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Neurochemical Evidence for Cholinergic-Adrenergic Coupling in Mammalian Central Nervous System

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NEUROCHEMICAL EVIDENCE FOR CHOLINERGIC-ADRENERGIC COUPLING

IN MAMMALIAN CENTRAL NERVOUS SYSTEM

by

SILAS N. GLISSON III

A Dissertation Submitted to the Faculty of the Department of Pharmacology, Loyola-Stritch School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

September 1971
BIOGRAPHY

Silas Nease Glisson III was born to Mr. and Mrs. Silas Nease Glisson Jr. on May 8, 1941 in Springfield, Illinois.

After graduating from Springfield High School (Illinois) in 1959, he attended Illinois College for two years. His final two years of college were spent at Southern Illinois University in Carbondale. In August 1964 he graduated with a Bachelor of Arts degree in Zoology.

The following two and one-half years he worked as a Medical Research Associate at Thudichum Research Laboratory, Galesburg State Research Hospital, Galesburg, Illinois. While there he was actively engaged in research concerning the developing mammalian brain.

He was accepted by the Department of Pharmacology and Experimental Therapeutics, Loyola-Stritch School of Medicine as a graduate student in September 1967.

On February 10, 1968, he joined the former Mary L. Mosimann in holy matrimony.

From that time to the present, Mr. Glisson has worked as a graduate student at Loyola-Stritch School of Medicine to fulfill requirements for a Doctor of Philosophy Degree in Pharmacology.
ACKNOWLEDGEMENTS

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In addition, I would like to thank Lionell Barnes and Gisela Kindel whose assistance was invaluable.

Finally, words cannot express the love I have for my wife, Mary, whose comfort and support have meant so very much over these four years.
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ABBREVIATIONS

ACh     ----- Acetylcholine
AChE    ----- Acetylcholinesterase
ATMN    ----- Atropine methyl nitrate
AtSO₄   ----- Atropine sulfate
DA      ----- Dopamine
DFP     ----- Diisopropylfluorophosphate
DOPA    ----- 3,4-Dihydroxyphenylalanine
HCl     ----- Hydrochloric acid
JB835   ----- 1-phenyl-3-hydrazinobutane
K₂CO₃   ----- Potassium carbonate
NE      ----- Norepinephrine
β       ----- Beta
g.      ----- Gravity
gm.     ----- Gram
i.p.    ----- Intraperitoneal
i.v.    ----- Intravenous
kg.     ----- Kilogram
μg.     ----- Microgram
mg.     ----- Milligram
ml.     ----- Milliliter
mm.     ----- Millimeter
No.     ----- Number
"P"     ----- Probability
s.c.    ----- Subcutaneous
S.E.    ----- Standard error
<       ----- Less than
I. INTRODUCTION AND REVIEW OF THE LITERATURE

PURPOSE:

The studies incorporated in this dissertation are designed to add to, using neurochemical techniques, the pharmacological evidence that cholinoceptive neurons in the central nervous system, under certain conditions, have the ability to modulate central adrenergic neurons, reflected as a change in the levels of norepinephrine and/or dopamine.

A. CHOLINOCEPTIVE PATHWAYS OF THE BRAIN:

The mapping of cholinoceptive neurons in the central nervous system has till now met with considerable difficulty. Lack of specific histochemical or of the more recently developed, fluorescent microscopy techniques, has made direct identification of cholinoceptive neurons impossible and therefore evidence obtained thus far has been indirect.

The presence of acetylcholine, choline acetylase, and acetylcholinesterase has been used to locate cholinoceptive neurons (Hebb, 1963 and Koelle, 1963). In addition, Feldberg (1957) and Machne (1963) conducted experiments with both systemically and topically applied cholinomimetics, cholinesterase inhibitors, and cholinergic blocking agents. Stimulation and
blocking of recorded responses was thought to indicate presence of cholinoceptive neurons. Another approach to the identification of central cholinoceptive neurons was to identify acetylcholine in the subcellular synaptic fraction from brain tissue. Whittaker (1963), DeRobertis (1963), Ryall (1963), and Curtis (1964), were among the first to conduct such experiments. Their findings demonstrated that acetylcholine was present in the subcellular fraction containing the synaptic vesicles. Although this type of evidence added support to the concept of the role of acetylcholine as a central neurotransmitter, it did not however, define specific cholinoceptive pathways.

Probably the most direct evidence of cholinoceptive pathways comes from studies using microelectrophoretic application of acetylcholine and cholinomimetic agents directly on central neurons while recording extra- or intracellularly. Single neurons responding to injected acetylcholine are considered to be cholinoceptive. Central mapping of cholinoceptive neurons has been done primarily in cat, however, neural and other physiological variances may exist between cat and rabbit. As these two species are similar in levels of transmitters found within specific brain areas, the nerve tracts in these two species could be considered as, also similar. Evidence by Curtis and Eccles (1958), Curtis et al. (1961), and Curtis (1965) demonstrated cholinoceptive nerve paths in the spinal cord of cat. Bradley and Wolstencroft (1962 and 1965) added evidence of cholinoceptive neurons in the midbrain of cat. Curtis and Andersen (1961), Curtis and Davis (1963), and
Phillis et al. (1968) demonstrated cholinoceptive neurons in cat thalamus. Margules and Stein (1967) reported the presence of cholinoceptive synapses in the medial hypothalamic area in cat. Curtis et al. (1963) and McLennan (1964) demonstrated the presence of cholinoceptive neurons innervating caudate nucleus in cat. Curtis and Crawford (1969) reported the presence of cholinoceptive neurons in spinal cord, thalamus, hypothalamus, basal ganglia, cerebellum, and in cortex. Extensive work has also been done on cortical acetylcholine content. Krnjevic and Phillis (1963) and Spehlmann (1963) demonstrated cholinoceptive nerve tracts in cat cerebral cortex. Other central areas where cholinoceptive neurons have been located include: the inferior colliculus (Curtis and Koizumi, 1961); both the mesencephalic and medullary reticular formations (Bradley and Wolstencroft, 1962; Salmoiraghi and Steiner, 1963); and the well-documented work of Eccles et al. (1954) and Eccles et al. (1956) demonstrating cholinergic innervation of the Renshaw cell, which is associated with the spinal motoneurons.

These studies, although far from providing a complete account of the cholinoceptive nerve tracts in the brain, do clearly demonstrate that cholinoceptive neurons are widely distributed from the spinal cord, rostrally, through the medulla, midbrain thalamus, cortex, and caudate nucleus; in addition to lesser structures like the inferior colliculus. Whether these are long nerve tracts passing through many areas before terminating
on a specific group of cells or short nerve tracts widely branching through one particular area is not known. The data does show, however, that within each of these areas there are nerves the postsynaptic membranes of which are sensitive to acetylcholine. Denisenko (1965) has further clarified the properties of these cholinceptive membranes. He has demonstrated that certain areas of the brain have nicotine-sensitive cholinceptive neurons, i.e. cortex, while some areas are more muscarine sensitive, i.e. midbrain. More recently, Krnjevic (1969) has reported that many more sites in the cortex, brainstem, and at other subcortical levels, in the cerebellum and in the medulla respond to acetylcholine muscarinically rather than nicotinically. Anden et al. (1966) in their studies on the effects of induced blockades and facilitations of dopamine transmission, found asymmetrical syndromes evoked from the intact corpus striatum. Previous evidence of MacIntosh (1941), Feldberg and Vogt (1948), and Burn and Chipman (1951) had shown that the neostriatum contained high concentrations of acetylcholine, choline acetylase, and acetylcholinesterase. In addition, Koelle (1954) indicated that neostriatal nerves, containing acetylcholinesterase, represented true acetylcholine nerve terminals within this structure. These cholinceptive nerves ascend uncrossed in the crus cerebri and in the internal capsule. Shute and Lewis (1963) found that there was a severe loss of ipsilateral
acetylcholinesterase activity in the neostriatum after unilateral lesions at the junction between the mesencephalon and the diencephalon. From these results, Anden et al. (1966) concluded that in their lesion experiments, neostriatal acetylcholine terminals were also removed; and they, in fact, had found a reduction of acetylcholinesterase activity in the ipsilateral neostriatum much as Shute and Lewis had. It was their conclusion that cholinceptive neurons to the neostriatum are functional antagonists of the neostriatal dopamine neurons.

Schematic diagrams of central cholinceptive pathways have frequently been published. Stein (1970) has proposed a cholinceptive pathway to explain his results on the pharmacology of the reward and punishment systems. It was found that application of the cholinomimetic agents, carbachol and physostigmine, to the medial hypothalamic area restored the inhibitory effect of punishment; and usually depressed the rate of unpunished behavior, as well. Stein concluded that the punishment system has a critical focus in the medial hypothalamus and that cholinceptive synapses are formed at this level.

At best we can state that the telencephalon and midbrain-diencephalon areas do contain the enzymatic systems for synthesis of acetylcholine, as well as, its degradation. Also contained within these areas are cholinceptive sites which can be stimulated by acetylcholine and other cholinomimetic agents;
and can be blocked by various cholinolytic drugs. It is evident that cholinoceptive neurons have wide distribution in the mammalian brain. Specific nerve tracts within the individual areas, as well as, their role in neuromodulation is at present still vague.

B. CATECHOLAMINE PATHWAYS IN THE BRAIN:

Unlike the case with the cholinergic system, recent technology has allowed for accurate mapping of the adrenergic nerve tracts in the central nervous system, using the fluorescence microscopy techniques developed by the Swedish group. These techniques not only localize adrenergic nerve paths, but also differentiate quantitatively between norepinephrine and dopamine containing neurons. Using histological sectioning, individual nerve tracts can be traced from origin to termination. However, in intact and untreated animals, it is not possible to demonstrate the parts of the neuron connecting the nerve cell body and the terminal portions. It was found by Anden et al. (1966) that by either using a monoamine oxidase inhibitor or by making a lesion in the various monoamine neuron systems, they were able to demonstrate and map out many of the ascending monoamine neuron tracts. These adrenergic tracts have so far only been demonstrated in the rat. One would assume with some variations that rabbit nerve tracts would closely parallel those found in the rat.
**Dopamine nerve tracts:**

Dopamine nerve cell bodies originating in the substantia nigra area of the mesencephalon project rostrally and become aggregated in a bundle which ascends just medial and dorsal-medial to the ventral part of the crus cerebri. At the level of the posterior part of the median eminence the bundle turns into the ventral rostral part of the crus cerebri and enters and diverges into the retrolenticular part of the internal capsule. Running rostrally and dorsally in the internal capsule the nerves then ascend in the fibrae capsulae internae to innervate the neostriatum (caudate nucleus and putamen). It has been found that the neostriatum contains close to 80 percent of the brain dopamine, which is correlated with the large nerve bundle innervating this region.

An additional large dopamine nerve tract was also found. Cell bodies for these nerves arise in the median/mesencephalon region and ascend medially to the crus cerebri into the medial forebrain bundle innervating the tuberculum olfactorium and the nucleus accumbens. These nerve bundles run lateral to the norepinephrine nerve tracts, also running rostrally, and ventromedially to those dopamine nerve fibers which innervate the neostriatum.

Dopamine neurons have also been shown to innervate the retina of the eye, with both the cell bodies and terminals located there.
Norepinephrine nerve tracts:

Initial studies by Dahlstrom et al. (1962), followed by the investigations of Dahlstrom and Fuxe (1964 and 1965), Anden et al. (1966), and Anden et al. (1965) have demonstrated the location of norepinephrine nerve tracts in the central nervous system. Using lesion-histochemical techniques, it was found that at least 75 percent of the ascending norepinephrine neurons originate in cell bodies of the pons and medulla oblongata. These fibers innervate various nuclei of the hypothalamus including the nucleus dorsomedialis hypothalamic, the nucleus paraventricularis, and the nucleus periventricularis hypothalamic. These neurons, in addition, innervate the thalamus, the preoptic area, the ventral part of the nucleus interstitialis striae terminalis, the septal area, the amygdaloid complex, the hippocampal formation, the gyrus cinguli, and the pyriform and neocortex. Fibers to the neocortex ascend from the medial forebrain bundle dorsally into the tractus diagonalis and then turn caudally into the superficial parts of the white substance. Cell bodies of these neurons were found in the pons and the nucleus reticularis lateralis of the medulla oblongata. Carlsson et al. (1964) had shown that bulbo-spinal norepinephrine neurons also arise from these cell bodies.

An additional 25 percent of the norepinephrine neurons innervating the telencephalon have their cell bodies in the
formatio reticularis of the mesencephalon. Norepinephrine cell bodies have not as yet been found in the neocortex itself, although norepinephrine neurons richly innervate this structure.

Apart from the identification of the specific dopamine and norepinephrine nerve tracts coursing through the brain, nerve terminals containing either norepinephrine and/or dopamine have been found in large numbers within all areas of the central nervous system. The density of nerve terminals, based on the relative intensity of the histochemically developed fluorescence, related directly to the levels of norepinephrine and/or dopamine in these structures.

In conclusion, adrenergic neurons, referring specifically to those containing norepinephrine and dopamine, maintain their cell bodies in the mesencephalic portion of the brain and send their nerve fibers rostrally and caudally innervating the spinal cord, diencephalon, telencephalon, and neostriatum. Although these studies indicate the major nerve bundles contained in the adrenergic system, minor complexities associated with these pathways, as well as, the exact neuro-modulating role of the neurotransmitters, norepinephrine and dopamine, are not yet well defined. Present evidence indicate that norepinephrine has an excitatory effect upon central neurons (Salmoiraghi, 1966) with influence upon "vegetative" functions, i.e. temperature control, neuroendocrine regulation of pituitary function, control of mood, as well as, "non-
vegetative" functions, i.e. control of posture and motor activity. In the case of dopamine, however, the effect upon central neurons, primarily in the basal ganglia, is that of inhibition (Wurtman, 1966 and Salmoiraghi, 1966), which is postulated to be antagonistic to the excitatory actions of acetylcholine in this region (Anden et al., 1966).

C. EVIDENCE OF CHOLINERGIC-ADRENERGIC COUPLING: PERIPHERAL AND CENTRAL

Peripheral:
Classification of the postganglionic nerve fibers of the entire autonomic nervous system was performed by Dale in 1934. He defined autonomic transmission as being either "adrenergic" or "cholinergic" based on the neurohumor involved in the transmission of impulses from the nerve endings to the effector organ. Otto Loewi, in 1921, had already postulated the role of acetylcholine in parasympathetic transmission; and Von Eular, in 1931, had implicated noradrenaline in sympathetic transmission. Thus Dale's classification was not speculative; the results of these earlier studies provided supportive evidence for his classification.

The concept of the cholinergic-adrenergic link was advanced in 1958 by Burn and Rand, although first mention of mixed
cholinergic and adrenergic postganglionic sympathetic fibers was made by Dale in his 1934 publication. Dale defined postganglionic parasympathetic fibers as "cholinergic" and added that, "postganglionic sympathetic fibers are predominantly though not entirely 'adrenergic'". Dale's inclusion of cholinergic fibers within the postganglionic sympathetic supply was later confirmed in part by the demonstration of cholinergic fibers in the postganglionic supply to the nictitating membrane of the cat (Bacq and Fredericq, 1935); to the dog uterus (Sherif, 1935); and from the stellate ganglion of the heart (Folkow et al., 1948). Koelle in 1955 demonstrated the presence of choline acetylase and cholinesterase in adrenergic neurons, both of which are involved in synthesis and degradation of acetylcholine. Thus, considerable evidence had accumulated prior to the hypothesis of Burn and Rand which strongly suggested that a cholinergic-adrenergic link existed. It was, however, the hypothesis put forth by Burn and Rand in 1958 which advanced the role of acetylcholine in the release of adrenergic neurotransmitters at the postganglionic sympathetic nerve endings. Their postulation was that, "all the postganglionic sympathetic fibers have cholinergic mechanisms involved in the release of noradrenaline after nerve stimulation, i.e. postganglionic sympathetic nerve stimulation first releases acetylcholine which triggers off the mechanism by which noradrenaline is liberated from the local tissue stores at the sympathetic
postganglionic nerve endings". Jaju, in 1969, discussed three possible neural mechanisms which would explain the Burn and Rand hypothesis. First, that postganglionic fibers might release only acetylcholine which would in turn release noradrenaline from the adjacent chromaffin cells, a mechanism similar to that operating in the adrenal medulla. Secondly, the release of acetylcholine by the postganglionic impulse might initiate or facilitate the liberation of noradrenaline from different nerve fibers. Thirdly, the release of acetylcholine from the postganglionic fibers might initiate or facilitate the liberation of noradrenaline from the same fiber. The second explanation seem most reasonable based on previous evidence of mixed cholinergic and adrenergic fibers in the postganglionic supply. Whether or not the Burn and Rand concept of cholinergic-adrenergic coupling will finally be accepted or rejected is still a mute question. What seems evident, is that cholinergic-adrenergic coupling does exist (cf. Karczmar, 1967).

One of the earliest observations of cholinergic-adrenergic linkage was reported by Brucke in 1935. It was observed that a small dose (5μg) of acetylcholine injected at the base of a tuft of hairs in cat's tail produced piloerection which was similar in response to that seen after sympathetic stimulation. Coon and Rothman, in 1940, observed results similar to those observed by Brucke, using acetylcholine and nicotine. Burn
et al. (1959) were able to demonstrate that reserpinization abolished the piloerection response to both nicotine and to a small dose of acetylcholine, reserpine having caused a marked decrease in the amount of norepinephrine, as well as the number of chromaffin cells and their granules in the skin of the cat's tail. Further support of the role of acetylcholine in sympathetic stimulation came from Burn and Rand's observations (1962) that hemicholinium, known to block the synthesis of acetylcholine, when injected into the base of the tufts of hairs caused a slower erection of the tufts of hairs to repeated sympathetic stimulation. Untreated tufts of hairs were still erect. The authors concluded that nicotine and small doses of acetylcholine liberated norepinephrine which produced the piloerection. This response was greatly reduced after blockade of acetylcholine synthesis by hemicholinium and absent after depletion of catecholamine stores by reserpine or nerve degeneration. More recently, Rand and Whaler (1965) have shown that botulinum toxin, which blocks release of acetylcholine from cholinergic neurons, prevents piloerection when injected into the base of tufts of hairs in cat's tail. The authors concluded that acetylcholine release was essential for piloerection, following sympathetic nerve stimulation.

Studies on the nictitating membrane have added evidence of a cholinergic-adrenergic link. Bacq and Fredericq (1935)
first demonstrated cholinergic fibers innervating the nictitating membrane of the cat. Burn and Rand (1960) found that in reserpinized cats, contraction of the nictitating membrane by stimulation of postganglionic sympathetic fibers was potentiated by eserine and blocked by atropine, suggesting release of an acetylcholine-like substance by sympathetic fibers. Burn et al. (1963) expanded on these findings using isolated nictitating membrane preparations. Further, Ambache (1951) using botulinum toxin, which blocks the release of acetylcholine from cholinergic neurons, observed greatly reduced contractions of the nictitating membrane to sympathetic stimulation. Jacobowitz et al. (1965) using neuronally induced contractions of the nictitating membrane in rabbits observed that hemicholinium, which inhibits synthesis of acetylcholine, produced a reversible reduction in isometric contractions of the nictitating membrane to supramaximal tetanic stimulation of the sympathetic postganglionic trunk. Similarly, Burn et al. (1963) using hyoscine demonstrated a hyoscine resistant component of nictitating membrane contractions stimulated postganglionically supramaximally at high frequencies. They concluded that the hyoscine resistant component was adrenergic in nature, while the hyoscine sensitive component was cholinergic. Burn et al. (1963) observed that in nictitating membrane of cat treated with hyoscine or atropine, contractions to acetylcholine which should have been blocked
were in fact potentiated with neostigmine far more at supramaximal postganglionic stimulation frequencies below 5/second, than above.

These studies on nictitating membrane indicate that acetylcholine is involved to some extent in the modification of responses to sympathetic stimulation, although the mechanisms involved are unclear from these results.

Involvement of acetylcholine in response to sympathetic stimulation have also been observed on intestine and colon. Gillespie and Mackenna (1959 and 1961) observed that in reserpinized animals electrical stimulation of the sympathetic neurons produced contraction of the colon. It was further demonstrated that this effect could be blocked by atropine. Burn and Rand (1962) continued these studies demonstrating that hemicholinium reduced the inhibitory effects of sympathetic stimulation on the isolated colon of untreated animals. In addition, they were able to demonstrate reversal of this effect with the addition of choline. It was concluded by the authors that the reduced adrenergic response was due to the reduction in acetylcholine by hemicholinium. Similarly, Rand and Whaler (1965) found that botulinum toxin blocked the inhibitory effect of nerve stimulation in isolated rabbit ileum. Burn et al. (1963) found a biphasic response to low and high frequency stimulation on the isolated rabbit ileum with mesentary attached. Their results suggested that at lower
frequency both acetylcholine and norepinephrine were released, while at the higher frequencies only norepinephrine was released. Their conclusions were based on evidence that hyoscine increased the inhibitory effect of nerve stimulation on the ileum at low frequencies, but was ineffective at the higher frequencies.

Studies by Sjostrand (1961) on the isolated vas deferens of guinea pig, which were previously thought to contain only sympathetic innervation, have shown that acetylcholine potentiates the response of the vas deferens to electrical stimulation. Boyd et al. (1960), Burn and Weetman (1963), Ohlin and Stromblad (1963), and Riley and Maanen (1962) found potentiation of the isolated vas deferens response to electrical stimulation using physostigmine and neostigmine. Chang and Rand (1960) demonstrated decreased responses of this preparation to nerve stimulation using hemicholinium. Rand and Whaler (1965) have additionally shown that botulinum toxin abolished the contractions of the vas deferens to sympathetic nerve stimulation. These studies suggest a role of acetylcholine in the sympathetic response of the vas deferens to nerve stimulation.

Cholinoceptive neurons were reported (Sherif, 1935) to be contained in the sympathetic supply to the uterus in dog. Later, Burn and Rand (1960) reported that in reserpinized cats, stimulation of the hypogastric nerve produced contraction of the uterus contrasting the inhibitory effect normally observed.
This stimulant effect, seen in reserpinized cats, was potentiated by eserine and blocked by atropine. It was concluded from the observations that acetylcholine was involved in the excitatory effect on uterus following postganglionic nerve stimulation in reserpinized cats.

Numerous investigators: Ahlquist et al. (1954), Grindly et al. (1939), Holtz et al. (1952), and Ottis et al. (1957) have demonstrated the ability of epinephrine to contract the spleen. Burn and Rand (1962) found that after stimulation of the post-ganglionic nerves to the spleen, the volume of the spleen either increased or decreased in reserpinized animals. In those cases where an increase in volume was observed, it was found that eserine would potentiate the response while atropine blocked the dilatation of the spleen. In those cases where contraction of the spleen was observed, indicated by the decrease in volume, eserine was without effect. Atropine did have an effect, converting the increase into decrease in volume of the spleen. The authors suggested the latter response to atropine resulted from incomplete depletion of catecholamines by reserpine and subsequent release following nerve stimulation producing the observed contraction. Further, Bradon and Rand (1961) demonstrated the release of acetylcholine-like substance following postganglionic nerve stimulation in reserpinized cats. Using perfusate collected after nerve stimulation they observed that this perfusate would cause
the isolated guinea pig ileum to contract and atropine was found to block the contraction produced by the perfusate. Application of neostigmine to the reserpinized cats caused an increase in the amount of acetylcholine-like substance released upon nerve stimulation.

Other investigators: Farber (1936), Daly et al. (1961), Bradon and Boyd (1961), and Bradon and Rand (1961) have also observed sympathomimetic effects of acetylcholine upon the spleen in both dog and cat. They found that upon close intra-arterial injection of acetylcholine into the spleen, contraction of the spleen occurred. Reserpinization, dibenzyline or hexamethonium pretreatment, or nerve degeneration of the sympathetic supply were found to block this response. Ferry (1963) has further shown that acetylcholine in the presence of atropine causes contraction of the spleen. This effect could be blocked by bretylium and hexamethonium. A direct excitation of post-ganglionic adrenergic neurons was therefore suggested for the action of acetylcholine. Bretylium's blocking action, it was suggested, was upon the release of catecholamines by acetylcholine while hexamethonium's blocking action prevented the entry of acetylcholine into the sympathetic neuron (Burn and Gibbons, 1964). More recently, Krauss et al. (1970) have shown that intra-arterial injection of acetylcholine evoked release of norepinephrine from the isolated perfused cat spleen and induced antidromic nerve impulses in the splenic nerve.
Tetrodotoxin, it was shown, prevents release of norepinephrine by splenic nerve stimulation and blocked the antidromic discharges, however, it did not block the release of norepinephrine by acetylcholine. Further, it was shown that a single dose of bretylium blocked the release of norepinephrine to splenic nerve stimulation but not release due to acetylcholine. Higher concentrations of bretylium were able to block the release of norepinephrine by acetylcholine in the tetrodotoxin treated spleen. Hemicholinium was shown by Bradon and Rand (1961) to block contraction of the spleen produced by stimulation of the sympathetic neurons; the addition of choline reverses the hemicholinium block. These studies suggest that after depletion of acetylcholine, nervous stimulation is ineffective in producing the sympathetic response in spleen. In the rat spleen, electron microscopic studies have revealed two types of vesicles within the terminations of sympathetic neurons; one containing dense granules and the other type which was not granular but homogeneous (De Robertis and Pellegrino de Iraldi, 1961). Following reserpine pretreatment, the granular vesicles disappeared. It has been shown by autoradiography combined with electron microscopy that the granular vesicles store 3H-norepinephrine within the terminals of the sympathetic nerves supplying the rat spleen.

Studies on cardiac tissues also favor the cholinergic-adrenergic concept. Hoffman et al. (1945) studies the effects
of acetylcholine upon isolated perfused and atropinized hearts from cat, rabbit, and guinea pig. He observed positive inotropic and chronotropic effects produced by acetylcholine which closely resembled those effects observed following sympathetic stimulation. Such evidence suggested release of catecholamine-like substances by acetylcholine to produce the effects. Their suggestion was confirmed when an epinephrine-like substance was identified in the perfusate. In addition, sympathetic denervation of the heart and reserpination either reduced or blocked the effect of acetylcholine (Cabera et al., 1966). Moreover, Hukovic (1960) demonstrated that both 2:6 xylyl ether and bretylium, both of which block release of catecholamines, blocked the acetylcholine induced positive inotropic effect on isolated atria in the presence of atropine. Similarly, nicotine has been reported to produce a biphasic response on isolated heart and atria of cat, rabbit, and guinea pig (Giotti, 1954; Kot tegoda, 1935; Leaders and Long, 1962; McDowall, 1946; and Trendelenburg, 1960). Chiang and Leaders (1965) demonstrated a similar biphasic effect of nicotine on isolated rat atria. Kot tegoda (1953) reported that in atropinized perfused rabbit atria, nicotine increased both the force and rate of atrial contraction and that hexamethonium, a ganglionic blocking agent, blocked these effects to ganglion stimulatory properties of nicotine; however, Lee and Shideman, in 1959, found similar responses
of nicotine on cat papillary muscles devoid of ganglia. Kottegoda's responses to nicotine, it appeared, were not due to ganglionic stimulation. Burn and Rand (1958) reported that reserpinization or denervation of the rabbit atria, depleting the nerve endings of catecholamines, decreased or abolished any stimulatory effects of nicotine on the atria. These studies suggest that like acetylcholine, nicotine is involved in catecholamine mediated responses of the atria.

Studies on the isolated rabbit atria following stimulation of sympathetic neurons innervating the atria have shown that in normal rabbits a positive chronotropic response was produced. However, in reserpinized rabbits a negative inotropic response was observed. In addition, eserine potentiated and atropine blocked this inhibitory effect (Burn and Rand, 1962). Similar studies by Day and Rand (1961) showed that after guanethidine, stimulation of postganglionic neurons of cat atria caused a cardio-inhibitory effect. Using the same preparation, Leaders, in 1963, found in normal cats that stimulation of the sympathetic fibers produced a classical cardio-stimulant effect followed by a small consistent cardio-inhibitory effect and this inhibitory effect could be potentiated by eserine and blocked by atropine. In addition, hemicholinium was able to block the inhibitory response in both normal and reserpinized atria suggesting that acetylcholine is directly involved in atrial responses seen after stimulation of
Evidence of cholinergic involvement in responses of blood vessels to sympathetic stimulation has been demonstrated by Burn and Dutta (1948) and by Kottegoda (1953). They reported that in the presence of atropine, both acetylcholine and nicotine produced vasoconstriction in rabbit's ear vessels. Moreover, tolazoline, an alpha adrenergic blocking agent, reversed the vasoconstriction response of acetylcholine and nicotine. From the results, it was concluded that acetylcholine released catecholamines from the sympathetic terminals which stimulated the beta receptors causing the vasodilatation. Burn and Rand in 1958 reported in addition, that in the presence of atropine, acetylcholine and nicotine failed to produce vasoconstriction of the rabbit ear vessels following reserpinization or denervation. Hukovic (1960) further demonstrated that 2:6 xylyl ether and bretylium both blocked the vasoconstrictor effect suggesting that the vasoconstriction response to acetylcholine is mediated through norepinephrine. Stimulation of postganglionic sympathetic neurons supplying rabbit ear vessels is known to produce vasoconstriction of the vessels. Horton and Rand (1962) demonstrated that the vasoconstriction response to postganglionic nerve stimulation caused a decrease in blood volume followed by vasodilatation. It was also shown that eserine potentiates this vasodilatation and that atropine blocks this response. Similarly, Burn and Rand (1962) reported that administration of hemicholinium blocked the vasoconstrictor
effect of nerve stimulation on rabbit ear vessels and that the hemicholinium block could be reversed by choline administration. It was concluded that nerve stimulation released ACh which in turn released norepinephrine producing the vasoconstriction. In support of their conclusions, evidence was presented showing that perfusate collected from the ear vessels contained a substance which caused the leech muscle to contract, presumably acetylcholine. More recently, Rand and Varma (1970) demonstrated that low doses of acetylcholine, muscarine, and pilocarpine enhanced the vasoconstriction of the rabbit ear artery in response to sympathetic nerve stimulation, while high doses depressed these effects and atropine was shown to block this depressant effect. In addition, it was reported that acetylcholine and methacholine enhanced the vasoconstrictor response to injected norepinephrine. It was suggested from these results that these cholinomimetic drugs act to facilitate or impair (depending upon dosage) the release and uptake of norepinephrine from adrenergic terminals associated with rabbit ear artery. Löffelholz and Muscholl (1970) demonstrated, using isolated rabbit atria perfused with Tyrode solution containing amphetamine, that electrical stimulation of the right postganglionic sympathetic neurons caused release of norepinephrine which could be markedly decreased by simultaneous stimulation of vagal nerves. The results, it was suggested, indicate an inhibitory cholinergic-adrenergic interaction.
Using hind limb vessels in dog, Burn in 1932, demonstrated that stimulation of sympathetic neurons to the hind limb produced vasoconstriction in some experiments and vasodilatation in others. Bulbring and Burn in 1935 showed that some of these fibers were cholinceptive in nature. Burn (1961) and McCubbin (1961) using guanethidine, which blocked norepinephrine release, found that sympathetic nerve stimulation to dog hind limb produced vasodilatation which could be blocked by atropine. Bernard and Schaepdryver (1964) were able to demonstrate that electrical stimulation of the nerve supply to dog hind limb produced vasoconstriction that in atropinized dogs was potentiated by anticholinesterases. Cholinergic involvement in sympathetic stimulation of dog hind limb vessels seemed indicated.

Studies using perfused accessory cephalic vein of the dog (Rice and Long, 1966) demonstrated that acetylcholine injected into the perfusing fluid produced vasoconstriction of the vein which could be inhibited by pretreatment with reserpine or phentolamine and could be potentiated with cocaine. Further, the venous effluent following acetylcholine could relax the chick rectal caecum and this effect could be blocked by pronethanol, an adrenergic beta blocker. Hexamethonium had no effect on the vasoconstriction produced by acetylcholine ruling out a ganglionic mechanism for the response. In reserpinized animals, tyramine has been shown by Chandra et al.
(1965), Maxwell et al. (1959), and Trendelenburg (1961) to produce a fall in systemic blood pressure. This depressor effect is potentiated by eserine and blocked by atropine suggesting acetylcholine involvement.

From the volume of evidence reviewed, one would be hard pressed to doubt the existence of a cholinergic-adrenergic linkage in the Autonomic Nervous System. One might, and rightfully so, question under what conditions such a coupling phenomena functions and for what purpose. Hopefully future studies will enlighten us on these points.

Yet, there are counter arguments to acetylcholine's role in synaptic transmission as proposed by Burn and Rand (Karczmar, 1969), as well as, other concepts involving acetylcholine in synaptic transmission. Koelle (1963) suggested a double step release of acetylcholine from nerve terminals. Acetylcholine released in the course of the first step would then impinge upon the presynaptic nerve terminal, this second step initiating mass liberation of the transmitter.

At one time, Feldberg (1948) suggested that cholinergic transmission may never embrace two successive relays, and that the cholinergic mechanism is followed by a noncholinergic step.
Evidence of cholinergic-adrenergic coupling within the central nervous system can be considered as sparse compared to the volume of evidence dealing with the peripheral nervous system. It should not be inferred from this that cholinergic-adrenergic coupling does not exist within the central nervous system, but rather it attests to the difficulty in obtaining meaningful data from so complex and inaccessible a system. Most of the evidence reported has come from recent studies. Srimal et al. (1969) demonstrated, using intracerebroventricular injections of choline in anesthetized dogs, that a biphasic blood pressure response was observed; an initial hypertensive phase followed by a delayed hypotensive phase. The initial hypertensive phase was felt to be due to choline and was blocked by atropine. The delayed hypotensive phase resembled that observed after intracerebroventricular injection of norepinephrine. This hypotensive phase was abolished by prior reserpinization and blocked by pronethalol and INPEA, both adrenergic beta receptor blocking agents. It was concluded that the intracerebroventricularly injected choline stimulated the central release of norepinephrine to cause the hypotensive phase.

Additional evidence reported by Przuntek and Philippu (1971) demonstrated that, in vitro, acetylcholine in the presence
of calcium accelerated the release of norepinephrine from intact hypothalamic cells. In a similar preparation, Philippu and Przuntek (1967) demonstrated that acetylcholine releases norepinephrine from the subcellular norepinephrine storing vesicles of the hypothalamus. Philippu (1970) further demonstrated that in cat hypothalamus labelled with 14C-norepinephrine, the radioactive norepinephrine was found to be taken up by the synaptosomal fraction. Moreover, during perfusion of the third ventricle of cat, labelled norepinephrine was found to be spontaneously released from the hypothalamus into the perfusing fluid. Addition of acetylcholine in the presence of calcium enhanced the release of labelled norepinephrine as did electrical stimulation of the hypothalamic nuclei.

Van Meter (1969) and Van Meter and Karczmar (1971) studying central EEG responses in rabbits to anticholinesterase agents confirmed the finding of Longo and Silvestrini (1957) that EEG recruitment could be blocked with eserine, and demonstrated that the pretreatment with reserpine abolished the anti-recruitment effects of eserine and that the replenishment of the norepinephrine levels following DOPA administration restored blocking action of eserine on EEG recruitment. In addition, this eserine block could not be obtained following depletion of the norepinephrine levels by alpha-methyl-para-tyrosine, but with replenishment of the norepinephrine stores following administration of DOPA and a monoamine oxidase
inhibitor, eserine was again able to block the EEG recruitment. Similarly, Karczmar and Longo (1969) and Karczmar et al. (1970) observed in reserpinized rabbits that eserine produced EEG and behavioral syndrome characteristics of the paradoxical sleep phenomena. Neither eserine nor reserpine alone produced these effects. It was concluded that an interplay between eserine and reserpine existed and such interplay was necessary for the paradoxical sleep phenomena to occur in rabbits.

These data, although few in numbers, clearly point to possible existence of a cholinergic-adrenergic coupling phenomena within the central nervous system. Present methodology, i.e. fluorescence microscopy, microelectrophoretic drug application, and ion-exchange chromatography, provides more exacting results allowing for a clearer interpretation of the results. The lack of sufficiently refined experimental methodology seems to be a major hindrance of earlier studies. The concept of central cholinergic-adrenergic coupling is even more convincing if one reflects on the diffuse neural network we presently understand as comprising the central nervous system. Unlike the more well-defined Autonomic nerve network, the Central network, with its opposing cholinergic and adrenergic systems, could employ such a coupling phenomena as a neuromodulating mechanism designed to maintain homeostatic balance within the central nervous system.
D. ACTIONS OF PERTINENT DRUGS:

1-phenyl-3-hydrazinobutane (JB835)

structure:

1-phenyl-3-hydrazinobutane (identified in the text by the more common nomenclature - JB835), one of a series of compounds synthesized by John Biel, was found to be a potent inhibitor of monoamine oxidase, the enzyme responsible for degradation of dopamine and norepinephrine as well as of epinephrine and serotonin. Spector et al. (1959) demonstrated in vitro that JB516, also a monoamine oxidase inhibitor, and JB835 were equally potent and completely inhibited the activity of monoamine oxidase at a concentration of $1 \times 10^{-5}$ M within 30 minutes. Studies with JB835 indicated that a single dose (3mg/kg, iv) produced 95 percent inhibition of monoamine oxidase in rabbit brain homogenates within 30 minutes, the inhibition lasting for 48 hours, subsequently, a 75 percent inhibition lasted for the next 48 hours. The subcutaneous route required longer periods for the peak effect to occur. Serotonin was observed to rise by 50 percent within 15 minutes while accumulation
for one-half turn-over of the brain pool of serotonin is about 15 minutes, while that of norepinephrine is several hours. The observed rapid rise in brain serotonin following JB835 suggests that it readily penetrates the brain, as does JB516. Similar observations were made by Brodie et al. (1960).

Although JB516, the hydrazine analogue of amphetamine, in doses of 10mg/kg,iv, or higher produced immediate amphetamine-like central excitation and mydriasis, JB835 in doses up to 40mg/kg,iv produced no such effects.

These results demonstrate JB835's ability to inhibit monoamine oxidase in brain in rabbits and to increase brain amine levels without producing central excitation typical of many monoamine oxidase inhibitors, i.e. JB516.

3,4-DL-Dihydroxyphenylalanine (DOPA)

Structure:
DOPA is synthesized in vivo within chromaffin cells and certain neurons by the hydroxylation of tyrosine (Nagatsu et al., 1964; Wurtman, 1966). DOPA is not readily abundant in blood or tissues in vivo (Anton and Sayre, 1964), suggesting that it is rapidly metabolized probably by decarboxylation. When injected, however, DOPA is capable of penetrating into the brain from the blood (Carlsson et al., 1958). Wurtman et al. (1970) injected small doses (2.5 mg/kg) of 14C labelled DL DOPA intraperitoneally into mice. It was found that the level of 14C-catecholamines in the whole carcass reached a peak within 20 minutes and that O-methylation was the main catabolic path of the labelled DOPA in the periphery. Almost all of the 14C label was found with the dopamine fraction, a much smaller amount with the norepinephrine fraction, and essentially none incorporated into protein. Further, it was found that only 0.01 percent of the injected label was found in brain 20 minutes following the injection of the labelled DOPA as 14C DOPA and 14C dopamine, the remainder of the label recovered in the periphery. However, Bartholini and Pletscher (1968) and Persson and Waldeck (1968) demonstrated, using larger doses of DOPA, that norepinephrine levels as well as dopamine levels were elevated in the brain of rats and mice. Similar observations were made in rabbits following DOPA pretreatment by Glisson and Karczmar (1970).

Behaviorally, DOPA produces central excitation which is
increased by previous inhibition of monoamine oxidase (Carlsson et al., 1960) as well as stimulate the spontaneous motility (Carlsson et al., 1957).

Diisopropylfluorophosphate (DFP)

Structure:

DFP's mechanism of cholinesterase inhibition is that of phosphorylating and thereby inactivating "serine enzymes", i.e. acetylcholinesterase and butrylcholinesterase. Since acetylcholinesterase is found in erythrocytes, at parasympathetically innervated effector organs (smooth muscle; glands), at the skeletal muscle end-plates, at autonomic ganglia, and in the central nervous system, administration of the lipid soluble DFP inhibits at these sites the action of acetylcholinesterase, which is to hydrolyze the neurotransmitter, acetylcholine. When the cholinesterase activity is reduced by one-half, symptoms of accumulation of acetylcholine begin to appear, i.e. salivation, lacrimation, defecation, and urination. Further reduction of the cholinesterase activity to below 10 percent normal activity will eventually result in death, usually
due to respiratory failure, caused in part by neuromuscular paralysis of the intercostal muscles and diaphragm, and in part by central depression (Goldstein et al., 1969). DFP's ability to penetrate the central nervous system is due to its high lipid solubility which allows for easy entry into the brain. Regarding the inhibition of various cholinesterases, in low concentrations (10\(^{-7}\) - 10\(^{-8}\)) DFP preferentially inhibits butrylcholinesterase as does physostigmine at low doses. At high doses (10\(^{-6}\)), however, DFP readily inactivates acetylcholinesterase; a dose of 2.5 mg/kg being more than sufficient for acetylcholinesterase inhibition (Koppanyi and Karczmar, 1951). Grieg and Holland (1949) and Greig and Mayberry (1951) reported that the permeability of the blood brain barrier of the central nervous system is increased by cholinesterase inhibitors. Fuchsine, barbital, and choralose were shown to have increased entry into the brain following administration of cholinesterase inhibitors. Such evidence is relevant since DFP could alter the blood brain barrier allowing quaternary drugs entry into the brain. Care must be taken to assure that the effects of quaternary drugs in the presence of DFP represent only peripheral responses (Albanus, 1970; Wills, 1970).
Atropine sulfate (AtSO)

Structure:

The muscarinic blocking action of the tertiary atropinic agents is highly specific in as much as the nicotinic sites such as the ganglia and neuromuscular end-plates are not appreciably blocked, even by doses greater than those necessary to produce complete blockade of susceptible muscarinic receptors. The mechanism of action of the muscarinic blocking agents, i.e. atropine sulfate, atropine methyl nitrate, etc., involves a decrease in the response of normally sensitive tissues, i.e. peripheral vascular smooth muscle, gastrointestinal and genitourinary smooth muscle, constrictor and ciliary muscles of the eye, as well as, secretions from glands such as bronchial, salivary, and gastric, to acetylcholine. The decreased response may be due to impaired access of acetylcholine to the muscarinic receptors. The
block is competitive in type since very large doses of acetylcholine may "break through" and produce a muscarinic effect. The primary central nervous system effect at moderate doses is sedation, while at toxic doses excitation predominates (Sutherland, 1970). Further studies using dogs (Albanus, 1970) indicate that atropine (0.5-2.5mg/kg) produced a decrease in general locomotor activity and ataxia, marked tachycardia, depressed salivation, marked mydriasis; Albanus was also able to produce complete inhibition of the normal blood pressure response to acetylcholine. Of importance was that the centrally induced ataxia seen with atropine could also be observed with atropine methyl nitrate, the quaternary atropinic, but only using 100 times the dose of atropine. This is consistent with the observations that massive doses of quaternary compounds will eventually "break through" the blood brain barrier and enter the central nervous system.

Hingtgen and Aprison (1970) reported that high doses (0.8mg/kg) of atropine blocked behavioral excitation in rats, which they had previously shown to be correlated with central acetylcholine concentrations (Aprison et al., 1968). Comparable doses of atropine methyl nitrate had no effect. Polak and Meeuws (1966) demonstrated that atropine caused a release of acetylcholine from isolated cerebral cortex in rats, which Hingtgen and Aprison considered was in part responsible for the block of behavioral excitation observed by them.
following atropine.

In addition to the above effects, atropine is able to offer some amount of protection from organophosphorus poisoning. Johnson and Stewart (1970) reported that in rabbits, atropine (1.2mg/kg, iv) was able to reverse the respiratory arrest produced by sarin without affecting the changes in nerve-muscle function. Douglass and Matthews (1952) reported that of atropine's actions on anticholinesterase poisoning, the most important one was the central effect in protecting the medullary respiratory center from the depressant effect of anticholinesterases.

These results indicate that a dose of 2.0mg/kg of atropine is adequate to produce blockade of peripheral muscarinic sites, as well as exert blocking actions within the central nervous system upon the cholinceptive sites.

Atropine methyl nitrate (ATMN)

\[
\text{Structure:} \quad \begin{array}{c}
\text{H}_2\text{C} \\
\text{CH}_3 \\
\text{C} \\
\text{CH}_3 \\
\text{CH}_2 \\
\end{array}
\quad \begin{array}{c}
\text{CH}_2 \\
\text{O} \\
\text{CO} \\
\text{CH} \\
\text{H}_2\text{O} \text{H} \\
\end{array}
\quad \text{NO}_3^-
\]

The actions of atropine methyl nitrate in the periphery are identical to those described for atropine sulfate, although ATMN at the same concentration as atropine sulfate
is more potent in its ability to block peripheral muscarinic
sites than atropine, probably due to atropine's wider central
and peripheral distribution as opposed to only the peripheral
distribution for ATMN (Goldstein et al., 1969). The quaternary
structure of ATMN excludes it from easy access into the
central nervous system, although entry can occur using
high doses, i.e. 100 times atropine's dose (Albanus, 1970).

Although central protection is of greatest importance
against respiratory failure in organophosphorus poisoning,
Coleman et al. (1963) have reported some protection against
organophosphorus poisoning with ATMN alone or with an oxime,
suggesting that the peripheral sites contribute to the
toxicity.

The evidence indicate that a dose of 2.0mg/kg of ATMN is
sufficient for blockade of peripheral muscarinic sites but
this low dose is incapable of entry into the central nervous
system without alteration of the normal entry mechanisms
(Albanus, 1970; Wills, 1970).

Although recent evidence (Van Meter, 1969; Van Meter and
Karczmar, 1970; Philippu, 1970) indicate anticholinesterase
effects upon central norepinephrine levels, the effects of
anticholinesterases upon central dopamine levels are unknown.
With this background of information, the importance of an
investigation into the effects of anticholinesterases on
dopamine, as well as norepinephrine levels seemed indicated. As norepinephrine is formed by the hydroxylation of dopamine, it would be inconsistent to consider that norepinephrine was affected by anticholinesterases but not dopamine.

The initial purpose, therefore, was to determine whether the anticholinesterase, diisopropylphosphofluoridate (DFP), would produce a demonstrable effect upon the levels of dopamine and or norepinephrine in rabbit brain. Midbrain-diencephalon and the caudate nucleus were selected, for their high amine contents, as the areas to be examined.

Following these initial experiments, additional studies would be carried out to clarify any observed responses. It would be important to determine whether the effects upon norepinephrine and dopamine levels due to DFP were dose dependent. In addition, the ability of atropinic agents, both tertiary and quaternary, to block the DFP-induced effects should be investigated.

Upon completion of these experiments, it would be necessary to define more precisely the specific brain areas involved. Using localized brain area, i.e. midbrain, thalamus, hypothalamus, and hippocampus, the information obtained would indicate whether the responses to DFP, as well as the sites of atropinic blockade, were localized or widespread.
Finally, with regard to norepinephrine and dopamine, studies using intraventricular injections of DFP would provide information as to the central responses to DFP, which should closely mimic those responses seen following peripheral blockade by the quaternary atropinic, ATMN.

Since the effects upon norepinephrine and dopamine levels are postulated to be due to the elevated acetylcholine levels resulting from DFP's inhibition of cholinesterase activity; the inhibition of cholinesterase activity and the resulting elevation of acetylcholine levels should be demonstrated. Therefore, assay of the percentage inhibition of the cholinesterase activity produced by DFP, as well as bioassay of the levels of acetylcholine will be carried out following the various drug pretreatments.

These experiments will provide pertinent information as to the neurochemical changes associated with the cholinergic-adrenergic coupling phenomena.
II. MATERIALS AND METHODS

Materials:

New Zealand albino rabbits were chosen as the biological material because of their readily availability, their ease of handling, and the availability of pertinent data obtained both in this laboratory and elsewhere. Only male animals were used in these experiments to avoid possible estrus effects on the results. In some of the experiments two brain areas were used, the midbrain-diencephalon and the caudate nucleus. The caudate nucleus in rabbit is of sufficient size and amine content to allow individual determinations. In other experiments four individual brain areas were used: the thalamus, the hypothalamus, the midbrain, and the hippocampus. In these latter experiments tissue samples were pooled from two rabbits to obtain a more accurate determination of the amine levels in these tissues. The weight of the rabbits was 2.5 + 0.5 kilograms. All animals were housed in individual rabbit cages located within the animal facilities. The room temperature was maintained at 74 degrees Fahrenheit and food and water was provided to the animals ad lib.

Drugs and dosage:
1-phenyl-3-hydrazinobutane (JB835) was administered at a constant dose of 6.0mg/kg whenever used alone or as part of a drug regime. The drug was dissolved in physiological saline and administered via the intraperitoneal route.

3,4-DL Dihydroxyphenylalanine (DOPA) was administered at a constant dose of 50mg/kg when used either alone or as part of a drug regime. The drug, as the hydrochloride, was heated in physiological saline until dissolved, allowed to cool, and administered via the intraperitoneal route. Although the DL form of this drug was used as it is readily available in this form it has been demonstrated that only the L form is capable of conversion to dopamine by DOPA decarboxylase (Cooper et al., 1970). The functional dosage would therefore be 25mg/kg.

Diisopropylfluorophosphate (DFP) was administered in three different dosages in the various experiments. Although in one experimental series 1.0mg/kg was used to demonstrate the dose effect relationship, the 2.5mg/kg dosage was the primary dose used. The DFP for these two dosages was prepared in peanut oil at a stock solution concentration of 1 percent. Both the 1.0 and 2.5mg/kg dosages were administered in the neck region via the sub-cutaneous route. LD 50 doses were determined on mice with each new DFP preparation to ensure constant potency from preparation to preparation. The third dose of DFP used was 200μg. This dosage was prepared in
physiological saline and injected via the intraventricular route within 15 minutes from preparation. The concentration of the stock solution was 2.0mg/ml from which a 100µl volume (200µg) was used for the injection into the ventricle. DFP prepared in saline was found to have a pH of 3.61. Injection of such a low pH solution into the ventricle is definitely not physiological. However, due to the rapid decomposition of DFP at the more basic pH's, no attempt was made to adjust the pH of the DFP solution to that of cerebral spinal fluid contained in the ventricle. Feldberg (1958) in his studies of intraventricular injection of DFP into cats, was made no mention of any pH correction. It was felt, however, that the effects, if any, due to the low pH should be controlled. Therefore, in the sham control rabbits receiving physiological saline in place of DFP, the pH of the saline was adjusted to that of the DFP solution, 3.61.

Atropine sulfate (AtSO) was used at a dosage of 2.0mg/kg or 4.0mg/kg when used as part of a drug regime. The drug was dissolved in physiological saline and administered via the intraperitoneal route.

Atropine methyl nitrate (ATMN) was used at a constant dosage of 2.0mg/kg when used as a part of a drug regime. The drug was dissolved in physiological saline and administered via the intraperitoneal route.
**Sequence of drug administration:**

Eight major drug administration sequences were used in these experiments; their sequences and their relationship to the sacrifice time can be described as follows. (1) Animals were sacrificed two hours after receiving either JB835 alone, DOPA, or ATMN alone. (2) Animals were sacrificed one hour following DFP given alone at the dose of either 1.0 or 2.5 mg/kg. (3) Animals receiving JB835 and DOPA were given JB835 followed after 1 hour by DOPA and sacrificed 2 hours following DOPA (3 hours after JB835). (4) Animals receiving either JB835-, DOPA-, or ATMN with DFP were given DFP one hour following the administration of one of the other members of the combination and sacrificed 1 hour after the administration of DFP (2 hours after JB835, DOPA, or ATMN). (5) Animals receiving the JB835-DOPA-DFP combination were given JB835 first followed at 1 hour intervals by DOPA and by DFP; they were sacrificed 1 hour later (3 hours after JB835). (6) Animals receiving JB835-DOPA-DFP combination with either AtSO or ATMN were given JB835 followed at 1 hour intervals by DOPA and either AtSO or ATMN; DFP was administered 15 minutes later and the animals were sacrificed 1 hour later (3 hours 15 minutes after JB835). (7) Animals receiving JB835-AtSO-DFP combination were given JB835 followed after 1 hour by AtSO and after 15 minutes by DFP; they were sacrificed 1 hour later (2 hours 15 minutes after JB835). (8) The last
group of animals were given JB835 followed at hourly intervals by DOPA and by DFP 1.0mg/kg; they were sacrificed at intervals of 1 hour, 2 hours, and 3 hours after DFP.

All of these dose cycles were adjusted so that animals were always sacrificed at 2 P.M. ± 30 minutes to avoid possible circadian effects on the results. In the case of the animals receiving AtSO or ATMN, the slightly longer dose cycle was found to be without effect on the amine levels.

Isolation and removal of tissue samples:

Following decapitation the caudate nucleus and midbrain-diencephalon were isolated and removed according to the following procedure. First, the left and right cerebral hemispheres were separated and cut was made through the left and right cerebral peduncles, liberating the left and right hemispheres. By carefully teasing the hippocampus back, the entire caudate nucleus from head to tail was exposed. Using a pair of toothed iris forceps, the caudate nucleus was teased away from the ventricular wall.

The midbrain-diencephalon region is then easily obtained by transecting the lower brain stem on a line from the caudal end of the inferior colliculus to the rostral end of the pons, thereby isolating the midbrain-diencephalon from the rest of the brain stem. Following removal of this brain part, any vascular or connective tissue still connected
to the parts was removed and the parts rinsed free of blood with distilled water.

For those studies in which individual brain parts were used, their removal was as follows: **Hippocampus:** the hippocampus was rolled back and teased away from the neocortex after the hemispheres have been removed from the rest of the brain. **Midbrain:** the midbrain-diencephalon section was placed on a glass plate and a sagittal cut was made down the center of the tissue. A transverse cut was then made along the caudal border of the thalamus, separating the midbrain from the diencephalon. **Thalamus and hypothalamus:** the diencephalon was placed with the exposed inner surface dorsally positioned on the glass plate. The border between the thalamus and hypothalamus was found and a transverse section made separating the dorsal thalamus from the more ventral hypothalamus. These dissections were easily made and identical areas were always removed from the various brains sampled.

**Catecholamine determinations:**

The method for the determination of norepinephrine and dopamine from a single tissue was described in part by Carlsson and Waldeck (1958); Bertler et al. (1958); and Carlsson and Lindqvist (1962). This method utilizes the principles of ion-exchange column chromatography in the purification and separation of norepinephrine and dopamine. Modifications of the original method were made for use in
these studies. The procedures used are as follows.

**Extraction of amines:**

Immediately following removal, each tissue was weighed and placed in a 15 ml ground glass hand homogenizer containing 8.0 mls. of 0.4 N. perchloric acid. The homogenizer and acid were prechilled and kept in an ice bath at all times to minimize destruction of the amines by temperature. Next, the tissue was thoroughly homogenized and the homogenate poured into a 12 ml. thick-walled conical centrifuge tube, also in an ice bath. An additional 3.0 mls. of the perchloric acid were then added to the homogenizing vessel as a wash. After moving the pestle up and down the vessel several times, the wash volume was poured into the homogenate.

At this time recovery standards are prepared. 4.0 mls. of a homogenate made from brain tissue and perchloric acid were added to each of two 12 ml. centrifuge tubes. One tube contained the standards and the other served as its tissue blank. To the standard tube, 3.0 mls. of the perchloric acid was added. Next, one ml. of norepinephrine standard at a concentration of 1 µg/ml. was added to the standard tube, as was one ml. of dopamine standard of the identical concentration. To the tissue blank tube, 5.0 mls. of the perchloric acid was added. These two tubes, one containing the homogenate plus standards and the other the homogenate alone, were treated
identically with the regular brain samples.

The ice-cold centrifuge tubes were next centrifuged for 15 minutes at 3800 × g. Following centrifugation, the supernatant was filtered using Watman #1 filter paper into a 15 ml. graduated conical tube kept in an ice bath. After 30 minutes, the volume of filtered extract was measured and recorded. The tubes were then sealed and placed overnight in a refrigerator.

**Column preparation:**

The columns to be packed with ion-exchange resin have been adapted from 1.0 ml. disposable syringes. It is necessary that the internal diameter of the syringe barrel be 4.2-4.5 mm. The upper lip of the syringe was cut off and a 2 cm. piece of tygon tubing affixed to the top of the syringe barrel in such a way that one centimeter of tubing extended above the top of the barrel. This "column assembly" was then submerged in a beaker containing distilled water. It is necessary that the actual packing of the column with resin be done under airtight conditions, as air bubbles will disrupt the uniform packing of the resin. Air bubbles were removed from the empty column by inserting a stirring rod inside the column and working it up and down a few times. With the column completely filled with water, a small ball of Pyrex glass wool was inserted into the column and forced to the tip with a stirring rod. This piece of glass wool serves to trap the resin within the
column. A two milliliter pipet is then filled with AG 50W x 8 200-400 mesh H+ form ion-exchange resin which has been suspended in 0.01 M phosphate buffer plus 1 percent EDTA for at least 24 hours. The tip of the filled pipet was inserted under water into the tygon tubing attached to the top of the column. The resin was then allowed to flow into the column. After the resin bed has settled and a height of 50 mm. of resin attained, another ball of Pyrex glass wool was inserted into the top of the column, the resin bed now being contained between the two glass wool retainers. The freshly packed columns were then mounted onto the "column board". The "column board" consisted of a 20 ml. graduated glass syringe mounted firmly through a horizontal board which is secured to ring stands. A 3-way plastic stopcock was attached to the tip of this syringe. To the left of this syringe, a 20 ml. plastic syringe barrel was mounted in similar fashion as the glass syringe. This plastic syringe barrel served as a reservoir through which the extract and other reagents can be added to the column. The reservoir and glass syringe are connected through a series of plastic stopcocks in a way that liquids can be drawn into the glass syringe from the reservoir. On the open end of the stopcock attached to the glass syringe, a plastic fitting was placed. This fitting allows for an air-tight seal to be made between the tygon tubing on the column and the stopcock on the glass syringe.
This fitting was made by removing the metal insert of a disposable needle and using as the fitting, the upper plastic portion.

Prior to attachment of the freshly packed resin column, distilled water was drawn through the stopcock into the glass syringe to remove any air from the stopcock. With the distilled water slowly dripping through the stopcock, the resin column was fitted tightly onto the stopcock fitting. The column was now mounted in such a way as to allow for liquids to be drawn into the glass syringe from the reservoir and then to flow through the resin column. The resin was next made ready for addition of the tissue extracts by cycling the resin from the H+ form to the Na+ form. The cycling was accomplished by passing 20 mls. of 0.01 M sodium phosphate buffer plus 1 percent EDTA at pH 6.5 through the resin at a flow rate of one drop every 10 seconds. After the buffer has passed through the column the pH of the effluent was checked. A pH of 6.5 indicated that the column was cycled and ready to receive the tissue extract.

Before adding the extract to the column, the pH was adjusted to 6.5 using 5 N. potassium carbonate. A brom phenol blue pH indicator (pH 6.0) was added to the extract to speed up the titration, although final pH was determined using short range pH paper. At pH 6.5, potassium perchlorate precipitates out of the neutralized extract. Storing for 10 minutes in the
refrigerator increased the precipitation rate. Following the cooling, the extract was centrifuged at slow speed for 3 minutes to allow the precipitate to settle. The extract was then poured into the reservoir on the "column board", care being taken not to allow the precipitate to enter the reservoir. The extract was drawn into the glass syringe and allowed to flow through the resin at a rate of one drop every 10 seconds.

After the extract has passed through the column, the resin was washed with two 20 ml. volumes of distilled water. The flow rate for these two washed can proceed at a faster rate than the extract.

Following the washes, the reservoirs were filled with 1 N. hydrochloric acid. During the elution of the norepinephrine and dopamine fractions, the flow rate for the hydrochloric acid was maintained at one drop every 10 seconds. The first 3 mls. of acid were passed through the column and discarded. The next 8 and 13 mls. of acid eluted the norepinephrine and dopamine fractions, respectively. Following collection of the 8 ml. norepinephrine fraction the flasks were sealed and stored in the refrigerator. Flasks containing the dopamine fractions were treated similarly.

Conversion of norepinephrine and dopamine to fluorophores:

It was necessary to convert both norepinephrine and dopamine to their respective fluorophores before their respective
concentrations could be determined using a spectrophotofluorometer. Separate conversion procedures were used for norepinephrine and dopamine, adding another degree of specificity to the method. The chemical conversion of norepinephrine to norepinephrinachrome to norepinephrinalutine (Bertler et al., 1958), the actual fluorophore, was carried out as follows.

<table>
<thead>
<tr>
<th></th>
<th>Std.</th>
<th>RB</th>
<th>S</th>
<th>IS</th>
<th>Tbf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>3.80</td>
<td>3.90</td>
<td>2.90</td>
<td>2.80</td>
<td>2.90</td>
</tr>
<tr>
<td>0.01 M NaPO_4</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>pH 6.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutralized eluate</td>
<td></td>
<td></td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>pH 6.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard NE (1µg/ml)</td>
<td>0.10</td>
<td></td>
<td></td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>0.5% Zinc sulfate</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>0.25% K Fe(CN)_3</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
</tbody>
</table>

2% ascorbic acid +

5N. NaOH

5N. NaOH

2% ascorbic acid

Upon completion of the above chemical procedure, the samples were poured into reading tubes and read in an AMINCO spectro-
photofluorometer at 400/505 nm uncorrected excitation/emission wavelengths. The three tubes: sample (S), internal standard (IS), and tissue blank faded (TBf) comprised a set and were used for each sample eluate. Potassium ferricyanide was used as the oxidant in this conversion procedure because of its inability to convert dopamine to its respective fluorophore. Such specificity added to the elimination of elution cross-over contamination, if any. The fluorophore was found to be stable for up to one hour.

Dopamine is similarly converted to dopaminachrome to dopaminalutine, the actual fluorophore (Carlsson and Waldeck, 1958). The conversion procedure for dopamine is as follows.

<table>
<thead>
<tr>
<th>Std.</th>
<th>RB</th>
<th>S</th>
<th>IS</th>
<th>TBu (mls.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>3.05</td>
<td>3.30</td>
<td>1.30</td>
<td>1.05</td>
</tr>
<tr>
<td>Citrate-PO buffer</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>4 pH 5.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard DA</td>
<td>0.25</td>
<td>----</td>
<td>----</td>
<td>0.25</td>
</tr>
<tr>
<td>(1µg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unneutralized</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>eluate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutralized eluate</td>
<td>----</td>
<td>2.0</td>
<td>2.0</td>
<td>----</td>
</tr>
<tr>
<td>pH 5.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na SO\textsubscript{2} - NaOH soln.</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.02 N. Iodine</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
</tbody>
</table>
After cooling the samples, they were poured into reading tubes and read in the fluorometer at 330/380 μm uncorrected excitation/emission wavelengths. Although the dopamine fluorophore is reported to be stable for 24 hours, all samples were read in the fluorometer within one hour. The boiling water bath is used to increase the intensity of the dopamine fluorophore in a shorter period than if it were left standing at room temperature.

Due to inherent variations from sample to sample the rate of oxidation from one sample to the next varied when compared to the oxidation rate of the standard which does not contain tissue extract. Since the standard is used as the reference in determining the amount of amine present in each sample, it is critical that the rate of oxidation of the standard
and each tissue sample are identical. Therefore, this method employs the use of an internal standard for the sole purpose of checking the sample rate of oxidation against that of the standard; the sample values were always corrected so that the standard and the sample had equal oxidation rates.

The formula for calculating the μg/gm concentrations of the amines is as follows:

\[
\frac{1}{5} \cdot \frac{2}{6} \cdot \frac{3}{7} \cdot \frac{4}{8} = \text{μg/gm wet weight tissue.}
\]

1. Net (sample - blank) sample fluorometer units corrected for 100% oxidation rate.
2. Standard concentration (μg).
3. Total eluate volume (ml) plus K CO volume (ml) used to neutralize the eluate.
4. Total volume of PCA (ml) used to homogenize tissue plus tissue water volume (ml) (75% of the tissue weight).
6. Volume (ml) of the eluate used in the sample.
7. Volume of the extract (ml) recovered after filtration.
8. Tissue weight (gm).

Additional reference may be found in the literature cited.

Using the fluorometer values obtained from the tissue standard samples prepared with the brain samples, the percent recovery of both norepinephrine and dopamine can be calculated.
Average recoveries for norepinephrine were found to vary between 70-80 percent, and those for dopamine between 50-60 percent. These recoveries are consistent with those reported in the literature for this method. Brain sample μg/gm concentrations were always corrected for 100 percent recovery based on the standard recoveries obtained with each series of brain samples.

Intraventricular studies:

The first series of intraventricular injections of DFP were performed on rabbits who had been anesthesized with pentobarbital (30mg/kg, iv). The second series of intraventricular injections of DFP were performed on unanesthesized rabbits, which were given 0.50 mls. of lidocaine (2.0mg/ml) locally into the scalp. With the exception of the anesthesia, both groups of rabbits were treated identically. With the animals held in place by hand, the hair was clipped from the top of the head. In the case of the second group of rabbits, lidocaine was now injected into the scalp. A longitudinal incision was made down the center of the scalp. Using a scalpel blade, the skull was cleared of muscle and connective tissue on the left side of the midline suture near the coronal suture. Following removal of the surface tissue, bone wax was applied to the skull to stop any bleeding from the bone itself. Next, using predetermined coordinates, a point
was found which was 4.0 mm. caudal to the coronal suture and 4.5 mm. lateral to the midline suture. At this point a small hole was drilled using an electric Dremel Moto drill containing a 1/32 inch drill bit. Care was made to penetrate only the bone. Bone wax was then applied to the opening. The rabbits were replaced in their cages for a one hour period; allowing them to settle down before beginning the drug regime. The actual surgical treatment did not produce any observable signs of stress; the unanesthesized and the anesthesized rabbits showed similar behavior following the recovery of the latter from anesthesia.

After one hour had elapsed, the rabbits were given the first in a series of drug treatments. There were three drug combinations used: JB835-DOPA-Saline; JB835-DOPA-DFP; and JB835-DOPA-ATMN-DFP. The doses for JB835, DOPA, and ATMN, as well as the administration sequences for all drugs including DFP were described earlier. The dose of DFP used in these studies was 200µg contained in a constant volume of 100µl. The preparation of the DFP and saline used in these experiments was described earlier. A 100µg dose of DFP in a 100µl volume were previously used by Feldberg (1958) in intraventricular injections. In preliminary trials it was found in these laboratories that the rabbits tolerated 100µg and 200µg of DFP equally well via the intraventricular route,
the dose volume being constant. It was decided on this basis and on that of preliminary neurochemical findings that 200µg of DFP in a dose volume of 100µl would be used in the actual experiments. In the case of the sham operated control rabbits receiving JB835-DOPA-Saline, the volume of 100µl was injected intraventricularly.

At the proper time in the administration cycle the intraventricular injection of either DFP or saline was carried out, the rabbit again held securely in place. Using a 250µl glass syringe and a 27 gauge stainless steel needle, which had been prepared to a length of 6.0mm., the 100µl volume was loaded into the syringe. Maintaining a vertical plane to the skull, the needle was lowered through the wax-filled hole. As soon as the needle was fully inserted (up to the flared portion of the needle acting as a stop) the dose volume was slowly injected into the lateral ventricle, the needle withdrawn, and the hole immediately refilled with bone wax. The animals were then replaced in their cages for one hour and sacrificed.

Preliminary trials using various coordinates and injection depths were performed to insure that the described procedure did place the dose volume within the left lateral ventricle. Using Brom Phenol Blue, which stains the tissues it comes in contact with, 100-500µl volumes were injected and the brains removed and dissected, the location of the staining was then
determined. The 4.0mm. X 4.5mm. coordinates were found to place the needle tip within the desired left lateral ventricle as indicated by the complete staining of the walls of the left lateral ventricle, as well as of the walls of the aqueduct and third ventricle walls using the 100µl volume of the dye. The 500µl dye volume stained the walls of all ventricles and the aqueducts associated with them. Repeated trials were performed and each time the dye was observed only on the walls of the ventricular system. Other coordinates do appear in the literature for intraventricular injections. A variety of coordinates could be used since the left lateral ventricle runs parallel to the midline suture; however, in these studies the 4.0mm. X 4.5mm. coordinates were found preferable. As an additional precaution, while dissecting the brain parts out, visual location of the entry point of the needle was evaluated to verify entry into the ventricle, the surrounding tissues were inspected as well to be sure that the needle did not enter one of these tissues. Injection into the left lateral ventricle was visually confirmed in all animals receiving an intraventricular injection.

Some comment should be made regarding the symptomatology of the rabbits following intraventricular injection of DFP. Due to the pretreatment of JB835-DOPA, the animals exhibited piloerection, hyperreflexia, and mydriasis. Following intraventricular administration of DFP, the animals began to exhibit
intermittent seizures beginning after about 5 minutes. These seizures consisted of leg jerking, rolling over, and spinning in a clockwise or counterclockwise direction which varied from rabbit to rabbit. This seizure period lasted for from 5-10 minutes after which the rabbits assumed a fixed position; some animals rocked back and forth while in this position. During the remaining time the rabbits exhibited a chewing jaw motion; one actually bit its tongue enough to cause bleeding. In addition, the rabbits seemed to have a lowered threshold to audiogenic seizures; a loud sound caused sudden short bursts of leg jerking. Most of the rabbits salivated during the latter half of the post-drug period. DFP did not produce miosis in any of the animals when given via this route. Piloerection disappeared and the hair remained flat. Skeletal muscle fasciculations were not observed. Feldberg (1958) reported similar behavioral symptoms in cats given DFP intraventricularly.

These symptoms differed in many respects to those seen following DFP via the subcutaneous route. When DFP was given subcutaneously, miosis always occurred (except when atropine treatment was employed), there was no apparent change in audiogenic seizure threshold, skeletal muscle fasciculations were observed, the rabbits did not assume a fixed position, did not rock back and forth or spin in either clockwise or counterclockwise direction. Sham-operated control rabbits
receiving 100µl of saline at pH 3.61 did not exhibit any of the symptoms seen following DFP in either anesthetized or unanesthetized rabbits; in fact their behavior was identical to that of rabbits receiving JB835-DOPA, but not treated surgically.

**Cholinesterase activity assay:**

Cholinesterase activity was measured in control rabbits (no drugs), in rabbits treated with JB835-DOPA; JB835-DOPA-DFP; and JB835-DOPA-AtSO -DFP combinations. The doses and drug administration sequences were already described. Cholinesterase activity was measured in midbrain, thalamus, hypothalamus, and caudate nucleus; two separate samples of each part were used in each drug group. The method used for measurement of the cholinesterase activity was previously described by Ellman et al. (1961). The procedure was as follows. The animals were pretreated with the above mentioned drug combinations or given no drugs in the case of the control rabbits; they were sacrificed and the brain areas removed as described earlier. After weighing the individual brain parts, they were homogenized in ice-cold 0.10 M phosphate buffer, pH 8.0. All tissues were homogenized to a final weight/volume concentration of 20mg/ml. The 20mg/ml. homogenates were kept in an ice bath until ready for the cholinesterase assay. In pretrial experiments it was found that the 400µl homo-
The homogenate volume described by Ellman et al. was not satisfactory for the control rabbit assays, as the cholinesterase activity in control rabbit brain parts was too high for an accurate measurement when the 400µl volume was used. 200µl's of homogenate was found to be acceptable and was used in these assays. To control for color formation due to other than cholinesterase materials, an eserinized blank was run with the tissue sample, both containing the same volume of homogenate. The 3 X 10⁻⁵ M dose of eserine was reported to inhibit 100 percent of the acetylcholinesterase activity while only inhibiting 25 percent of the butrylcholinesterase activity, thus after subtraction of the blank activity from the sample activity, the cholinesterase activity remaining would represent primarily only that due to acetylcholinesterase. During each enzymatic assay, a DFP-treated brain part and its respective blank were run together with an identical non-DFP treated brain part. Thus, not all DFP brain samples were run together, a system that could induce error. Over the course of the enzymatic study, brain parts from animals treated with JB835-DOPA-DFP and JB835-DOPA-AtSO -DFP drug combinations were run against identical brain parts from both control (no drug) animals, as well as JB835-DOPA treated animals.

The sequence of events for each enzymatic measurement were as follows. The Beckman DU spectrophotometer absorbancy wave-
length was set for 412 mµ as described by Ellman et al. After an adequate warm up period (1 hour), the sensitivity and dark current was adjusted such that the null indicator read zero. A standard quartz photocell containing 0.10 M phosphate buffer pH 8.0 was used to make these adjustments. Next, the photocells were removed and 200µl of homogenate was added to the sample and to blank photocells. To the blank photocell 100µl of eserine was added producing a final volume concentration of \(3 \times 10^{-5}\) M, which inhibited all of the acetylcholinesterase activity and 25 percent of the butrylcholinesterase activity. To each photocell 100µl of DTNB (Di-thio-bis nitrobenzoic acid) was added and the reagents in the cell mixed by blowing air bubbles through the cell. The photocells were replaced in the cell housing of the photometer. The increasing absorbance, owing to DTNB's reaction with non-specific cholinesterases, i.e. thiolesterases, and protein, was monitored until a peak absorbance was reached. Using the slit adjustment knob, the null needle was returned to zero. The photocells were again removed from the cell-housing and 200µl of acetylthiocholine substrate was added to each cell and mixed with air bubbles. A 10 second interval was maintained between the cell sequence so that a proper timing sequence could be maintained during subsequent absorbancy measurements. The photocells were quickly replaced in the photometer and absorbancy measurements.
of each cell made at 1, 2, 4, and 6 minute intervals. When all samples had been run for that day (3 hours after the first measurement), the first sample was rerun to check for loss of enzyme activity. No loss of enzyme activity was observed over the testing period.

It was found that the caudate nucleus tissue samples from control and JB835-DOPA treated rabbits had, at six minutes, surpassed the maximum absorbancy measurable. To stay within the linear portion of the absorbancy curve, the four minute absorbancy value, minus the respective blank value, was used to calculate the mean change in absorbance/minute for all samples. From this value the average change in absorbance/minute was determined for each pair of brain parts within each drug group.

The JB835-DOPA treated rabbits served as the sham control group and the mean acetylcholinesterase activity for each part within this group was considered to represent 100 percent cholinesterase activity with respect to the two DFP treated groups. The rate of hydrolysis (moles of substrate hydrolyzed/minute/gram of tissue) of acetylcholinesterase was calculated using the formula described by Ellman et al. (1961).

**Acetylcholine bioassay:**

Acetylcholine levels of rabbit brain thalamus, hypothalamus, midbrain, and caudate nucleus were measured using bioassay
techniques on guinea pig ileum. Animals were pretreated with JB835-DOPA; JB835-DOPA-DFP; JB835-DOPA-AtSO -DFP; JB835-DOPA-4 ATMN-DFP drug combinations; control animals (no drugs) were also used. The doses and administration sequences have been described earlier. The bioassay procedures used were based on methods described by Magnus (1904); Turner (1965); and Livingston (1968) with modifications by Kindel (1971). Following drug pretreatment, the animals were sacrificed and the brain parts were removed as described earlier. Once removed the individual brain parts were quick-frozen in liquid nitrogen. To prechilled homogenizing vessels, 4 mls. of eserinized (20µg/ml) bicarbonate-free Locke Ringer solution with 1/15 M sodium phosphate monobasic buffer pH 6.68-7.30 are added. The ratio of bicarbonate-free Locke Ringer and 1/15 M sodium phosphate monobasic buffer was 3.333/1.000. To the 4 mls. of buffer solution, 0.5 mls. of 0.333 N. hydrochloric acid was added and the two solutions mixed. The first brain part was removed from the liquid nitrogen, weighed, and placed into the homogenizing vessel. The tissue was homogenized using a power driven pestle for 2-3 minutes keeping the homogenizing tube in an ice bath during the entire procedure. After homogenization, the homogenate was placed in a boiling water bath for 10 minutes. Upon removing the vessel from the water bath, the homogenate was mixed using a Vortex mixer
so that any tissue residue on the upper walls of the vessel was mixed in with the homogenate. The homogenate was then allowed to cool. Once cooled, the homogenate was poured into a thick-walled centrifuge tube. 1.0 ml. of cold distilled water was then added to the homogenizing vessel as a wash. After swirling in the vessel, the wash was added to the homogenate in the centrifuge tube. The homogenate was then centrifuged for 15 minutes at 3800 X g. Following centrifugation, the supernatant was poured into another centrifuge tube and the tissue pellet re-extracted with 1.0 ml. of the bicarbonate-free Locke Ringer solution without eserine. The re-extracted homogenate was centrifuged for 15 minutes at 3800 X g. The supernatants from both the first extraction and the re-extraction were pooled and neutralized to pH 6.8-7.0 with 0.333 N. sodium hydroxide. The neutralized extract was then centrifuged at 3800 X g. The supernatant was poured into a centrifuge tube containing alpha chymotrypsin (400μg/ml of extract). The extract and chymotrypsin were mixed, the centrifuge tube stoppered, and the extract incubated for 10 minutes at 38 degrees Centigrade in a dry incubator. After the incubation, the volume of the extract was measured and recorded. 2.5 mls. of the extract was removed and added to another centrifuge tube to be used later with acetylcholine standard. The sample extract was then stoppered and frozen.
until bioassayed.

0.40 mls. of 0.333 N. sodium hydroxide was then added to the 2.5 mls. of extract isolated for use with acetylcholine. The two solutions were mixed and the extract incubated in a boiling water bath for 10 minutes. This procedure destroys any acetylcholine present. After boiling, the extract was cooled and centrifuged for 15 minutes at 3800 X g. The supernatant was poured into a centrifuge tube and neutralized with 0.333 N. hydrochloric acid to pH 6.8-7.0. The neutralized extract was stoppered and frozen for later use.

Simply stated, the extraction steps include: (1) homogenization of the tissue and acid extraction of the acetylcholine. (2) heating of the homogenate to destroy all cholinesterase present in the tissue. (3) centrifugation and re-extraction of the tissue pellet. (4) neutralization of the extract. (5) addition of chymotrypsin to the extract which destroys substance "P", aided by incubation. (6) bioassay of the acetylcholine present in the tissue extract on guinea pig ileum.

Bioassay:

Tyrode solution was prepared with 1µg/ml. of morphine, 0.2µg/ml. of pyrilamine, and 0.03µg/ml. of dibenzyline. Morphine was used to decrease spontaneous activity of the ileum; pyrilamine and dibenzyline blocked any histamine and serotonin, respectively, still present in the extract.
The guinea pig was sacrificed by a blow on the head. The ileum was isolated, removed, and flushed with Tyrode solution. A piece of the ileum was then mounted in the 5 ml. organ bath aerated with 5 percent CO₂ and 95 percent O₂. A 100µg dose of acetylcholine was used to test the sensitivity of the preparation. If sensitive, the preparation was allowed to stand for one hour to allow its accommodation to the bath.

Using acetylcholine, a dose producing a maximal response of the ileum was found as well as a dose producing 75 percent of maximal response. Repeated doses of the latter dose were given to assure a constant response. Using the tissue extract, which has been allowed to thaw, the volumes necessary to induce a high and low effect were found; the high "dose" was capable of double the response seen with the low "dose". With these two extract responses established, doses of authentic acetylcholine were found which mimicked the two responses produced by the tissue extract. These two doses of authentic acetylcholine were used repeatedly to assure constant ileum responses. An amount of acetylcholine is then added to the 2.5 mls. of extract which had been put aside for the acetylcholine standard, the high and low "dose" volumes from this standard-extract preparation capable of inducing approximately the high and low "dose" (volume) responses produced by the tissue extract we've selected. After establishing that the responses to the acetylcholine-extract standard and the tissue extract
were constant, a latin square was completed. From the latin square data, acetylcholine levels in the various brain parts were calculated and expressed as \( \mu g/gm \) (Livingston, 1968).

Upon completion of the latin square, serotonin and histamine were added to the bath to assure that responses to both were still blocked by the blocking agents added to the Tyrode solution.

Statistics:

The statistical analysis of the data presented in this dissertation was carried out by means of the unpaired Student "t" test, the samplings coming from two groups of rabbits. The degrees of freedom used in the establishment of the probability \( (P) \) values were \( n-1 \) for each group with the total degrees of freedom being \( n-2 \). Statistical significance of the data was accepted when the "P" value was 0.05 or less. In some cases where the sample size was small and there was a drug-induced shift in the mean value, "P" values of 0.10-0.05 were shown. Although this value does not indicate statistical significance as per standards established for statistical analysis, it was felt that a clear trend was indicated in the case of the data in question; had large sample sizes been used, statistical significance would have been proven.
RESULTS

The effects of JB835 and DOPA on norepinephrine and dopamine levels.

As expected, it was found that either JB835 or DOPA increased the levels of norepinephrine over their control concentrations in both caudate nucleus and in the midbrain-diencephalon; the dopamine levels were increased at least in the case of the former (Table I). In the case of the caudate nucleus, JB835 increased the levels of norepinephrine and dopamine by 52 and 19 percent respectively. Similar increases in norepinephrine occurred in the case of the midbrain-diencephalon, JB835 inducing a 52 percent increase and DOPA- a 16 percent increase. However, the concentration of midbrain-diencephalon dopamine was not increased above control levels by either JB835 or DOPA (Table II).

Furthermore, DOPA-JB835 combinations induced even more pronounced increases in the levels of norepinephrine in both the caudate nucleus and in the midbrain-diencephalon area (by 55 and 270 percent, respectively). Also, this dose combination induced an increase of dopamine levels in the case of the caudate nucleus which was significantly greater than that induced by JB835 or DOPA alone (Table I; Figure I).
Moreover, while neither the monoamine inhibitor nor DOPA, employed alone, increased the level of dopamine in the case of the midbrain-diencephalon, their combination induced a significant increase of dopamine in this area (Table II; Figure 2).

The effect of DFP on norepinephrine and on the dopamine levels in otherwise non-treated and pretreated animals.

With this background of information, DFP was employed at two dose levels, 1.0 and 2.5 mg/kg, in rabbits otherwise not treated, or pretreated with the DOPA-JB835 combination. It was of interest that DFP affected significantly dopamine and norepinephrine levels only in pretreated animals, i.e. in the rabbits which were "loaded", following the appropriate pharmacological maneuvers, with the catecholamines.

In animals which were not pretreated with DOPA and/or the monoamine oxidase inhibitor, neither dose of DFP induced a significant change in norepinephrine and dopamine, whether in the case of the caudate nucleus or of the midbrain-diencephalon region (Figures 3 and 4). Slight increases in norepinephrine were recorded with both doses of DFP in the case of the midbrain-diencephalon, and with the larger dose of DFP in that of the caudate nucleus; a relatively large but statistically not significant decrease in norepinephrine was found following 1.0 mg/kg dose of DFP in the case of the
TABLE I

Effects of DFP in the Caudate Nucleus on Norepinephrine (NE) and Dopamine (DA) Levels.

<table>
<thead>
<tr>
<th>Drug*</th>
<th>Mean NE ± S.E.</th>
<th>No.**</th>
<th>Mean DA ± S.E.</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPA</td>
<td>0.438 ± 0.032</td>
<td>4</td>
<td>8.913 ± 1.339</td>
<td>6</td>
</tr>
<tr>
<td>DOPA + DFP</td>
<td>0.322 ± 0.083</td>
<td>6</td>
<td>6.841 ± 0.938</td>
<td>6</td>
</tr>
<tr>
<td>JB835</td>
<td>0.498 ± 0.117</td>
<td>4</td>
<td>7.162 ± 0.762</td>
<td>4</td>
</tr>
<tr>
<td>JB835 + DFP</td>
<td>0.238 ± 0.016</td>
<td>4</td>
<td>5.904 ± 0.497</td>
<td>5</td>
</tr>
<tr>
<td>JB835 + DOPA</td>
<td>0.592 ± 0.054</td>
<td>8</td>
<td>8.306 ± 0.568</td>
<td>10</td>
</tr>
<tr>
<td>JB835 + DOPA + DFP</td>
<td>0.221 ± 0.028</td>
<td>9</td>
<td>9.110 ± 0.809</td>
<td>10</td>
</tr>
<tr>
<td>Control</td>
<td>0.328 ± 0.069</td>
<td>8</td>
<td>6.004 ± 0.579</td>
<td>15</td>
</tr>
</tbody>
</table>

* JB835, 6 mg/kg (ip); DOPA, 50 mg/kg (ip); DFP, 2.5 mg/kg (sc). The controls were given saline.

** S.E. = standard error; No. = number of individual determinations.
TABLE II

Effects of DFP in the Midbrain-Diencephalon on Norepinephrine (NE) and Dopamine (DA) Levels.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mean NE ± S.E.</th>
<th>No.</th>
<th>Mean DA ± S.E.</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPA</td>
<td>0.393 ± 0.035</td>
<td>6</td>
<td>0.225 ± 0.047</td>
<td>4</td>
</tr>
<tr>
<td>DOPA + DFP</td>
<td>0.226 ± 0.033</td>
<td>6</td>
<td>0.199 ± 0.053</td>
<td>6</td>
</tr>
<tr>
<td>JB835</td>
<td>0.513 ± 0.100</td>
<td>4</td>
<td>0.142 ± 0.024</td>
<td>4</td>
</tr>
<tr>
<td>JB835 + DFP</td>
<td>0.243 ± 0.084</td>
<td>6</td>
<td>0.394 ± 0.107</td>
<td>5</td>
</tr>
<tr>
<td>JB835 + DOPA</td>
<td>0.910 ± 0.141</td>
<td>8</td>
<td>0.377 ± 0.073</td>
<td>8</td>
</tr>
<tr>
<td>JB835 + DOPA + DFP</td>
<td>0.362 ± 0.055</td>
<td>11</td>
<td>0.875 ± 0.151</td>
<td>10</td>
</tr>
<tr>
<td>Control</td>
<td>0.336 ± 0.035</td>
<td>19</td>
<td>0.257 ± 0.045</td>
<td>11</td>
</tr>
</tbody>
</table>

For other explanations, cf. Table I.
CAUDATE NUCLEUS

NOREPINEPHRINE

DOPAMINE

FIGURE I
LEGEND FOR FIGURE I

Effect of JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), with or without DFP (2.5 mg/kg, sc); and DFP alone (2.5 mg/kg, sc) on caudate nucleus norepinephrine and dopamine levels. Control values are indicated with the horizontal line. Values are expressed as the Mean ± the standard error and represent 4-10 individual determinations. The level of significance was calculated using Student's test.

o indicates P < 0.001 for the difference between bargraphs 2 and 3.
FIGURE 2
LEGEND FOR FIGURE 2

Effect of JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), with or without DFP (2.5 mg/kg, sc); and DFP alone (2.5 mg/kg, sc) on midbrain-diencephalon norepinephrine and dopamine levels. Control values are indicated by the horizontal line. Values are expressed as the Mean ± the standard error and represent 4-10 individual determinations. The level of significance was calculated using Student's test. + indicates P<0.005 and • indicates P<0.025 for the difference between bargraphs 2 and 3 in the upper and lower horizontal rows, respectively.
FIGURE 3
LEGEND FOR FIGURE 3

Effect of DFP (1.0 and 2.5 mg/kg, sc); and JB835 (6 mg/kg, ip) plus DOPA (50 mg/kg, ip), with and without DFP (1.0 and 2.5 mg/kg, sc) on caudate nucleus norepinephrine and dopamine levels. Values are expressed as the Mean ± the standard error and represent 4-10 individual determinations. Control levels (not shown) are 0.328 ± 0.069 and 6.004 ± 0.579 for norepinephrine and dopamine respectively. Significance was calculated using Student's test.

• indicates P<0.005 for the difference between bargraphs 3 and 4; o indicates P<0.001 for the difference between bargraphs 3 and 5.
FIGURE 4
Effect of DFP (1.0 and 2.5 mg/kg, sc); and JB835 (6 mg/kg, ip) plus DOPA (50 mg/kg, ip), with and without DFP (1.0 and 2.5 mg/kg, sc) on midbrain-diencephalon norepinephrine and dopamine levels. Values are expressed as the Mean ± the standard error and represent 4-10 individual determinations. Control levels (not shown) are 0.336 ± 0.035 and 0.257 ± 0.045 for norepinephrine and dopamine respectively. Significance was calculated using Student's test. • indicates P<0.025 for the difference between upper bargraphs 3 and 4, and also lower bargraphs 3 and 5. o indicates P<0.005 for the difference between upper bargraphs 3 and 5.
latter. The changes in dopamine levels induced by DFP whether in the case of the midbrain-diencephalon area or of the caudate nucleus were even less marked than those in norepinephrine concentrations (Figures 3 and 4).

To the contrary, in "loaded" animals, i.e. in the animals in which the levels of norepinephrine and of dopamine were increased by the pretreatment, DFP (2.5 mg/kg) produced a significant change with respect to the "loaded" state in both the monoamines in the case of the caudate nucleus as well as in that of the midbrain-diencephalon region when employed at the larger dose; the smaller dose of DFP exerted less of an effect, although the trend of the data was similar with both doses of the anticholinesterases.

Perhaps surprisingly, the general direction of the changes was toward increased levels of dopamine and decreased levels of norepinephrine. These changes were generally more pronounced in the case of the midbrain-diencephalon area than in that of the caudate nucleus; moreover, the magnitude of the change induced by DFP relative to the "loaded" state was generally greater in the case of the animals pretreated by JB835 than in that of the animals pretreated by DOPA (Figures 5 and 6), while the maximal change due to DFP was found in the case of animals pretreated with the JB835-DOPA combination (Figures 1 and 2). It should be noted that this was also the order of the increase in norepinephrine or dopamine levels induced by DOPA, JB835, and JB835-DOPA combination (Table II,
Figures 2 and 6).

Specifically, the results in question were as follows (the percent change in monoamines induced by DFP after DOPA, JB835, or JB835-DOPA treatment represent the change relative to the monoamine levels in similarly pretreated animals that were not given DFP): In the case of the midbrain-diencephalon area, DFP, at 2.5 mg/kg, induced a 53 percent decrease in norepinephrine level in the animals pretreated by JB835, while 43 percent decrease in norepinephrine was noted following the pretreatment with DOPA. In the case of the animals pretreated with DOPA and JB835 a 60 percent decrease was noted following 2.5 mg/kg DFP. Somewhat less consistent data were obtained with regard to dopamine, although generally an increase in this monoamine was noted. A small (12 percent) decrease was noted after DFP (2.5 mg/kg) in the case of the DOPA-pretreated rabbits, while a very marked increase in dopamine occurred in the case of the animals pretreated with JB835 or with the JB835-DOPA combination: a 177 percent increase was found in the former case following the 2.5 mg/kg dose of DFP, and a 135 percent increase was recorded with the 2.5 mg/kg dose of DFP following the JB835-DOPA pretreatment. It should be noted that in this case the change following the JB835-DOPA pretreatment was somewhat smaller than that following pretreatment with JB835 alone. However, the increase in dopamine levels of the midbrain-
FIGURE 5

CAUDATE NUCLEUS

NOREPINEPHRINE

DOPAMINE

\[ \mu g/gm \]
LEGEND FOR FIGURE 5

Effect of JB835 (6 mg/kg, ip); and DOPA (50 mg/kg, ip), with or without DFP (2.5 mg/kg, sc) on caudate nucleus norepinephrine and dopamine levels. Values are expressed as the Mean ± the standard error and represent 4-6 individual determinations. Control values (not shown) are 0.328 ± 0.069 and 6.004 ± 0.579 for norepinephrine and dopamine respectively. The level of significance was calculated using Student’s test.

• indicates P<0.10-0.05 for the difference between bargraphs 3 and 4.
FIGURE 6

**MIDBRAIN - DIENCEPHALON**

**NOREPINEPHRINE**

<table>
<thead>
<tr>
<th>µg/gm</th>
<th>1.00</th>
<th>0.80</th>
<th>0.60</th>
<th>0.40</th>
<th>0.20</th>
<th>0.00</th>
</tr>
</thead>
</table>

**DOPAMINE**

<table>
<thead>
<tr>
<th></th>
<th>1.00</th>
<th>0.80</th>
<th>0.60</th>
<th>0.40</th>
<th>0.20</th>
<th>0.00</th>
</tr>
</thead>
</table>

- < 0.01
- 0.10 - 0.05
LEGEND FOR FIGURE 6

Effect of JB835 (6 mg/kg, ip); and DOPA (50 mg/kg, ip), with or without DFP (2.5 mg/kg, sc) on midbrain-diencephalon norepinephrine and dopamine levels. Values are expressed as the Mean ± standard error and represent 4-6 individual determinations. Control values (not shown) are 0.336 ± 0.035 and 0.257 ± 0.045 for norepinephrine and dopamine respectively. The level of significance was calculated using Student's test.

● indicates P<0.01 for the difference between bargraphs 1 and 2; ○ indicates P<0.10-0.05 for the difference between bargraphs 3 and 4 in both the upper and lower horizontal rows.
diencephalon area in the "loaded" state of the animal was very pronounced; it corresponded to almost a two-fold change compared to the controls and it was much more marked than that recorded in the "loaded" animals in the case of the caudate nucleus (Table 1). In this situation, DFP may have saturated the capacity of the midbrain-diencephalon area as it elevated the dopamine levels to 0.875 microgm./gm. compared to the 0.257 microgm./gm. level of the controls (Table 2).

In the case of caudate nucleus, DFP induced, similarly, marked decreases in norepinephrine in pretreated rabbits. A 26 and a 52 percent decrease were found with the 2.5 mg/kg dose of DFP in animals pretreated with DOPA and JB835, respectively; an even greater change occurred in animals pretreated with a JB835-DOPA combination, as the decrease with this dose of DFP amounted to 63 percent; a 13 percent decrease was noted following the smaller dose of 1.0 mg/kg. The DFP-induced changes in dopamine levels of the caudate nucleus were less consistent than those in the norepinephrine levels. DFP (2.5 mg/kg) induced relatively small decreases in dopamine level following either DOPA (23 percent) or JB835 (18 percent); however, under maximal loading conditions, i.e. after JB835-DOPA pretreatment, a small increase (9 percent) in the levels of dopamine was noticed (Table 1); none of these changes were statistically significant.
The effects of atropine and atropine methyl nitrate.

It may be expected that the effects of DFP on dopamine and/or norepinephrine were due to accumulated acetylcholine; in fact, results indicating the occurrence of such an accumulation have been obtained (cf. below section "acetylcholine levels"). It may be then further surmised that atropine should block the effects of DFP on the monoamines, while the quaternary atropine, atropine methyl nitrate, which does not readily penetrate into the brain, should be less effective in this respect. Pertinent experiments were therefore carried out under maximal "loading" conditions, i.e. following the pretreatment with JB835-DOPA combination.

The results were not always as expected. The DFP-induced decrease in the norepinephrine levels of either the caudate nucleus or of the midbrain-diencephalon area was partially blocked by 48 and 64 percent, respectively with atropine, 2.0 mg/kg, given i.p. prior to DFP (Figures 7 and 8). Similarly, atropine prevented, at the same dose, most of the increase in dopamine due to DFP in the case of the midbrain-diencephalon level (Figure 8). However, the effect of DFP on the dopamine levels of the caudate nucleus was not effected by atropine (Figure 7). It should be pointed out however that, in the case of the caudate nucleus, the effect of DFP on the levels of dopamine following pretreatment with JB835-DOPA combinations was statistically not significant (cf. above,
FIGURE 7

CAUDATE NUCLEUS

NOREPINEPHRINE

DOPAMINE

μg/gm

JB835+DL-DOPA
JB835+DL-DOPA+DFP
JB835+DL-DOPA+ATROPINE SULFATE

• <0.05
○ <0.001
LEGEND FOR FIGURE 7

Effect of JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), with and without DFP (2.5 mg/kg, sc) or atropine sulfate (2.0 mg/kg, ip) and DFP (2.5 mg/kg, sc) on caudate nucleus norepinephrine and dopamine levels. Values are expressed as the Mean ± the standard error and represent 4-10 individual determinations. Control values are indicated by the horizontal line. Significance was calculated using Student's test. o indicates P<0.001 for the difference between bargraphs 1 and 2; • indicates P<0.05 for the difference between bargraphs 2 and 3.
FIGURE 8
Effect of JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), with and without DFP (2.5 mg/kg, sc) or Atropine sulfate (2.0 mg/kg, ip) and DFP (2.5 mg/kg, sc) on midbrain-diencephalon norepinephrine and dopamine levels. Values are expressed as the Mean ± the standard error and represent 4-10 individual determinations. Control values are indicated by the horizontal line. Significance was calculated using Student's test.

• indicates P<0.01 for the difference between bargraphs 2 and 3. The bargraphs 1 and 2 in both horizontal rows also differed significantly (cf. Figure 1).
Even more surprising were the results obtained with atropine methyl nitrate (2.0 mg/kg), given i.p. prior to DFP, to rabbits under maximal "loading" conditions. While the quaternary atropine did not, as expected, block significantly the DFP effect on the levels of norepinephrine and of dopamine in the case of the caudate nucleus (figure 9), and while similarly it did not prevent the DFP-induced decrease of the level of norepinephrine in that of the midbrain-diencephalon region, most unexpectedly it afforded complete protection against DFP-induced, otherwise marked increase in the midbrain-diencephalon dopamine (Figure 10). In fact, while both atropine and quaternary atropine exhibited a similar blocking action upon the DFP-induced increase of dopamine in the midbrain-diencephalon region, the effect of the latter was much more pronounced.

It should be finally added that methylatropine (cf. Figures 9 and 10) given alone (without DFP) to rabbits did not have any significant affect on the levels of either monoamines.

Time characteristics of the effect of DFP on norepinephrine and on dopamine.

A possible explanation regarding the DFP-induced decrease in norepinephrine and elevation of dopamine observed in animals pretreated with JB835-DOPA combinations (Figures 1 and 2) could be the inhibition of dopamine-β-hydroxylase, the
FIGURE 9

CAUDATE NUCLEUS

NOREPINEPHRINE

DOPAMINE

µg/gm

0.00
0.20
0.40
0.60
0.80
1.00

0.00
2.00
4.00
6.00
8.00
10.00

J6835+DL
J6835+DL
J6835+DL+AT
J6835+DL+AT
AT

METHYL
METHYL
NITRATE
NITRATE

< 0.001

< 0.001
LEGEND FOR FIGURE 9

Effect of atropine methyl nitrate (2.0 mg/kg, ip); and JB835 (6 mg/kg, ip) plus DOPA (50 mg/kg, ip), with or without DFP (2.5 mg/kg, sc) or atropine methyl nitrate (2.0 mg/kg, ip); and DFP (2.5 mg/kg, sc) on caudate nucleus norepinephrine and dopamine levels. Values are expressed as the Mean ± the standard error and represent 4-10 individual determinations. Control levels are indicated by the horizontal line. Statistical significance was calculated using Student's test. o indicates P<0.001 for the difference between bargraphs 1 and 2.
FIGURE 10
LEGEND FOR FIGURE 10

Effect of atropine methyl nitrate (2.0 mg/kg, ip); and JB835 (6 mg/kg, ip) plus DOPA (50 mg/kg, ip), with or without DFP (2.5 mg/kg, sc) or atropine methyl nitrate (2.0 mg/kg, ip); and DFP (2.5 mg/kg, sc) on midbrain-diencephalon norepinephrine and dopamine levels. Values are expressed as the Mean ± the standard error and represent 4-10 individual determinations. Control levels are indicated by the horizontal line. Significance was calculated using Student's test.

• indicates P<0.05 for the difference between bargraphs 2 and 3. Bargraphs 1 and 2 in both horizontal rows also differed significantly (cf. Figure 1).
enzyme responsible for the conversion of dopamine to norepinephrine within the storage granules (Weiner, 1970). By inhibiting this enzyme, DFP would allow dopamine levels to increase while norepinephrine levels, after norepinephrine was released following the DFP treatment, would be lowered. If such conditions were occurring, norepinephrine levels would approach a depleted state over a period of time, while dopamine levels would be elevated. To investigate this possibility, a study was undertaken in which norepinephrine and dopamine levels were measured in animals pretreated with JB835-DOPA combination given DFP, 1.0 mg/kg, at intervals of one, two and three hours following DFP. The 1.0 mg/kg dose of DFP was used as in the course of this long-time experiment, the larger dose of DFP induced delayed toxicity which may have effected, nonspecifically, the results.

In the case of the midbrain-diencephalon, one hour following DFP, norepinephrine levels were 75 percent of those levels in rabbits pretreated with only JB835 and DOPA (Figure 11). Dopamine levels at one hour were only slightly elevated over those treated with JB835 and DOPA. Two hours following DFP, however, both the decrease in norepinephrine and elevation in dopamine levels became more apparent; norepinephrine levels 20 percent less than one hour after DFP, while dopamine levels were 45 percent above the one hour levels. It would appear that the time shift on the maximal effect due to 1.0 mg/kg of
FIGURE 11
LEGEND FOR FIGURE 11

Effect of JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), with or without DFP (1.0 mg/kg, sc) on midbrain-diencephalon norepinephrine and dopamine levels. Amine levels were measured at 1, 2, and 3 hours following DFP. Values are expressed as the Mean ± the standard error and represent 3-8 individual determinations. Control levels (not shown) are 0.336 ± 0.035 and 0.257 ± 0.045 for norepinephrine and dopamine respectively.
DFP as compared to that of 2.5 mg/kg dose of DFP, might reflect a slower rate of cholinesterase inhibition and relatively lower acetylcholine levels. Three hours following DFP, the nor-epinephrine levels were no longer decreasing, but rather increasing (Figures 11 and 12). Similarly, dopamine levels stopped increasing and were on the decline (Figures 11 and 12). It has been reported that the time required for conversion of dopamine to norepinephrine by dopamine-\(\beta\)-hydroxylase is much longer, than that needed for the conversion of dopamine from DOPA, (hours versus minutes, Spector et al., 1959). It is interesting that one hour following the maximum dopamine elevation the norepinephrine levels began to increase. Since norepinephrine levels did not continue to decrease to a depleted state and dopamine levels declined three hours following DFP, it may be concluded that the DFP-induced decrease in norepinephrine and elevation in dopamine is not due to inhibition of dopamine-\(\beta\)-hydroxylase by DFP.

The effects observed in the caudate nucleus (Figure 12) at various times after 1.0 mg/kg of DFP in rabbits pretreated with JB835 and DOPA differed markedly from the effects seen in the midbrain-diencephalon region. Norepinephrine levels continuously decreased one, two, and three hours following DFP. One hour following DFP, the caudate nucleus dopamine levels were slightly lower than the levels in rabbits treated with only JB835 and DOPA. Two hours following DFP a 40 percent
FIGURE 12
LEGEND FOR FIGURE 12

Effect of JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), with or without DFP (1.0 mg/kg, sc) on caudate nucleus norepinephrine and dopamine levels. Amine levels were measured at 1, 2, and 3 hours following DFP. Values are expressed as the Mean ± the standard error and represent 3-10 individual determinations. Control levels (not shown) are 0.328 ± 0.069 and 6.004 ± 0.579 for norepinephrine and dopamine respectively.
reduction from the one hour levels was observed. Three hours following DFP, the dopamine levels stopped decreasing and a 30 percent increase over the two hour levels occurred. It is interesting that the lowered dopamine levels in the caudate nucleus at two hours were further reflected in the lowered norepinephrine levels at three hours. If one were to compare the time course of the changes in caudate nucleus dopamine with the time course of the changes in norepinephrine in the midbrain-diencephalon (cf. Figures 11 and 12), a striking similarity can be seen. As dopamine is particularly present in the caudate nucleus and norepinephrine in the midbrain-diencephalon, one may suggest that the DFP-induced response, observed with caudate nucleus dopamine and midbrain-diencephalon norepinephrine, is characteristic of the transmitter most readily released.

Further studies with quaternary atropine.

Studies using atropine methyl nitrate alone or with DFP in midbrain-diencephalon indicate (Figure 13) that ATMN alone caused a slight elevation in norepinephrine levels compared to levels of norepinephrine in control (no drug) animals; on the other hand, ATMN had no effect on dopamine. Similar results were also observed in the case of the caudate nucleus; norepinephrine and dopamine levels with ATMN were similar to those levels observed in control rabbits (Figure 14). From
MIDBRAIN-DIENCEPHALON

NOREPINEPHRINE

DOPAMINE

μg/gm

DFP
ATROPINE METHYL NITRATE + DFP
ATROPINE METHYL NITRATE

<0.025
±0.10–0.05

FIGURE 13
LEGEND FOR FIGURE 13

Effect of DFP (2.5 mg/kg, sc); and Atropine methyl nitrate (2.0 mg/kg, ip), with or without DFP (2.5 mg/kg, sc) on midbrain-diencephalon norepinephrine and dopamine levels. Values are expressed as the Mean ± the standard error and represent 2-4 individual determinations. Control levels are indicated by the horizontal line. Significance was calculated using Student's test.

• indicates P<0.025 for the difference between lower bargraphs 2 and 3; + indicates P<0.10-0.05 for the difference between upper bargraphs 2 and 3.
LEGEND FOR FIGURE 14

Effect of DFP (2.5 mg/kg, sc); and Atropine methyl nitrate (2.0 mg/kg, ip), with or without DFP (2.5 mg/kg, sc) on caudate nucleus norepinephrine and dopamine levels. Values are expressed as the Mean + the standard error and represent 2-4 individual determinations. Control levels are indicated by the horizontal line.
these results it can be concluded that ATMN block of the DFP-induced elevation of dopamine observed in the midbrain-diencephalon of rabbits pretreated with JB835-DOPA-ATMN-DFP (2.5 mg/kg) combination was not due to ATMN itself, but rather to the combination ATMN-DFP. Whether JB835 with DOPA influenced this response is yet to be seen.

ATMN was therefore combined with DFP (2.5 mg/kg) to determine the validity of the above conclusion. The results indicate that in the case of the midbrain-diencephalon, the DFP-induced decrease of norepinephrine levels were unaffected by ATMN treatment which was similar to the observations with ATMN in the case of the JB835-DOPA-DFP combination (Figures 10 and 13). Moreover, ATMN was able to block the DFP-induced elevation of dopamine (Figure 13), in the absence of the JB835-DOPA pre-treatment in confirmation of the earlier findings (Figure 10); and this fact rules out in addition, any influence of JB835 and DOPA on this response. ATMN had little, if any, effect upon dopamine levels in caudate nucleus in response to DFP (Figure 14).

It could be then concluded, that in the case of the midbrain-diencephalon region the action of the quaternary methylatropine is responsible for the block in the DFP-induced elevation in dopamine levels.

The above results with ATMN-DFP combinations suggest that a peripheral involvement is responsible for the elevation
in dopamine by DFP; on the other hand, the decrease in norepinephrine levels by DFP, which was unaffected by ATMN, seems to be the result of central actions of DFP. If a central release mechanism is the means by which DFP produces the observed decrease in norepinephrine levels in midbrain-diencephalon, it seems unlikely that this effect should concern only one catecholamine, and dopamine would be expected to be susceptible to this releasing effect also. However, any release in dopamine centrally by DFP would be masked by the postulated peripheral effect of DFP leading to elevated central levels of dopamine. The possibility, in addition, has not been eliminated that DFP itself may stimulate dopamine synthesis in the brain and thus produce the elevated levels of dopamine.

In the presence of the blockade of both the central and peripheral cholinoceptive sites an elevation in dopamine levels following this block and a decrease in norepinephrine levels by DFP would indicate a direct action of DFP itself.

Experiments were therefore carried out to determine DFP's central actions. Whether DFP, centrally, induces (1) the direct release of both norepinephrine and dopamine, the original level of the latter being elevated due to DFP's proposed peripheral influence, or (2) the release of only norepinephrine while stimulating dopamine synthesis leading to elevated levels of dopamine, should be determined from these experiments. DFP's peripheral influence on dopamine levels is proposed
to be the result of increased synthesis of dopamine from DOPA. Therefore, in the absence of an excess of DOPA, this peripheral influence would be minimal. Further, if both the central and peripheral atropine-sensitive sites were blocked by atropine, thereby eliminating both the central actions of DFP (release of norepinephrine), as well as the peripheral influences of DFP (elevation of dopamine levels), then any actions of DFP on the monoamines would indicate that DFP, itself, was responsible for the monoamine responses and that these responses were not due to Ach acting on the atropine-sensitive cholinceptive sites.

Midbrain-diencephalon norepinephrine levels were decreased by 50 percent in the case of animals pretreated with JB835 prior to the administration of DFP, compared to the norepinephrine levels in those receiving only JB835 (Figure 15). Furthermore, DFP caused a greater than 50 percent increase in dopamine levels in the midbrain-diencephalon compared to that observed in "loaded" rabbits given DFP (cf. Figure 2). In the rabbits pretreated with JB835, AtSO (4.0 mg/kg) given prior to DFP blocked the DFP-induced decrease in norepinephrine, the norepinephrine levels in the atropine-treated animals being comparable to the norepinephrine levels in JB835-treated animals (Figure 15). From these results it is concluded that the DFP-induced norepinephrine decrease is not a direct action of DFP itself, as blockade by atropine would not occur if
FIGURE 15

MIDBRAIN—DIENCEPHALON

NOREPINEPHRINE

DOPAMINE

$\mu g/gm$
LEGEND FOR FIGURE 15

Effect of JB835 (6 mg/kg, ip) with or without DFP (2.5 mg/kg, sc), or Atropine sulfate (4.0 mg/kg, ip) and DFP (2.5 mg/kg, sc) on midbrain-diencephalon norepinephrine and dopamine levels. Values are expressed as the Mean ± the standard error and represent 4-6 individual determinations. Control levels (not shown) are 0.336 ± 0.035 and 0.257 ± 0.045 for norepinephrine and dopamine, respectively. Significance was calculated using Student\'s test.

* indicates $P<0.10-0.05$ for the difference between both the upper and lower bargraphs 1 and 2.
DPF's action was direct.

In addition, the midbrain-diencephalon dopamine levels in rabbits pretreated with JB835 and given AtSO (4.0 mg/kg) prior to DFP were not as elevated as in the case of the JB835-DFP treated rabbits. The dopamine levels in the atropine treated animals were similar to the dopamine levels in the JB835 treated animals. As with norepinephrine, the DFP-induced elevation of dopamine in the midbrain-diencephalon appears then not to result from direct stimulation of dopamine itself, but to be induced by the peripheral action of DFP.

Results concerning the caudate nucleus were not so clear. Norepinephrine levels following the various treatments were similar to those observed in the case of the midbrain-diencephalon. DFP caused a decrease in norepinephrine levels in JB835 pretreated animals compared to norepinephrine levels of the rabbits treated with JB835 alone. In addition, AtSO (4.0 mg/kg) blocked this DFP-induced decrease, the norepinephrine levels being similar to that observed in the case of the "loaded" animals given DFP (cf. Figure 1). However, in the case of the caudate nucleus, DFP given to JB835-pretreated rabbits caused not an elevation but a slight decrease in dopamine levels. Further, AtSO given prior to DFP in JB835 pretreated rabbits caused dopamine levels in the caudate nucleus to decrease even more (Figure 16). This response to atropine is unlike that observed in the case of the "loaded" rabbits given AtSO (2.0 mg/kg) prior to DFP (cf. Figure 7),
CAUDATE NUCLEUS

NOREPINEPHRINE

DOPAMINE

FIGURE 16
LEGEND FOR FIGURE 16

Effect of JB835 (6 mg/kg, ip) with or without DFP (2.5 mg/kg, sc), or Atropine sulfate (4.0 mg/kg, ip) and DFP (2.5 mg/kg, sc) on caudate nucleus norepinephrine and dopamine levels. Values are expressed as the Mean ± the standard error and represent 4-5 individual determinations. Control levels (not shown) are 0.328 ± 0.069 and 6.004 ± 0.579 for norepinephrine and dopamine, respectively. Significance was calculated using Student's test.

• indicated \( P \leq 0.10-0.05 \) for the difference between bargraphs 1 and 2.
where a slight block of the decrease in dopamine occurred. These observed differences may be due to the difference in the pretreatments.

The effects of DFP on catecholamines of brain-parts.

Thus far all experiments have been carried out with regard to midbrain-diencephalon and caudate nucleus. It has been demonstrated that DFP given to animals pretreated with JB835 and DOPA caused a significant decrease in norepinephrine levels in the midbrain-diencephalon, while at the same time significantly elevated dopamine levels in this area. Atropine sulfate given prior to the DFP in JB835-DOPA pretreated animals blocked both the norepinephrine decrease and the dopamine increase. Atropine methyl nitrate given prior to DFP in animals pretreated with JB835 and DOPA blocked only the dopamine elevation, suggestive of peripheral involvement. These responses were most pronounced in the midbrain-diencephalon area compared to those obtained in the case of the caudate nucleus. Therefore, additional experiments were carried out to determine whether specific areas within the midbrain-diencephalon region, i.e. midbrain, thalamus, and hypothalamus, could all exhibit the DFP responses, namely a decrease in norepinephrine levels and an increase in dopamine levels. An additional area, the hippocampus, was also selected to demonstrate DFP's effects on a non-midbrain-diencephalon area.
Mean norepinephrine levels in the thalamus, the hypothalamus, the midbrain, and the hippocampus were decreased by DFP (2.5 mg/kg) in rabbits pretreated with JB835 and DOPA when compared to norepinephrine levels in the animals receiving only JB835 and DOPA. Forty two, 13, 25, and 16 percent decreases in norepinephrine were observed in the thalamus, hypothalamus, midbrain, and in the hippocampus, respectively (Figure 17). Thus, the response to DFP was not restricted to any one specific area. The fact that this response to DFP was non-localized might have been predicted based on the reported presence of both cholinoceptive and adrenergic neurons within these brain areas.

Addition of ATMN prior to DFP in rabbits pretreated with JB835 and DOPA had no effect upon DFP's ability to decrease norepinephrine levels. In both the thalamus and midbrain, the levels of norepinephrine following the JB835-DOPA-ATMN-DFP combination were nearly identical with those in the case of rabbits treated with the JB835-DOPA-DFP combination. In the hypothalamus and hippocampus ATMN given prior to DFP in JB835-DOPA pretreated rabbits caused a further reduction in norepinephrine levels (25 and 21 percent, respectively) compared to the norepinephrine levels following JB835-DOPA-DFP combinations. The order of responses observed in these four brain areas is identical with that of the norepinephrine responses in the midbrain-diencephalon (Figure 10).
FIGURE 17

NOREPINEPHRINE

<table>
<thead>
<tr>
<th>µg/gm</th>
<th>THALAMUS</th>
<th>HYPOTHALAMUS</th>
<th>MIDBRAIN</th>
<th>HIPPOCAMPUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.20</td>
<td>0.40</td>
<td>0.60</td>
<td>0.80</td>
</tr>
<tr>
<td>0.00</td>
<td>0.20</td>
<td>0.40</td>
<td>0.60</td>
<td>0.80</td>
</tr>
<tr>
<td>0.00</td>
<td>0.20</td>
<td>0.40</td>
<td>0.60</td>
<td>0.80</td>
</tr>
<tr>
<td>0.00</td>
<td>0.20</td>
<td>0.40</td>
<td>0.60</td>
<td>0.80</td>
</tr>
</tbody>
</table>

+ < 0.05

○ 0.10-0.05
LEGEND FOR FIGURE 17

Effect of JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), with or without DFP (2.5 mg/kg, sc), or Atropine methyl nitrate (2.0 mg/kg, ip) and DFP (2.5 mg/kg, sc) on norepinephrine levels in the thalamus, the hypothalamus, the midbrain, and the hippocampus. Values are expressed as the Mean ± the standard error and represent 3-4 individual determinations. Control levels for each brain part are indicated by the horizontal line. Statistical significance was calculated using Student's test. + indicates P<0.05 for the difference between bargraphs 1 and 2 in the thalamus; • indicates P<0.10-0.05 for the difference between bargraphs 1 and 2 in the midbrain.
DFP given to animals pretreated with JB835 and DOPA caused an elevation of dopamine levels in the thalamus, the hypothalamus, the midbrain, and the hippocampus compared to dopamine levels of the animals treated with JB835 and DOPA (Figure 18). A 35, 31, 57, and 6 percent increase was observed in the case of the thalamus, hypothalamus, midbrain, and hippocampus respectively; the effects recorded in the case of the hypothalamus and midbrain were statistically significant. As in the case of norepinephrine, the DFP-induced elevation in dopamine levels within these four brain areas was of identical order with that of the dopamine responses due to DFP in the midbrain-diencephalon (Figure 10). Moreover, the DFP-induced dopamine elevation occurred in the midbrain-diencephalon, as well as in the non-midbrain-diencephalon area, indicating a non-localized effect of DFP.

The administration of ATMN prior to DFP in rabbits pretreated with JB835 and DOPA caused a block in the elevation of dopamine by DFP in all four brain areas (Figure 18). In the thalamus, a significant 87 percent reduction in dopamine levels occurred compared to the dopamine levels in the case of the rabbits treated with JB835-DOPA-DFP combination; in the hypothalamus, midbrain, and hippocampus, decreases of 55, a significant 58, and 34 percent, respectively, were noted. Again, the degree of the blocking effect of ATMN on the DFP-induced elevation in dopamine levels in these four brain areas was identical with
DOPAMINE

- JB835 + DL DopA
- JB835 + DL DopA + DFP
- JB835 + DL DopA + ATMN + DFP

THALAMUS
HYPOTHALAMUS
MIDBRAIN
HIPPOCAMPUS

+ < 0.05
0 < 0.01
⊕ 0.10 - 0.05
● < 0.025

FIGURE 18
LEGEND FOR FIGURE 18

Effect of JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), with or without DFP (2.5 mg/kg, sc), or Atropine methyl nitrate (2.0 mg/kg, ip) and DFP (2.5 mg/kg, sc) on dopamine levels in the thalamus, the hypothalamus, the midbrain, and the hippocampus. Values are expressed as the Mean ± the standard error and represent 3-4 individual determinations. Control levels for each brain part are indicated by the horizontal line. Statistical significance was calculated using Student's test. o indicates P<0.01 for the difference between bargraphs 2 and 3 in the thalamus; * indicates P<0.10-0.05 for the difference between bargraphs 1 and 2 in the hypothalamus; ± and • indicates P<0.05 and P<0.025 for the differences between bargraphs 1 and 2, and 2 and 3, respectively, in the midbrain.
that observed in the case of the midbrain-diencephalon (cf. Figure 10).

These and earlier data presented strongly suggest that the blocking action of ATMN on the DFP-induced elevation in dopamine levels observed in the midbrain-diencephalon occurs at the peripheral cholinoceptive sites otherwise stimulated by the DFP-induced elevation in acetylcholine.

**The effects of the intraventricular administration of DFP.**

Data thus far obtained suggest a central, as well as a peripheral action of DFP. However, it is difficult to separate DFP's central releasing effect from its peripheral dopamine elevating effect. Using ATMN, it was possible to block the peripheral elevating effect of DFP, but it could not be determined whether DFP, in addition to this action, was influencing the central amines through stimulation of non-atropine sensitive sites. If DFP were administered via the intraventricular rather than the subcutaneous route, the central actions of DFP could be more easily observed, being "divorced" under these circumstances from the peripheral influences.

Previous investigators have used intraventricular injection of drugs, i.e. acetylcholine, carbachol, norepinephrine, which have both peripheral and central actions, to separate the central from the peripheral responses to these agents (Feldberg, 1963;
Feldberg, 1958; Grossman, 1968; and Stein, 1970). The majority of these studies were investigating behavioral responses to central stimulation by the various drugs, in an attempt to identify the brain areas involved in producing these responses and, in addition, the types of neurons, i.e. cholinergic or adrenergic, involved.

Injection of 100µg of DFP into the lateral ventricle of cat was carried out by Feldberg (1958). Three symptomomological stages, which overlapped, were reported. The first stage had consisted of severe itching, wiping of the face and forelegs, and vigorous licking and chewing motion of the jaws. Itching is a characteristic sign of anticholinesterase action, probably resulting from inhibition of cholinesterase and subsequent accumulation of acetylcholine in the tissues lining the ventricular walls. During the second stage, increased tone and tremor of skeletal muscle occurred. The first two stages were relatively short in appearance while the third stage lasted longer. The third stage was characterized by an alteration of awareness and the development of stupor and catatonia. In this condition, the cats could be placed in abnormal positions which they retained for some minutes. Similar signs of catatonia were observed when eserine or large doses of acetylcholine were injected intraventricularly.

Rabbits given 200µg of DFP via the intraventricular route in the present study exhibited similar signs. The initial
excitement stage was characterized by either clockwise or counter-clockwise spinning movements; each animal spinning in only one direction. The rabbits subsequently developed a tonic type of kicking, and tremors. The last stage, beginning about 10 minutes after the injection, was characterized by a chewing jaw motion while the animals were lying in a resting position. Frequently the animals would rock from side to side. In a few cases the rabbits were up on all four legs in a catatonic-like stance moving their head from side to side. This stance lasted, however, only for 5 or 10 minutes. Pinpoint pupils, severe salivation, and foreleg muscle fasciculations were not as apparent as they were when DFP was given by the subcutaneous route. These behavioral responses in rabbits to intraventricular injection of DFP were identical in many respects to those responses observed in cats treated similarly.

In the initial experiments, the procedures of which are described in the Methods, the rabbits were anesthetized with sodium pentobarbital. The results obtained (Figures 19 and 20) with regard to the midbrain-diencephalon and caudate nucleus in the rabbits given JB835 and DOPA and not DFP, indicate that the pentobarbital was interfering with the normal uptake and release mechanisms for both norepinephrine and dopamine. As can readily be seen, the norepinephrine levels in both brain areas in the case of the sham controls (given JB835-DOPA-Saline combination) were considerably more elevated than the norepinephrine levels in animals similarly treated but not anesthetized (Tables
MIDBRAIN-DIENCEPHALON

WITHOUT PENTOBARBITAL

WITH PENTOBARBITAL

HOREPINEPHRINE

DOPAMINE

FIGURE 19
LEGEND FOR FIGURE 19

Effect of intraventricular injection of DFP (200 µg) or Saline on norepinephrine and dopamine levels in rabbit midbrain-diencephalon with and without pentobarbital anesthesia following pretreatment with JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), or JB835-DOPA-and ATMN (2.0 mg/kg, ip). Values are expressed as the Mean ± the standard error and represent 3-4 individual determinations. Statistical significance was calculated using Student's test.

"o" indicates P<0.025 for the difference between bargraphs 1 and 2; "~" indicated P<0.005 for the difference between bargraphs 1 and 3.
FIGURE 20
LEGEND FOR FIGURE 20

Effect of intraventricular injection of DFP (200μg) or saline on norepinephrine and dopamine levels in rabbit caudate nucleus with and without pentobarbital anesthesia following pretreatment with JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), or JB835-DOPA- and ATMN (2.0 mg/kg, ip). Values are expressed as the Mean ± the standard error and represent 3-4 individual determinations. Statistical significance was calculated using Student's test. Statistical significance (P<0.05) was found for the difference between the upper bargraphs 2 and 3.
similar results were observed in the case of the
dopamine levels. Maynert and Levi (1964) reported that sodium
pentobarbital prevented the morphine-induced decline in brain-
stem norepinephrine in cats, which led to elevated norepinephrine
levels. It appeared that in the case of our intraventricular
studies, pentobarbital acted in a similar manner. Therefore,
additional experiments were performed using unanesthetized
rabbits.

DFP (200μg) injected into the left lateral ventricle in
rabbits pretreated with JB835 and DOPA, caused a marked 33
percent decline in norepinephrine levels in the midbrain-
diencephalon (Figure 19) relative to the norepinephrine levels
observed in the case of the rabbits treated with JB835-DOPA-
Saline combinations (sham controls). Although the intra-
ventricular dose of DFP differed from that previously given
s.c. (2.5 mg/kg), the response induced by DFP was identical,
indicating that the releasing action of DFP was centrally
mediated. A similar decrease in norepinephrine levels was
observed in the pentobarbital anesthetized rabbits, although
the overall levels of norepinephrine were higher. ATMN given
prior to the intraventricular administration of DFP to rabbits
pretreated with JB835 and DOPA had no effect on the DFP-induced
decrease in norepinephrine levels observed in the case of the
rabbits treated with JB835-DOPA-DFP (intraventricular) combinations.
This inability of ATMN to block the decrease in norepinephrine
produced by DFP via the intraventricular route is identical with
the effects observed of ATMN on the DFP-induced decrease in
norepinephrine observed in the midbrain-diencephalon when DFP
was given s.c. (cf. Figure 10). ATMN was incapable of blocking
the actions of DFP on norepinephrine levels, whether DFP was
administered via the s.c. or intraventricular routes.

Dopamine levels in the midbrain-diencephalon of non-anesthetized
rabbits, pretreated with JB835 and DOPA were not significantly
different from the dopamine levels in the case of the rabbits
given JB835-DOPA-Saline combinations; the dopamine levels
were slightly but not significantly higher (Figure 19)
following DFP. In similarly treated rabbits anesthetized with
pentobarbital, a marked decrease in dopamine levels by DFP
was observed; however, it should be remembered that the dopamine
levels in anesthetized rabbits were higher than those observed
in unanesthetized rabbits.

The lack of elevated dopamine levels induced by DFP via the
intraventricular route, as opposed to the significant elevation
in dopamine levels induced by DFP via the subcutaneous route,
seems to indicate that the latter effect is due to the peripheral,
not central, action of DFP.

Further support for this conclusion was obtained in the
experiments in which ATMN was given prior to the intraventricular
administration of DFP in rabbits pretreated with JB835 and DOPA.
The levels of dopamine in the midbrain-diencephalon did not
differ significantly from the levels of dopamine in either the
rabbits given the JB835-DOPA-Saline combination, or the JB835-
DOPA-DFP (intraventricular) combination. Thus, DFP administered via the intraventricular route had no effect upon the dopamine levels in either JB835, DOPA pretreated animals or in those receiving, in addition, ATMN. These results were in sharp opposition to those observed in the experiments in which DFP was administered via the subcutaneous route; they firmly support the notion of the peripheral action of DFP involved in producing the elevation in dopamine levels within the midbrain-diencephalon.

As expected, in the caudate nucleus, the response to the intraventricular injection of DFP (200µg) was not well defined. This was typical of the caudate nucleus as the lack of conformity with those results obtained in the case of the midbrain-diencephalon was observed in the earlier experiments. Norepinephrine levels, in unanesthetized rabbits pretreated with JB835 and DOPA given DFP (200µg) intraventricularly, were not different from norepinephrine levels in animals treated with JB835-DOPA-Saline combination (Figure 20). In animals treated similarly, but not anesthetized with pentobarbital, there was a 29 percent though not significant, decrease in the norepinephrine levels in the DFP treated animals compared to those without DFP. ATMN given to unanesthetized animals pretreated with JB835 and DOPA followed by DFP intraventricularly produced a significant (P<0.05) 38 percent decrease in norepinephrine levels compared to the norepinephrine levels in the case of the animals treated
with the JB835-DOPA-DFP (intraventricular) combination. Dopamine levels in caudate nucleus were not significantly different in rabbits treated with: JB835-DOPA-Saline combination; JB835-DOPA-DFP (intraventricular) combination; or JB835-DOPA-ATMN-DFP (intraventricular) combination. However, there was a 15 percent (not significant) increase in dopamine levels observed in the case of the rabbits treated with JB835-DOPA-DFP combination compared with the animals receiving JB835-DOPA-Saline combination (Figure 20). A similar increase in dopamine levels was observed in anesthetized rabbits treated with JB835-DOPA-DFP (intraventricular) combination.

The effect of DFP on cholinesterases and on acetylcholine.

The assumption that because DFP is a potent anticholinesterase the rabbit brain cholinesterases will be inhibited following DFP administration is not a valid assumption without direct evidence of such inhibition. It was for this purpose that experiments, measuring the percent of cholinesterase (total acetylcholinesterase and 25 percent of butrylcholinesterase) inhibition produced by DFP (2.5 mg/kg) in the thalamus, the hypothalamus, the midbrain, and the caudate nucleus were performed. Rabbits were treated, as described in the Methods, with the following combinations: JB835-DOPA; JB835-DOPA-DFP; JB835-DOPA-AtSO-DFP; and a no-drug control group was also employed. Their cholinesterase activity was measured in terms of the Rate of Hydrolysis (Moles of substrate hydrolyzed/minute
Cholinesterase activity in the thalamus, the hypothalamus, the midbrain, and the caudate nucleus of rabbits treated with JB835 and DOPA was almost identical (not significantly different) to the cholinesterase activity in control (no drug) animals (Table 3). These results indicate that JB835 and DOPA are without effect upon the activity of cholinesterase in these four brain areas. The data further confirms the reported findings that the cholinesterase activity is highest in the caudate nucleus and mesencephalon (midbrain) regions of the brain (Votava, 1967 and Karczmar, 1967), the mean rate being $0.22 \times 10^{-4}$ for the caudate nucleus and $0.12 \times 10^{-4}$ for the midbrain as compared to $0.07 \times 10^{-4}$ for the thalamus and $0.10 \times 10^{-4}$ for the hypothalamus (for S.E.'s cf. Table 3).

Using the cholinesterase activity in the rabbits treated with JB835 and DOPA (sham controls) to represent zero percent inhibition it was found that the addition of DFP (2.5 mg/kg) to rabbits pretreated with JB835 and DOPA produced a 96.30 percent inhibition of cholinesterase activity, or greater, in the caudate nucleus, the midbrain, the thalamus, and in the hypothalamus (Figures 21 and 22). Similar results were obtained when AtSO was given prior to DFP in rabbits pretreated with JB835 and DOPA, the percent of brain cholinesterase inhibition being 97.50 or greater in the four brain areas. Thus the administration of DFP (2.5 mg/kg) did produce nearly 100
### TABLE 3

CHOLINESTERASE ACTIVITY IN RABBIT BRAIN PARTS

<table>
<thead>
<tr>
<th>Drug</th>
<th>Caudate N.</th>
<th>Midbrain</th>
<th>Thalamus</th>
<th>Hypothalamus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no drug)</td>
<td>0.22± 0.01</td>
<td>0.12± 0.01</td>
<td>0.07± 0.00</td>
<td>0.10± 0.02</td>
</tr>
<tr>
<td>JB835-DOPA</td>
<td>0.22± 0.01</td>
<td>0.13± 0.01</td>
<td>0.06± 0.02</td>
<td>0.08± 0.02</td>
</tr>
<tr>
<td>JB835-DOPA-DFP</td>
<td>0.00± 0.00</td>
<td>0.00± 0.00</td>
<td>0.00± 0.00</td>
<td>0.00± 0.00</td>
</tr>
<tr>
<td>JB835-DOPA-AtSO-DFP</td>
<td>0.00± 0.00</td>
<td>0.00± 0.00</td>
<td>0.00± 0.00</td>
<td>0.00± 0.00</td>
</tr>
</tbody>
</table>

+ All values are X 10 and represent the mean of two determinations.

* Rate= Moles of substrate hydrolyzed/minute/gm. tissue.

Doses used: JB835, 6 mg/kg, ip; DOPA, 50 mg/kg, ip; DFP, 2.5 mg/kg, sc; AtSO, 2.0 mg/kg, ip.

No statistically significant difference was found between the cholinesterase activity of the control and JB835-DOPA combinations in any of the four brain areas. Statistical significance was found between the JB835-DOPA combination and each of the DFP containing combinations; the significance being in the caudate nucleus and midbrain P<0.005; in the thalamus P<0.10-0.05; and in the hypothalamus P<0.05.
FIGURE 21

CHOLINESTERASE INHIBITION BY DFP

% INHIBITION

CAUDATE NUCLEUS

MIDBRAIN

100
80
60
40
20
0

1855 + DOA
1855 + DOA+
1855 + DOA+
1855 + DOA+
1855 + DOA+
1855 + DOA+
1855 + DOA+
1855 + DOA+

FIGURE 21
LEGEND FOR FIGURE 21

Percent of cholinesterase inhibition by DFP (2.5 mg/kg, sc) in the caudate nucleus and midbrain of rabbit following pretreatment with JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), or JB835-DOPA- and Atropine sulfate (2.0 mg/kg, ip). JB835-DOPA combination represents zero percent inhibition of cholinesterase activity. Values represent the mean of two assays. Statistical significance was calculated using Student's test. P<0.005 significance was found for the difference between bargraphs 1 and 2, and 1 and 3 for the caudate nucleus and the midbrain.
FIGURE 22

CHOLINESTERASE INHIBITION BY DFP

% INHIBITION

THALAMUS

HYPOTHALAMUS

FIGURE 22
Percent of cholinesterase inhibition by DFP (2.5 mg/kg, sc) in the thalamus and the hypothalamus of rabbit following pretreatment with JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), or JB835-DOPA- and Atropine sulfate (2.0 mg/kg, ip). JB835-DOPA combination represents zero percent inhibition of cholinesterase activity. Values represent the mean of two assays. Statistical significance was calculated using Student's test. $P<0.10-0.05$ was found for the difference between bargraphs 1 and 2, and 1 and 3 for thalamus; $P<0.05$ was found for the difference between bargraphs 1 and 2, and 1 and 3 for the hypothalamus.
percent cholinesterase inhibition in rabbits pretreated with JB835 and DOPA, or with JB835, DOPA, and AtSO. It is also evident that AtSO does not interfere with DFP's ability to inhibit cholinesterase.

While the above data suggest that DFP should have elevated acetylcholine levels, as cholinesterases are the enzymes responsible for the hydrolysis of the latter, it was still necessary to demonstrate directly that indeed DFP (2.5 mg/kg, sc) elevated the levels of acetylcholine in the caudate nucleus, midbrain, thalamus, and hypothalamus in rabbit. Using bioassay techniques, acetylcholine levels were assayed in two samples each of the four brain areas for each drug combination. There was little difference between acetylcholine levels of the rabbits receiving the JB835 and DOPA treatment as compared to the levels found in the control rabbits in the case of the caudate nucleus, midbrain, thalamus, or hypothalamus (Table 4). Taking the acetylcholine levels in the caudate nucleus, midbrain, thalamus, and hypothalamus of control (no drug) rabbits as representing 100 percent of acetylcholine it was found that, of the four brain areas from the animals treated with JB835 and DOPA, the midbrain exhibited the greatest deviation from its respective control acetylcholine level; this deviation was only 11 percent (Figures 23-26). It was, in addition, found that neither JB835 nor DOPA had any significant effect, compared to the control rabbits, upon
### TABLE 4

**EFFECT OF DRUGS ON ACETYLCHELOLINE LEVELS IN RABBIT BRAIN PARTS**

<table>
<thead>
<tr>
<th>Acetylcholine ug/gm.</th>
<th>Caudate N.</th>
<th>Midbrain</th>
<th>Thalamus</th>
<th>Hypothalamus</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control (no drugs)</strong></td>
<td>2.02</td>
<td>1.35</td>
<td>1.35</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td>2.19</td>
<td>1.21</td>
<td>1.53</td>
<td>1.00</td>
</tr>
<tr>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td><strong>JB835-DOPA</strong></td>
<td>1.88</td>
<td>1.40</td>
<td>1.82</td>
<td>1.01</td>
</tr>
<tr>
<td></td>
<td>1.93</td>
<td>1.43</td>
<td>1.38</td>
<td>1.03</td>
</tr>
<tr>
<td>+</td>
<td>o</td>
<td>o</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DFP</strong></td>
<td>5.59</td>
<td>4.39</td>
<td>4.08</td>
<td>4.13</td>
</tr>
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<td></td>
<td>4.36</td>
<td>5.76</td>
<td>4.70</td>
<td>4.64</td>
</tr>
<tr>
<td><strong>JB835-DOPA-DFP</strong></td>
<td>5.00</td>
<td>4.78</td>
<td>3.62</td>
<td>3.25</td>
</tr>
<tr>
<td></td>
<td>6.30</td>
<td>4.08</td>
<td>3.47</td>
<td>3.50</td>
</tr>
<tr>
<td><strong>JB835-DOPA-AtSO-DFP</strong></td>
<td>5.89</td>
<td>3.67</td>
<td>2.81</td>
<td>2.68</td>
</tr>
<tr>
<td></td>
<td>4.80</td>
<td>3.56</td>
<td>3.17</td>
<td>3.01</td>
</tr>
<tr>
<td><strong>JB835-DOPA-ATMN-DFP</strong></td>
<td>4.73</td>
<td>4.39</td>
<td>3.96</td>
<td>3.49</td>
</tr>
<tr>
<td></td>
<td>4.28</td>
<td>5.10</td>
<td>3.07</td>
<td>3.46</td>
</tr>
</tbody>
</table>

Values represent individual bioassays. Doses used: JB835, 6 mg/kg, ip; DOPA, 50 mg/kg, ip; DFP, 2.5 mg/kg, sc; AtSO, 2.0 mg/kg, ip; ATMN, 2.0 mg/kg, ip. Statistical significance was calculated using Student's test.

+ indicates $P<0.05$ for the difference between the two groups;

• indicates $P<0.005$ for the difference between the two groups.
FIGURE 23

CAUDATE NUCLEUS  ACETYLCHOLINE

% INCREASE

CONTROL  1883 + DOPA  DFP  1883 + DOPA  +  1883 + DOPA  +  1883 + DOPA  +  1883 + DOPA  +
LEGEND FOR FIGURE 23

Percent of acetylcholine increase over control (no drug) acetylcholine levels in the rabbit caudate nucleus following JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), or DFP (2.5 mg/kg, sc) with or without JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), JB835-DOPA- and Atropine sulfate (2.0 mg/kg, ip), or JB835-DOPA- and Atropine methyl nitrate (2.0 mg/kg, ip). Percent increases are determined from the mean of two individual bioassays ± the standard error. Control levels are representative of 100 percent acetylcholine. Statistical significance was calculated using Student's test. The difference between the mean values of bargraphs 2 and 3 was found to be significant (P<0.05).
FIGURE 24

MIDBRAIN ACETYLCHELONE

% INCREASE

0 100 200 300 400

CONTROL JBE3S + DOPA OFF JBE3S + DOPA + ATAN + DOPA OFF + DOPA +
LEGEND FOR FIGURE 24

Percent increase of acetylcholine over control (no drug) acetylcholine levels in the rabbit midbrain following JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), or DFP (2.5 mg/kg, sc) with or without JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), JB835-DOPA- and Atropine methyl nitrate (2.0 mg/kg, ip), or JB835-DOPA- and Atropine sulfate (2.0 mg/kg, ip). Percent increases are determined from the mean of two individual bioassays ± the standard error. Control levels are representative of 100 percent acetylcholine. Statistical significance was calculated using Student's test. The difference between the mean values of bargraphs 2 and 3 was found to be significant (P < 0.025).
THALAMUS  ACETYLCHOLINE

% INCREASE

400

300

200

100

0

CONTROL  JB35 + DOFA  DPP  JB35 + DOFA +  ATMN + DPP  ALSO4 + DPP

FIGURE 25
Percent increase of acetylcholine over control (no drug) acetylcholine levels in the rabbit thalamus following JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), or DFP (2.5 mg/kg, sc) with or without JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), JB835-DOPA- and Atropine methyl nitrate (2.0 mg/kg, ip), or JB835-DOPA- and Atropine sulfate (2.0 mg/kg, ip). Percent increases are determined from the mean of two individual bioassays ± the standard error. Control levels are representative of 100 percent acetylcholine. Statistical significance was calculated using Student's test. The difference between the mean values of bargraphs 2 and 3 was found to be significant (P<0.025).
FIGURE 26

% INCREASE

HYPOTHALAMUS ACETYLCHOLINE

CONTROL JHB3 + DOPA DFP JHB3 + DOPA + DFP JHB3 + DOPA + ATMN + DFP + ATSO + DFP +

400
300
200
100
0

FIGURE 26
LEGEND FOR FIGURE 26

Percent increase of acetylcholine over control (no drug) acetylcholine levels in the rabbit hypothalamus following JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), or DFP (2.5 mg/kg, sc) with or without JB835 (6 mg/kg, ip) and DOPA (50 mg/kg, ip), JB835-DOPA- and Atropine methyl nitrate (2.0 mg/kg, ip), or JB835-DOPA- and Atropine sulfate (2.0 mg/kg, ip). Percent increases are determined from the mean of two individual bioassays + the standard error. Control levels are representative of 100 percent acetylcholine. Statistical significance was calculated using Student's test. The difference between the mean values of bargraphs 2 and 3 was found to be significant (P<0.005).
acetylcholine levels in any of the four brain areas.

DFP (2.5 mg/kg) alone produced increases in acetylcholine content compared to its effect in the animals pretreated with the JB835-DOPA combination. The percent increases, all of which were significant, due to DFP alone were: 297, 205, 330, and 136 in the midbrain, the thalamus, the hypothalamus, and the caudate nucleus, respectively, over the acetylcholine levels in identical areas of the JB835-DOPA treated rabbits.

DFP given to rabbits pretreated with JB835 and DOPA caused an elevation in the acetylcholine levels over control acetylcholine levels by 168, 246, 146, and 228 percent in the caudate nucleus, the midbrain, the thalamus, and the hypothalamus, respectively. When ATMN was given prior to DFP to JB835 and DOPA pretreated animals acetylcholine levels elevated by the DFP as compared to control levels were: 114, 271, 144, and 238 percent in the caudate nucleus, midbrain, thalamus, and in the hypothalamus, respectively.

It is evident that DFP, whether alone or following pretreatment with JB835 and DOPA or JB835, DOPA, and ATMN, produced marked elevations in acetylcholine levels in these four brain areas in rabbit; this then relates well with the demonstration of cholinesterase inhibition produced by DFP in these four areas (cf. Figures 21 and 22).

A special comment is required with regard to the last drug combination; in this case, atropine sulfate, given prior to DFP in rabbits pretreated with JB835 and DOPA, was observed
to interfere somewhat with the elevation of acetylcholine levels by DFP. Although this interference was not found to be statistically significant, a trend was evident. In the midbrain, thalamus, and hypothalamus acetylcholine levels in atropine sulfate treated animals were lower following DFP than all other DFP containing drug combinations (Figures 24-26). The only brain area where this effect was not observed was the caudate nucleus, where the acetylcholine levels of the atropine sulfate treated animals were approximately the same as the other drug groups containing DFP (Figure 23).

The observed lessening of DFP-induced elevation of acetylcholine levels was, however, anticipated. Mitchell (1963), Szerb (1964), and Polak and Meeuws (1966) demonstrated that atropine sulfate enhanced the release of acetylcholine from the cerebral cortex of the cat, rabbit, sheep, and rat. Similar results were also observed in rats by Hingtgen and Aprison (1970). Thus, the observation that in the presence of AtSO, acetylcholine levels following DFP were less than those observed in animals not pretreated with AtSO, seem reasonable. It would seem that AtSO caused a lowering of acetylcholine levels through release, and subsequent degradation of acetylcholine by cholinesterase, prior to the administration of DFP. Thus, with an initial lowered level of acetylcholine, the elevation of acetylcholine levels caused by DFP was less in those drug combinations where the acetylcholine levels were not initially lowered by atropine. It should also be pointed out that this
effect was not observed following DFP in animals pretreated with drug combinations which included ATMN.

Regardless of the individual variations within each brain area, all drug combinations containing DFP (2.5 mg/kg) showed acetylcholine levels which were elevated a minimum of 100 percent over control acetylcholine levels. Altogether, we could demonstrate that DFP at a dose of 2.5 mg/kg, s.c. adequately inhibited brain cholinesterase in all four brain areas (Table 3), causing marked elevation in the acetylcholine levels within these areas (Table 4).
DISCUSSION

The concept of cholinergic-adrenergic coupling is not by any means novel. Burn and Rand in 1959 had proposed for the peripheral nervous system, a neural mechanism by which acetylcholine was able to cause the release of norepinephrine from sympathetic nerve terminals. Koelle (1963) modified this concept to allow for acetylcholine, having been released from a nerve terminal, to act back upon that nerve terminal from which it was released, to cause a "percussive" release of norepinephrine.

Considerable evidence has been presented over the years which seems to support these concepts. Only recently, however, has attention been focused upon the possibility of a cholinergic-adrenergic mechanism existing in the central nervous system.

Both electrophysiological and neurochemical evidence obtained by Van Meter (1969) and Van Meter and Karczmar (1970) suggested an interplay between the central neurotransmitters, acetylcholine and norepinephrine, in the anticholinesterase (eserine) produced blockade of EEG recruitment. Specifically, in rabbits whose norepinephrine levels had been depleted by αMPT, and related drugs, eserine administration was unable to produce blockade of the EEG recruitment. However, following replenishment of the norepinephrine stores with DOPA administration, the ability of eserine to block EEG recruitment was restored.
Additional evidence of Karczmar and Longo (1970) and Karczmar et al. (1971) demonstrated a similar relationship in rabbits in the case of the paradoxical sleep phenomena.

More recently, Philippu (1970) has provided evidence which indicates that acetylcholine does, in fact, cause the release of norepinephrine from cerebral cortex in cat, provided calcium is present.

Although these findings indicate the possibility for the existence of a central cholinergic-adrenergic link, they do not provide comprehensive evidence on the transmitter systems involved, and the results dealing with acetylcholine, norepinephrine, and dopamine, as well as cholinesterases, i.e. acetyl-, butrylcholinesterase, etc., remain incomplete.

The present studies were therefore undertaken to provide evidence as to the central effects produced by the anticholinesterase, DFP. The questions to be answered relate to:

1. the effect of DFP upon the activity of cholinesterase;
2. the subsequent effects upon the levels of acetylcholine due to cholinesterase inhibition by DFP; and
3. the effect of DFP and/or acetylcholine upon the norepinephrine and dopamine levels. In addition, these findings would hopefully provide a base from which an understanding of the mechanisms involved in central cholinergic-adrenergic coupling could evolve.

Measurement of the cholinesterase levels in the midbrain, the thalamus, the hypothalamus, and the caudate nucleus confirmed
that nearly 100 percent inhibition of cholinesterase activity was obtained using the 2.5 mg/kg, sc, dose of DFP, with or without pretreatment with JB835 and DOPA. Moreover, results on the activity of cholinesterase in control rabbits confirms previous findings (Karczmar, 1967, and Votava, 1967) that cholinesterase activity is highest within the caudate nucleus and the mesencephalon (midbrain) areas in cat, rat, mouse, and rabbit.

With the marked inhibition of cholinesterase activity, acetylcholine levels were found to be elevated by this dose of DFP. In the midbrain, the thalamus, the hypothalamus, and the caudate nucleus, DFP caused a minimum of 100 percent increase in the levels of acetylcholine. In no case did the JB835 and DOPA pretreatment cause significant differences in the effect of DFP on either the cholinesterase activity or on levels of acetylcholine. As such, the role of JB835 and DOPA in these experiments was restricted to elevating the levels of norepinephrine and dopamine which allowed the DFP-induced responses to be more readily observed.

Of possibly greater significance was the lack of effect on the acetylcholine levels in the thalamus, the hypothalamus, the midbrain, and the caudate nucleus, of either norepinephrine or dopamine, levels of which were elevated by the administration of the JB835-DOPA combination. The postulation of Burn and Rand, as well as that of Koelle, provided for the action of
acetylcholine upon the sympathetic nerve terminals to cause
the release of norepinephrine. As such, their proposal
provided for cholinergic (acetylcholine) actions upon the
sympathetic adrenergic (norepinephrine) nerve terminals. However,
the possibility of adrenergic actions upon cholinergic nerve
terminals was not considered in either the Burn and Rand or
Koelle proposals. Until now there has been no reported
evidence concerning the possibility of an adrenergic-cholinergic
link. In the present experiments it was demonstrated in
rabbits that norepinephrine and dopamine in a "loaded" state (that
is after the monoamine levels were elevated by JB835-DOPA
pretreatment) did not cause significant changes in the levels
of acetylcholine in the midbrain, the thalamus, the hypothalamus,
or the caudate nucleus. These findings are highly significant
and suggest that the possible mechanism of adrenergic-cholinergic
coupling does not exist in these rabbit brain areas.

It should be pointed out, however, that the above conclusion
was based upon data obtained from animals whose levels of
monoamines were in a "loaded" state, but whose acetylcholine
levels were not. The possibility should not be excluded that
monoamines can exert some type of influence other than a
coupling phenomena, i.e. competition of the transmitters for
available storage sites, upon the levels of acetylcholine.
Evidence obtained from animals given DFP alone or JB835-DOPA-
DFP indicate that in the latter case, the levels of acetylcholine
were lower than in the case of the former in the thalamus, the hypothalamus, and the midbrain. Similar responses were observed in the case of rabbits pretreated with JB835-DOPA-ATMN- and given DFP compared to those given DFP alone. Although these differences were not significant, their trend suggest the possibility of monoamine influence upon acetylcholine levels when both transmitters are in the "loaded" state.

The questions that arise are concerned with the mechanisms involved in the acetylcholine-catecholamines coupling and particularly in the differential effects on dopamine and on norepinephrine.

The present findings demonstrate that in rabbits, DFP given systemically, induces significant increments of dopamine levels and significant decrements of norepinephrine levels in the midbrain-diencephalon and to some extent, the caudate nucleus regions. These changes, however, could be noted only after the levels of these monoamines were increased by appropriate pharmacological maneuvers, as DFP did not affect the monoamines without prior JB835 and/or DOPA pretreatment. This finding suggest that the effects of DFP on the monoamines are best observed under optimal conditions for their neuronal synthesis and storage. Conversely, in the studies on amine uptake and release the results were best observed in animals depleted of monoamines by reserpinization (Anden et al., 1964). Of course, DFP may have induced less pronounced changes under less favorable conditions as well, which escaped detection.
by our methods; they may be however revealed by more sensitive
techniques, as well as, be reflected more readily in the
change in the monoamine turnover values, which were not studied
at present.

One would expect that acetylcholine would exert a similar
effect on both the monoamines. However, the rate of synthesis
of dopamine is much higher than that of norepinephrine (Weiner,
1970), while perhaps the opposite may be true with regard
to their rates of release and elimination. It may be then
speculated that these two mechanisms may favor increased
synthesis and storage of dopamine and increased dissipation
of norepinephrine in the presence of accumulated acetylcholine.

It should be pointed out that the acetylcholine-induced
increase in dopamine in "loaded" rabbits was conspicuous in the
case of the diencephalon-mesencephalon area, and inconsistent
and much less pronounced in that of the caudate nucleus. This
may be related in some way to the high dopamine level in the
latter compared to the former (cf. Tables 1 and 2). It might
be considered that since the highest levels of dopamine in the
brain are found in the caudate nucleus, a much greater
stimulatory effect upon the dopamine levels is needed by
acetylcholine to produce a significant dopamine increase,
the levels of which are already high, compared to the amount
of stimulation needed by acetylcholine to produce a significant
dopamine increase in the case of the midbrain-diencephalon, whose
levels of dopamine are much lower. Speculatively, it is also possible that acetylcholine activation embraces only norepinephrine-rich neurons in the caudate, and both norepinephrine- and dopamine-rich neurons in the midbrain and related areas.

It is difficult to explain why the responses observed in the case of the midbrain-diencephalon are not always observed in the case of the caudate nucleus. The above suggestion regarding the dopamine levels may serve to provide a possible explanation as to the lack of response to acetylcholine by this transmitter in the caudate. With regard to norepinephrine, the lack of response to acetylcholine by this amine could be due to the fact that norepinephrine levels in the caudate nucleus are extremely low, if not almost undetectable. It is possible that of the two monoamines in the caudate, dopamine serves as the actual transmitter, while norepinephrine serves only as a metabolic biproduct formed in the degradation of dopamine by dopamine-β-hydroxylase. Still another possibility could be that the cholinergic and adrenergic neurons within the caudate nucleus might not be compatible for a cholinergic-adrenergic coupling, i.e. the proposed acetylcholine stimulated interneuron is not present in the caudate nucleus.

Clarification should be made as to the mechanism by which DFP administration produces the elevation in dopamine levels and the decrease in norepinephrine levels in the midbrain-diencephalon, and to a lesser extent in the caudate nucleus.
There are three possible explanations of DFP's actions on norepinephrine and dopamine. First, DFP as a drug could, itself, produce these effects by directly stimulating the adrenergic neuron, in a yet unknown manner, producing the elevation of dopamine levels, probably through stimulation of dopamine synthesis from DOPA, as well as causing the release of norepinephrine and a subsequent lowering of the levels of this transmitter. Further, these direct actions of DFP should be entirely independent of any mediation by acetylcholine, whether through DFP stimulation of nearby cholinoceptive neurons or interneurons, causing the release of acetylcholine which would be indirectly responsible for the actions of DFP on both norepinephrine and dopamine levels. The second proposed possibility to explain DFP's actions upon norepinephrine and dopamine levels relates to an indirect action by DFP. This action would be due to acetylcholine, the levels of which are elevated by DFP's inhibition of cholinesterases (acetyl-, butrylcholinesterase, etc; cf. Methods). Acetylcholine would then act on the adrenergic neuron or an interneuron, to cause an elevation in dopamine levels, as well as to cause the release and lowering of the norepinephrine levels. The third proposed possibility concerns the inhibition of dopamine-β-hydroxylase by DFP or by the accumulated acetylcholine to produce the norepinephrine and dopamine responses.

The results of experiments on the effects of DFP on
norepinephrine and dopamine at various intervals following DFP (cf. Figures 11 and 12), were interpreted as not supporting the third possibility. Had dopamine-α-hydroxylase been inhibited by DFP, norepinephrine levels would have continued to decrease, and in fact Billiet et al. (1970) found a decrease in norepinephrine levels in rabbit caudate nucleus following dopamine-α-hydroxylase inhibition. No such effects were observed in the present studies. Moreover, by three hours both the norepinephrine and the dopamine levels were approaching their respective control levels.

Furthermore, there is no supportive evidence for the first possibility, i.e. the direct action of DFP. However, supportive evidence does exist with regard to the second possibility. The findings that DFP (2.5 mg/kg) caused nearly 100 percent inhibition of cholinesterase activity in the thalamus, the hypothalamus, the midbrain, and the caudate nucleus, as well as the subsequent elevation in the levels of acetylcholine levels in these four brain areas, allow, at present, the conclusion that the DFP-induced effects upon both norepinephrine and dopamine, i.e. the elevation in dopamine levels and the decrease in norepinephrine levels, should be due to the elevated acetylcholine levels resulting from DFP inhibition of cholinesterase.

Consistent with the above proposal is that, cholinoceptive neurons and high levels of acetylcholine and acetylcholinesterase were found by many investigators in the midbrain-diencephalon
area and in the caudate nucleus (for references, cf. Curtis and Crawford, 1969; Karczmar, 1967, 1969, and 1970, as well as in the present studies, cf. Tables 3 and 4). Both dopamine and norepinephrine are also present in these two areas, dopamine being particularly high in the latter (Bertler and Rosengren, 1959; cf. also Tables 1 and 2). These areas are richly interconnected, and the direct dopaminergic nigro-striatal pathway (Anden et al., 1966) is particularly important in the present context. Cell bodies of both norepinephrine and dopamine containing neurons have been shown to originate in the mesencephalic (midbrain) region and project rostrally in nerve bundles to the striatum (caudate nucleus and putamen) in the case of the dopamine containing neurons, and to the diencephalon (thalamus and hypothalamus) and telencephalon (cortex) in the case of the norepinephrine containing neurons (Anden et al., 1966). While many of the pathways in question may be cholinceptive (for references, cf. Silver, 1967; Karczmar, 1970), it is not known whether this is also true for the important nigro-striatal connection, although recent findings indicate the presence of acetylcholinesterase in several important nigro-striatal fascicles (Olivier et al., 1970). The presence of both cholinceptive and adrenergic neural pathways in the midbrain-diencephalon and nigro-striatal regions is supportive of the proposal that acetylcholine may induce the observed effects
either by activating the catecholamine neurons of the mesencephalon-diencephalon as well as of the striate area (possibly via cholinceptive interneurons, cf. Glisson and Karczmar, 1970), or via the cholinceptive participation in the pathways which connect these two areas. Of course, DFP administered systemically will cause acetylcholine accumulation outside of these areas as well, and diffusing or circulating acetylcholine, if it should escape cholinesterase, may cause similar effects on the monoamines elsewhere in the brain, which then would not be due to local, synaptic actions.

Interesting additional conceptualizations may be offered on the basis of the results obtained with the tertiary and quaternary atropine. The blocking actions of atropine on the acetylcholine-induced norepinephrine depletion and dopamine accumulation were not unexpected, and they supported the notion of the central site of the acetylcholine action on the monoamines. Even without the combined JB835-DOPA pretreatment, acetylcholine produced an elevation in dopamine levels and a decrement in norepinephrine levels in rabbits receiving only JB835, although the augmentation of response was much less than that observed after the addition of DOPA. Under these conditions, atropine (4.0 mg/kg) blocked nearly completely the acetylcholine-induced decrease in norepinephrine. The fact that AT.MN was incapable of blocking the norepinephrine depletion induced by acetylcholine in the case of the mesencephalon-diencephalon further strengthens this conceptualization. On the
other hand, the conspicuous effectiveness of the quaternary atropine in blocking the dopamine increase induced by acetylcholine (particularly in the case of the diencephalon-mesencephalon area) seems to suggest that the periphery is involved also. Indeed, the effects of acetylcholine are not restricted to the central nervous system. The two doses of DFP employed in this study would produce lethality in at least 50 percent of the rabbits in a 24 hour period (cf. Karczmar, 1967), and they are capable of inducing marked peripheral effects which in the rabbit are of the muscarinic nature (cf. Holmstedt, 1959). These effects are readily blocked by the quaternary atropinics, although the tertiary mother compound may be expected to antagonize them to an extent also. It may be speculated that, in the "loaded" animals the acetylcholine-induced increase in dopamine levels— and in the rate of its synthesis— may depend on their peripheral actions and the more marked action of ATMN may be due to its particular effectiveness at the parasympathetic periphery. Moreover, atropine at a higher dose (4.0 mg/kg), given to rabbits pretreated with JB835, was able to produce a more marked block of the acetylcholine-induced elevation in dopamine levels than at the lower (2.0 mg/kg) dose in the JB835-DOPA treated animals; the block produced by the higher dose of atropine closely resembled the block of the acetylcholine-induced dopamine elevation seen with the administration of ATMN (2.0 mg/kg),
indicating atropine's peripheral effectiveness in blocking the acetylcholine-induced elevation in the dopamine levels, provided doses greater than those of ATMN needed to produce this block are used. On the other had, as atropine, but not the quaternary atropine affected the acetylcholine-induced changes in norepinephrine in the "loaded" animals, this particular mechanism may be entirely central in its location.

On the basis of these findings, a working neural model was constructed to explain the effects of acetylcholine on the central norepinephrine and dopamine levels (Figure 27).

It is proposed that administration of DFP leads to the accumulation of acetylcholine within the central and autonomic nervous systems by inhibiting the activity of cholinesterases. Further investigations may, in fact, show that acetylcholinesterase is the cholinesterase particularly involved. Centrally, acetylcholine, acting either through an interneuron (labelled (1) in the figure) or directly through cholinceptive sites on the adrenergic neuron (labelled (2) in the figure) causes the release of norepinephrine from the adrenergic terminals. In addition, peripheral effects of acetylcholine, relayed via the afferent pathways to the adrenergic neuron, cause the elevation in dopamine levels, probably through stimulation of the synthesis of dopamine from DOPA. The effect postulated to depend on the stimulation of sensory afferents by the accumulation of acetylcholine, is not, at present, well
FIGURE 27 Hypothetical model of central cholinergic involvement in adrenergic nerve transmission.
understood. The data indicate that it is sensitive to the blocking actions of ATMN and AtSO (cf. below for further discussion).

It was apparent that the most prominent effects of acetylcholine upon the norepinephrine and the dopamine levels occurred in the midbrain-diencephalon. The additional studies demonstrated that the acetylcholine-induced elevation in dopamine levels and decrease in norepinephrine levels was widespread, as it occurred in the midbrain, the thalamus, the hypothalamus, and also in the non-midbrain-diencephalon area, the hippocampus. These data were very much in agreement with findings obtained with regard to the midbrain-diencephalon region.

In addition, it was found that ATMN blocked the elevation of the dopamine levels within these four brain areas, but was ineffective in preventing the acetylcholine-induced decrease in norepinephrine levels. This suggests that the sensory afferents, i.e. the lateral spinothalamic tract, are in close contact with the adrenergic neurons of the individual areas. Supportive of this concept is the fact that sensory afferents from the lateral spinothalamic tract are reported to distribute through the reticular formation and thalamus from which branching collaterals arise invading these regions. Moreover, various peripheral sensory receptors, i.e. pain, touch, etc., are reported to be stimulated by acetylcholine (personal communication, R. Wurster). Additional evidence of widespread sensory influence
comes from EEG and evoked potential experiments in which enviromental stimuli, i.e. sound, pain, light, often trigger widespread central alerting (Himwich, 1962).

To expand further upon this point, one might conclude that the cholinergically mediated peripheral influence upon the central nervous system reflected in the effect upon dopamine levels- could well be one of the mechanisms by which behavioral stresses act to influence central responses. In fact, it is most probable that the same sensory afferents are involved in both the acetylcholine- and the stress-induced effects upon the central nervous system. The question which remains to be answered is whether certain types of stressful conditions produce similar effects, as acetylcholine does, upon the dopamine levels.

Principal criticism of the studies purporting to show central drug responses such as these is that the drugs, particularly DFP, were administered via a peripheral route. Such routes of administration allow for stimulation of peripheral sites which contribute to, or modify, the central effect of the drug. This type of contribution was observed in these studies with regard to the ATMN sensitive, acetylcholine-induced elevation in dopamine levels. By using the quaternary atropinic, ATMN, it was attempted to demonstrate those responses to acetylcholine which were central in origin, as ATMN was reportedly unable to pass through the blood brain barrier at the dose used,
2.0 mg/kg (Albanus, 1970). However, it was possible that peripheral sites other than those blocked by ATMN also contributed to the central actions of acetylcholine, although such additional actions of acetylcholine seemed remote. Nevertheless, demonstration of the central actions of acetylcholine divorced from any peripheral influence was necessary if contribution to acetylcholine's central actions by non-ATMN sensitive sites was to be ruled out.

The results of the intraventricular injection of DFP indicated that norepinephrine was released by this mechanism and these results obtained following the intraventricular DFP administration resembled those due to s.c. DFP. On the other hand, there was an absence of any elevation in dopamine levels, contrary to the response to s.c. administered DFP. That no dopamine elevation occurred in these experiments, indicates an extracentral mechanism. Further, ATMN given prior to the intraventricular injection of DFP had no effect on dopamine levels, compared to the ability of ATMN to block the acetylcholine-induced elevation of dopamine levels when DFP was administered via the s.c. route.

Thus, it would appear that the central effects of acetylcholine on the central monoamine levels could be divorced from those actions of acetylcholine which are peripherally mediated. Moreover, the possibility of acetylcholine stimulating the ATMN-insensitive peripheral sites, which could contribute...
to the acetylcholine-induced release of norepinephrine, can be eliminated. Had the acetylcholine-induced release of norepinephrine been due to such peripheral stimulation of ATMN-insensitive sites, then lowering of the norepinephrine levels would not have been observed following the intra-ventricular injection of DFP. As such, a lowering of the norepinephrine levels was observed, a central rather than peripheral mechanism may be suggested for this action of acetylcholine.

Before drawing final conclusions, mention should be made of the many other uninvestigated, but not forgotten, mechanisms which might have been involved. The possibility of acetylcholine actions on enzyme systems such as catechol-O-methyl transferase and/or DOPA decarboxylase exists. Similarly, turnover studies of norepinephrine and dopamine would lend additional understanding to the observed responses, as would evoked potential and fluorescence microscopy studies. The possibility of other transmitter involvement, i.e. serotonin, tryptamine, histamine, is very real. It is impossible to investigate all possibilities in a single dissertation, but hopefully it will inspire additional investigations.
SUMMARY

In the thalamus, the hypothalamus, the midbrain, and the caudate nucleus in rabbit brain, DFP (2.5 mg/kg) was found to cause nearly 100 percent inhibition of cholinesterase activity. In addition, pretreatment of the animals with JB835-DOPA combinations had no effect upon cholinesterase activity in these four brain areas compared to cholinesterase activity in identical brain areas of control (no drug) animals, indicating that the monoamines norepinephrine and dopamine, elevated by the pretreatment, are not active on this enzyme system.

Data was also presented showing that acetylcholine levels in the thalamus, the hypothalamus, the midbrain, and the caudate nucleus in rabbit brain were elevated more than 100 percent over appropriate control acetylcholine levels by this dose of DFP (2.5 mg/kg). Of importance was the finding that elevated norepinephrine and dopamine levels were without effect upon the acetylcholine levels, indicating that adrenergic actions upon cholinceptive neurons do not exist in these rabbit brain parts.

Studies were subsequently carried out to demonstrate the central effects induced by the elevated central levels of acetylcholine as a result of cholinesterase inhibition by DFP.
Data were presented which link peripheral administration of DFP and the subsequent elevation in acetylcholine levels to a significant decrement of central norepinephrine in the midbrain-diencephalon, and to some extent in the caudate nucleus. These findings are supported by evidence of identical responses occurring in the midbrain, the thalamus, the hypothalamus, and the hippocampus due to acetylcholine.

Dopamine levels, unlike norepinephrine levels, were significantly elevated in the midbrain-diencephalon by acetylcholine and to a lesser extent in the caudate nucleus. Acetylcholine induced a similar effect on dopamine levels in the midbrain, the thalamus, the hypothalamus, and the hippocampus. Interestingly, however, acetylcholine's actions on dopamine and on norepinephrine occurred only following pretreatment with JB835, DOPA, or the JB835 and DOPA combination; this suggests that acetylcholine's actions are best observed under optimal conditions for monoamine storage and synthesis. Yet, acetylcholine may induce less pronounced changes under less favorable conditions as well.

Additional conceptualizations were offered on the basis of the results obtained with the tertiary and quaternary atropine. The blocking actions of atropine on the acetylcholine-induced norepinephrine depletion and dopamine accumulation were not unexpected, and they supported the notion of the central site of action of acetylcholine. The fact that ATMN was incapable of blocking the norepinephrine depletion induced by acetylcholine
in the case of the midbrain-diencephalon, further strengthens this conceptulization. However, the ability of ATMN to block the dopamine accumulation induced by acetylcholine suggested peripheral rather than central involvement for this action.

DFP (200µg) injected intraventricularly into rabbits caused a decrease in norepinephrine levels in the midbrain-diencephalon which ATMN was unable to block. Significant accumulation of dopamine in the midbrain-diencephalon was not, however, observed following intraventricular injections of DFP; nor did ATMN produce any significant effects on dopamine levels.

The conclusions arrived at from these studies led to the postulation of a neural model to explain the elevation in dopamine levels and the decrease in norepinephrine levels resulting from the accumulation of acetylcholine, resulting from DFP's inhibition of cholinesterase activity in rabbit brain.

It is proposed, based upon data obtained in these experiments, that elevated central acetylcholine levels, resulting from DFP's inhibition of cholinesterase activity, acts either through an interneuron or directly through cholinoceptive sites on the adrenergic neuron to cause the release of norepinephrine. In addition, DFP-induced elevation of acetylcholine leads through stimulation of the atropine and ATMN sensitive sites, to an elevation of the dopamine levels,
probably through stimulation of the synthesis of dopamine from DOPA.

Under the experimental conditions employed in these studies, neurochemical evidence of cholinergic-adrenergic coupling phenomena was thus demonstrated. The role of such a mechanism is, however, unclear.

It is hopeful that these observations will provide a foundation upon which future investigations of the cholinergic-adrenergic linkage will begin.
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APPROVAL SHEET

The dissertation submitted by Silas N. Glisson, III has been read and approved by a committee composed of Dr. A.H. Friedman, Dr. W.A. Himwich, Dr. Y.T. Oester, Dr. M.A. Collins and Dr. A.G. Karczmar (Chairman).

The final copies of the dissertation have been examined by the chairman of the examining committee and his signature which appears below verifies that all the 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 requirement for the Degree of Doctor of Philosophy.

11/4/1972
Date

Signature of Committee Chairman