descending influences, the locus caeruleus has been suggested to mediate visceral activity, such as possibly serving as a pneumotaxic center, for stimulation of this nucleus has been reported to change the pattern of respiration in the cat (Johnson and Russell, 1952).
In consideration of the great functional significance of the locus caeruleus, it is expected that an increasing amount of experimental work will be performed on this structure, particularly in primates such as the squirrel monkey. It is felt that the findings reported in this study may provide a useful anatomical basis for these studies.

Of the dopamine-containing groups in the rat, A9 and possible A8 ascend to terminate in the nucleus caudatus putamen (Ungerstedt, 1971a). This pathway is of considerable importance in discussing the clinical implications of l-dopa treatment of Parkinsonism in man. This neurological disorder, characterized biochemically by decreased levels of dopamine in the caudate and putamen nuclei, was first reported to be alleviated by l-dopa treatment in 1961 (Birkmayer and Hornykiewicz, 1961). Since that time, a substantial number of investigations have been reported in the literature covering experimental and clinical aspects of l-dopa treatment (see, for example, the excellent symposium chaired by O'Malley, 1970). A recent study using 6-hydroxydopamine to cause selective lesions in the nucleus caudatus putamen of the rat (Ungerstedt, 1971d) suggests that postsynaptic supersensitivity after degeneration of a nigrostriatal dopaminergic system may contribute an important factor to the effectiveness of l-dopa therapy for Parkinsonism. Although this dopaminergic pathway has been demonstrated in the rat (Andén et al., 1964b, 1965, 1966a; Hökfelt and Ungerstedt, 1969) and lesions of the substantia nigra have been shown to diminish the level of striatal dopamine (Sourkes and Poirier, 1966), the significance of this particular pathway in relation to the etiology of Parkinsonism has been questioned by several workers who feel that other routes than the nigrostri-
atal pathway are of greater importance. Mettler (1970), for example, working with rhesus monkeys, observed that the nigrostriatal fibers are of minor importance when compared with the number of nigropallidal fibers and, further, that the striatum receives a very large input from the centromedian nucleus as well. The author concluded that this finding with the fact that experimental lesions in the substantia nigra of animals do not produce the Parkinsonian symptomatology, suggested that the complex clinical picture of Parkinsonism could not be explained primarily on the basis of degeneration of the substantia nigra. Certainly the numerous catecholamine-containing cell bodies found in the pars compacta of the substantia nigra of the squirrel monkey in the present study as well as the relatively high dopamine concentrations in the caudate nucleus of this animal (Neff et al. 1969a) indicate the possibility of a significant dopaminergic nigrostriatal pathway in the squirrel monkey. Such a dopaminergic route was demonstrated in another primate (Macacus rhesus) by Poirier and Sourkes (1965). These workers found that after post-operative periods of one to nine months, ipsilateral lesions of the pars compacta of the substantia nigra resulting in severe cell loss was associated with a low concentration of dopamine in the corresponding striatum, while in those animals in which the pars compacta was not affected, the concentration of dopamine in the striatum on that side was not significantly altered. The axons from another dopamine-containing group in the rat, group A10 located dorsal to the nucleus interpeduncularis, have been described to ascend with the nigrostriatal system (Ungerstedt, 1971a). However, these fibers leave this bundle and innervate the nucleus accumbens, nucleus interstitialis striae terminalis, and the tuberculum olfactorium.
Serothonin-containing Groups: As described by Ungerstedt (1971a), in the rat serotonin-containing groups B1, B2, and B3 in the raphe give rise to a descending serotoninergic system which parallels the descending noradren­ergic pathway from groups A1 and A2. An ascending serotoninergic pathway originating from the raphe groups B7 and B8 was also described by the same author. This pathway was found to run in the medial forebrain bundle to innervate the cingulum, amygdala, hippocampus, and possibly the cerebral cortex. In the amygdala and hippocampus of the cat, serotoninergic fibers were described as making axosomatic contacts with neurons in these regions (Eidelberg et al., 1967). As with the other neurotransmitters such as dopamine, norepinephrine, and acetylcholine, the exact nature of the function of sero­tonin with relation to the operation of a given region or system of the brain is not known. Evidence from work on the cat, however, indicates that sero­tonin may serve as an inhibitory neurotransmitter on cells in the amygdaloid complex (Eidelberg et al., 1967). As indicated by Taber et al. (1960), the general organization of the raphe complex is rather similar throughout the phylogenetic scale, suggesting that this region is a rather primitive portion of the brain stem and, as such, may be involved with relatively simple but important and fundamental functions of the brain. Recent studies, particular­ly by Jouvet (1967, 1969), have revealed that the serotonin-containing raphe cells are necessary for the production of slow wave sleep. That is, just as the reticular activating system located within the midbrain appears to be responsible for maintaining the waking state (Moruzzi and Magoun, 1949), so are the raphe neurons thought to actively induce sleep. Pharmacologically, this action of serotonin may be demonstrated by administration of the trypto­
phane hydroxylase inhibitor, p-chlorophenylalanine. Sleep behavior in cats administered this drug is abolished but may be returned almost immediately by 5-hydroxytryptophan (Jouvet, 1966). Further, in a series of experiments, Jouvet and his co-workers (Jouvet and Renault, 1966; Jouvet et al., 1967) demonstrated the relationship between the raphe neurons and the sleep/wake cycle in cats. These investigators found that selective lesions of the raphe system resulted in persistent wakefulness with a concomittant decrease in the level of serotonin in the raphe region. Significant correlations were found between the extent of the lesion, the decrease in serotonin, and the degree of wakefulness.

Recent investigations have revealed other important functional implications of the raphe neurons such as a close correlation between the activity of hallucinogenic drugs and serotonin metabolism (Freedman, 1961, 1963). For example, it has been reported that when lysergic acid diethylamide (LSD) is given by general route to rats, the raphe neurons are specifically inhibited from firing (Aghajanian et al., 1968, 1970). Also, experimental evidence indicates that serotonin may be involved in heat regulation and, further, that serotonin and norepinephrine are reciprocally related in this body function. Simmond (1970), for example, reported that when various areas of the brain were heated, the turnover of serotonin increased with the increasing temperatures. Weiss and Aghajanian (1971) have recently demonstrated that the serotonin-containing raphe neurons are definitely involved in thermoregulation. In two different sets of experiments on rats, these researchers found that: 1) lesions destroying a large portion of the midbrain raphe nuclei completely prevented the heat-induced increase in brain 5-hydroxy-
indoleacetic acid concentration and 2) as body temperatures of the animals were increased, there was a simultaneous increase in the rate of firing of the individual raphe neurons.

Very recently, after the research for this dissertation had been completed, two reports appeared in the literature which suggested that serotonin may not be the only substance which fluoresces and is found in the raphe system. In one study on the rat, Aghajanian and Asher (1971) reported that, as expected, tryptophan loading caused an increase in the fluorescence of the raphe neurons as determined by fluorescence microscopy and microspectrofluorometric scanning. However, contrary to expectation, administration of p-chlorophenylalanine, which blocks serotonin synthesis from tryptophan, did not decrease the serotonin-induced fluorescence. These authors concluded that a substance other than serotonin which is also a derivative of tryptophan may be located within the raphe neurons. In another study of the rat, Björklund et al. (1971) reported a new type of indolamine-containing neuron in the raphe particularly within group B8 with some found in B7 and B9. The fluorescence reaction product of this monoamine had lower excitation and emission maxima than that of serotonin, it had a slower rate of photodecomposition upon exposure to ultraviolet light which was intermediate between the rates for serotonin and the catecholamines, and, unlike serotonin, its fluorescence was not affected by the tyrosine hydroxylase inhibitor p-chlorophenylalanine. These authors concluded that the indoleamine found in these cells might be 5,6-dihydroxytryptamine or 5-methoxytryptamine.

It is quite likely that this new indoleamine system also occurs in the raphe system of the squirrel monkey. During observations of brain stem
sections under the fluorescence microscope, particularly from animals not treated with a monoamine oxidase inhibitor, it was noted that the larger cells in S6, S8, and S9 appeared to fluoresce rather strongly while the fluorescence of the cells in the other groups in the raphe was weaker. This difference in fluorescence intensity may reflect the difference in sensitivity to photodecomposition between the newly determined indoleamine-containing cells and serotonin-containing cells, respectively. Certainly in view of the significance of the raphe system and in light of these new findings, this region in the squirrel monkey should be re-examined with the appropriate pharmacological manipulations and microspectrofluorometric analysis in order to determine the distribution of these new indoleamine-containing cells.
SUMMARY AND CONCLUSIONS

The present study, aimed at the localization and identification of the monoamine-containing cell bodies in the brain stem of the squirrel monkey (Saimiri sciureus) by fluorescence histochemistry, has revealed the following:

1) While no marked differences were found between the rat and the squirrel monkey with respect to the distribution of the monoamine-containing neurons in the brain stems of these two animals, two significant differences were found between the squirrel monkey and the cat.

2) The results concerning the identification of the locus caeruleus in the squirrel monkey suggest a revision of the stereotaxic atlas of the brain stem of this animal by Emmers and Akert (1963), changing the structure which is identified as the nucleus tr. mesenceph. n. trigemini to the locus caeruleus. Such a revision would prove to be significant for any studies (particularly stereotaxic and histochemical investigations) of this nucleus in the squirrel monkey.

3) On the basis of similar cytological and fluorescence features, the suggestion is made to combine the catecholamine-fluorescing groups C4, C6, and C7 (A4, A6, and A7, respectively, in the rat) into one group which characterizes and identifies the locus caeruleus. For the same reasons, groups C8 and C9 (A8 and A9 in the rat) may be grouped as the pars compacta of the substantia nigra. Of the serotonin-containing groups, S6, S8, and S9 (B6, B8, and B9 in the rat) could also be considered together due to their similar characteristics.
4) Although most of the monoamine-containing cells have similar cyto-
logical characteristics as observed on toluidine blue and hematoxylin
stained sections, no single feature of these cells appeared to be unique
to the monoamine-containing cells.
5) The cause of the fluorescing "dot" which is observed in many of the
nuclei of the monoamine-containing cells should be investigated.
6) With the possibility of a new indoleaminergic system in the raphe
as indicated in the recent literature, this region should be re-evaluat-
ed in the squirrel monkey, especially those cells in groups S6, S8, and
S9.

The finding of similar monoamine-containing cell groups in the brain
stem of the squirrel monkey as compared with the rat provides a greater degree
of validity for extrapolation of pharmacological and physiological studies
concerning monoamine functions and activities in the brain of the rat to that
of man. Although one might have expected significant differences between the
rodent and the primate, such a similarity is not so surprising in considera-
tion of the relatively basic position of the brain stem in the course of
mammalian neuroanatomical development. Perhaps a far more revealing aspect
of this problem will be investigations to map out the pathways from the groups
of cell bodies described in the brain stem of the squirrel monkey and com-
pare them with those of the rat. The information which has been presented
in this dissertation will provide the necessary morphological baseline for
such investigations.
APPENDIX I

Composition of Wayne Monkey Diet (Allied Mills, Inc.)

The ingredients of the diet given to the squirrel monkeys used in this study are listed below as provided by the manufacturer: dried skimmed milk, sugar (sucrose), soybean meal, corn gluten meal, oat groats, ground wheat, animal liver meal, dehydrated alfalfa meal preserved with ethoxyquin, soybean oil, dried beet pulp, dried bakery product, wheat germ meal, brewers dried yeast, irradiated dried yeast (source of vitamin D2), D-activated animal sterol (source of vitamin D3), vitamin A palmitate, vitamin B12 supplement, vitamin E supplement, menadione sodium bisulfite (source of vitamin K activity), riboflavin, niacin, calcium pantothenate, choline chloride, thiamine, ascorbic acid, folic acid, calcium carbonate, dicalcium phosphate, salt, manganous oxide, copper sulfate, iron carbonate, potassium iodate, cobalt sulfate and zinc oxide.
APPENDIX II

Detailed Description of the Freeze-drying Apparatus and Procedure

A. Apparatus

The freeze-dryer used in this study, as illustrated in text-figure 9 in the Materials and Methods section, consisted of a specimen tube cooled by a refrigeration unit and evacuated by a two-stage mechanical vacuum pump. Water vapors are collected by a chemical desiccant, phosphorous pentoxide, placed on the bottom of the tube as well as in the vacuum trap. The refrigeration unit, adapted from an early model freeze-dryer (Aloe Scientific Co.), consists of four cylindrical metal tubes placed vertically in a closed refrigerated box; each refrigeration tube holds one glass specimen tube. The specimen tube (20mm. - outside diameter) contains the sections of brain to be freeze-dried. The tissues are supported in the tube on a series of six copper-mesh shelves, with one tissue per shelf. The specimen tube is connected to the vacuum trap (Kontes Glass Co.; no. K-926J00, size 24) by pressure-vacuum tubing and the trap, in turn, connected to a two-stage mechanical vacuum pump (Welch Duo-Seal, model 1400). All connections are sealed with high vacuum grease (Dow-Corning) and, except at the specimen tube, clamped with a worm drive tubing clamp. This arrangement allows twenty-four blocks of tissue to be simultaneously freeze-dried.

B. Procedure

1. A small amount of phosphorous pentoxide (Fisher Scientific Co.) is placed in the bottom of the specimen tube, which had been previously dried in an oven (110°C). A copper mesh grid is placed between the desiccant and the
APPENDIX II cont'd

base of the copper shelves to provide support for the shelves. The tube is capped with aluminum foil and placed in the freezer (-72° to -78°C).

2. The frozen brain specimens are transferred to the pre-cooled specimen tube with pre-cooled entomologist's forceps, placing one tissue per shelf.

3. The specimen tube with the tissues is re-capped and transferred to the freeze-drying apparatus in a Dewar flask containing a mixture of acetone and dry ice (-70°C).

4. Using gloves, the specimen tube is quickly connected to a pre-cooled (-30°C) end of vacuum tubing and placed at the bottom of the refrigeration tube (-30° to -32°C); the other end of the vacuum tubing is connected to the inlet side of the vacuum pump. Prior to being integrated into the freeze-dryer, the vacuum trap is oven dried (110°C) and, after, cooling to room temperature, is filled with enough phosphorous pentoxide to be evenly spread in a layer one-half inch thick along its inside surface. Any phosphorous pentoxide which is accidently spilled on the ground glass of the vacuum trap is immediately removed in order to assure a tight seal. Fresh desiccant is used with every set of tissues that is freeze-dried.

5. The vacuum pump is turned on and allowed to run for eleven days. Periodically during this time, the vacuum is checked by a high frequency generator held close to the glass wall of the vacuum trap. A greenish glow on the glass indicates a vacuum better than 10^{-3} Torr (Pearse, 1968).
6. During the ninth and tenth days of freeze-drying, the tissues are slowly warmed to room temperature. Due to graded temperature zones in the refrigeration tubes, with the coldest at the bottom and the warmest at the top, warming the tissues merely involves raising the specimen tube over a period of time, i.e., five centimeters every four hours starting on the morning of the ninth day. The following table summarizes this warming procedure, indicating the distance from the bottom of the refrigeration tube and the corresponding temperature:

<table>
<thead>
<tr>
<th>Distance from the bottom of the tube (cm)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-30±2</td>
</tr>
<tr>
<td>5</td>
<td>-27±2</td>
</tr>
<tr>
<td>10</td>
<td>-24±2</td>
</tr>
<tr>
<td>15</td>
<td>-21±1</td>
</tr>
<tr>
<td>20</td>
<td>-16±3</td>
</tr>
<tr>
<td>25</td>
<td>-10±2</td>
</tr>
<tr>
<td>30</td>
<td>-4±2</td>
</tr>
<tr>
<td>35</td>
<td>+2±2</td>
</tr>
</tbody>
</table>

7. On the evening of the tenth day, the tissues are brought to room temperature and maintained at that temperature over-night. On the morning of the eleventh day, the specimen tube is placed in a warm water bath (50°C) for four hours to ensure that all of the tissue will be above room temperature and completely dehydrated when the vacuum is broken.
APPENDIX II cont'd

8. The specimen tube is removed from the water bath and the pump is stopped. Air is slowly let into the system at the vacuum pump end so that it passes over the desiccant before it reaches the tissues.

9. The specimen tube is disconnected from the rest of the freeze-dryer and the tissues are rapidly transferred to a jar containing paraformaldehyde for the gas-phase histochemical reaction to occur.
Standardization of the Relative Humidity of Paraformaldehyde

The relative humidity of paraformaldehyde may be standardized by its exposure to sulfuric acid of a known density for seven days within an air-tight container. Initially, paraformaldehyde of various humidities (50-80%) were employed to determine the optimal humidity for the tissues used in this study. In order to determine the concentration of sulfuric acid required to achieve the desired relative humidity, the following information concerning the relationship among constant humidity, density, and concentration of sulfuric acid was utilized (Weast, 1968):

<table>
<thead>
<tr>
<th>Relative Humidity of Sulfuric Acid</th>
<th>Density of Sulfuric Acid</th>
<th>Concentration of Sulfuric Acid (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>1.34</td>
<td>586</td>
</tr>
<tr>
<td>60</td>
<td>1.29</td>
<td>503</td>
</tr>
<tr>
<td>70</td>
<td>1.25</td>
<td>428</td>
</tr>
<tr>
<td>80</td>
<td>1.20</td>
<td>341</td>
</tr>
</tbody>
</table>

The sulfuric acid used in this standardization had a specific gravity of 1.84. In order to determine the volume of acid to use per given concentration of acid, the following formula was employed:

\[
\text{grams of sulfuric acid} = \frac{\text{volume of sulfuric acid (ml)}}{\text{specific gravity of sulfuric acid}}
\]
APPENDIX III cont'd

In the following table, these calculations reveal the volume of sulfuric acid which is required to produce the desired corresponding relative humidity:

<table>
<thead>
<tr>
<th>Relative Humidity</th>
<th>Grams of Sulfuric Acid</th>
<th>Volume of Sulfuric Acid (ml) per 100ml of water</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>586/1.84</td>
<td>31.9</td>
</tr>
<tr>
<td>60</td>
<td>503/1.84</td>
<td>27.3</td>
</tr>
<tr>
<td>70</td>
<td>428/1.84</td>
<td>23.3</td>
</tr>
<tr>
<td>80</td>
<td>341/1.84</td>
<td>18.5</td>
</tr>
</tbody>
</table>

As indicated in the Materials and Methods section of this dissertation, sulfuric acid of a relative humidity of 60% was found to produce the optimal results.
APPENDIX IV

Histological Staining Procedures

In both staining procedures used in this study, the tissues were initially treated similarly in the following manner:

1. deparaffinize in xylene - two changes, 2 minutes each
2. xylene:100% ethyl alcohol (50:50) - 30 seconds
3. 100% ethyl alcohol - 30 seconds
4. celloidin solution - 5 minutes
   formula for celloidin solution: nitrocellulose - 2g
   ether - 100ml
   100% ethyl alcohol - 100ml
5. 80% ethyl alcohol

Toluidine Blue:
(Dahlström and Fuxe, 1965a)
6. 70% ethyl alcohol - 30 seconds
7. 50% ethyl alcohol - 30 seconds
8. distilled water - two changes, 30 seconds each
9. stain in 0.5% aqueous toluidine blue - 2 minutes
10. wash in several changes of distilled water
11. differentiate in 95% ethyl alcohol until the stain appears slightly heavier than one would like; inspect microscopically
12. rinse in distilled water
13. dehydrate through the graded ethyl alcohols (50%, 70%, 95%, 100%) - 10 seconds each
14. xylene:100% ethyl alcohol - 30 seconds
15. xylene - two changes, 2 minutes each
16. mount in synthetic resin and cover-slip
APPENDIX IV cont’d

Results: cytoplasm - blue (intensity depends upon the individual Nissl substance staining characteristics of the neurons)
  nucleolus - blue to dark blue

Modified Klüver and Barrera stain using Darrow Red:
(Klüver and Barrera, 1953; Powers et al., 1960; Powers and Clark, 1963)

6. 95% ethyl alcohol - 30 seconds
7. stain in luxol fast blue (0.1% in 95% ethyl alcohol) at 57°C overnight
8. rinse in distilled water; several changes
9. dip quickly in lithium carbonate (0.05% in distilled water)
10. differentiate in 70% ethyl alcohol until the gray and white matter can be distinguished; do not over-differentiate
11. wash in distilled water
12. brief dip in lithium carbonate; differentiate in several changes of 70% ethyl alcohol until the greenish blue of the white matter contrasts sharply with the colorless gray matter
13. rinse thoroughly in distilled water
14. stain in Darrow red (0.025% in 0.2M acetic acid) - 30 minutes
15. rinse in distilled water
16. dehydrate through the graded ethyl alcohols (50%, 70%, 95%, 100%) - 10 seconds each
17. xylene; 100% ethyl alcohol - 30 seconds
18. xylene - two changes, 2 minutes each
19. mount in synthetic resin and coverslip

Results: Myelin - blue to blue-green
  cell bodies - light red
Preparation of the Brain for Histological Examination

A. Perfusion Fixation of the Brain

1. With the animal under sodium pentobarbital anesthesia (50mg/kg),
   the thoracic cavity was opened and the severed internal thoracic artery
   was clamped.
2. The aorta was isolated; a ligature was passed under the vessel and
   loosely tied.
3. An incision was made in the left ventricle. A polyethylene cannula
   (Intramedic, PE#160) with a plastic tubing adapter (tubing to female
   Luer) attached at the opposite end was inserted through the incision,
   passed up the aorta to the initial portion of the aortic arch, and
   secured with the ligature.
4. After severing the superior vena cava, the brain was flushed with
   50ml of 0.85% sodium chloride through the cannula with a syringe in order
   to clear the cerebral vessels of blood and prevent clotting.
5. The brain was then perfused with one liter of 4% formaldehyde in
   1.25% sodium chloride (neutralized with marble chips) through the
   cannula. In order to achieve sufficient perfusion pressure, a 50ml
   syringe was used with firm but steady pressure being applied. The
   perfusate was removed from the thoracic cavity by means of a vacuum
   aspirator.
6. After its removal from the cranial cavity, the brain was placed in
   fresh fixative for one week.
APPENDIX V cont'd

B. Dehydration and Embedding

After its removal from the fixative, the brain stem was isolated by a transverse cut through the cerebral peduncles. Most of the cerebellar cortex was removed by cutting the vermis and much of the hemispheres with a wedge-shaped section. The brain stem was cut into three one-centimeter thick blocks and prepared according to the following schedule:

1. washed in distilled water - two changes - one hour each
2. dehydrated through a graded series of ethyl alcohol (25%, 35%, 50%, 70%, 80%) for one hour each except for 70% ethyl alcohol which was overnight
3. 95% ethyl alcohol - two changes - 30 minutes each
4. 100% ethyl alcohol - two changes - 30 minutes each
5. 100% ethyl alcohol:benzene (50:50) - 30 minutes
6. benzene - two changes - 30 minutes each
7. benzene:paraffin (50:50) - over-night at 45°C
8. infiltrate with filtered Paraplast (m.p.:56-57°C); fresh paraffin each time - three changes
   paraffin #1 - 20 minutes
   paraffin #2 - 20 minutes
   paraffin #3 - 10 minutes in vacuo
9. embed with fresh paraffin

The blocks were trimmed and sectioned at a thickness of 10µ. Every tenth section was collected for toluidine blue-staining and the adjacent section was designated to be stained for myelin. The staining procedures were the same as indicated in Appendix IV.
APPENDIX VI

Sodium Borohydride Reduction: Monoamine Fluorescence Specificity Test
(Corrodi et al., 1964)

1. deparaffinize the sections by flooding the slide several times with xylene and carefully pouring off the excess containing dissolved paraffin; the tissue is thoroughly deparaffinized when it appears white

2. 100% isopropyl alcohol - two changes - 30 seconds each

3. 80% isopropyl alcohol - 30 seconds

4. 0.1% sodium borohydride in 80% isopropyl alcohol - 5 minutes

5. 80% isopropyl alcohol - three changes - 30 seconds each

6. 100% isopropyl alcohol - two changes - 30 seconds each

Allow the sections to dry; mount with xylene and view under the fluorescence microscope. With this procedure, monoamine-containing sites lose their fluorescence, while the autofluorescence is not affected.


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The dissertation submitted by Jack Edward Hubbard has been read and approved by the members of the dissertation committee.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form, and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

January 17, 1972  
Signature of Adviser