THE INTERRELATIONSHIPS BETWEEN CHRONIC RESTRAINT STRESS AND RESERPINE SEDATION: EFFECTS ON PITUITARY-ADRENAL FUNCTION AND BRAIN SEROTONIN AND NOREPINEPHRINE LEVELS IN MALE ALBINO RATS

BY

JOHN ADAM ROSECRANS

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JOHN ADAM ROSECRANS

Approved:

Thesis Committee:

Chairman _____

_____

_____

_____

Dean of Graduate Studies _____

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ABSTRACT

The General Adaptation Syndrome (G.A.S.) was characterized for chronic forced restraint stress in normal and reserpinized (1 mg/kg, I.P.) male albino rats. This was accomplished by analyzing the interrelationships among brain neurohumoral levels (serotonin (5-HT) and norepinephrine (NE)), serum corticosterone (KS) levels, and various organ weights such as thymus, testes, pituitaries, and adrenals.

Reserpine was observed to prevent normal rats from adapting to this stress (mortality rate was 50%) possibly via inanition. It was suggested that the overall non-adaptive effects produced could ultimately be due to the ability of reserpine to induce a chemical sympathectomy. Thus, by depleting the A.N.S. of accessible NE, an animal would be unable to respond to a severe change in environment.

Control animals demonstrated both behavioral and neurochemical adaptation in response to this stress. Initial excitation associated with restraint was related to increased brain 5-HT levels and decreased brain NE levels. As the experiment progressed, stress animals became less excitable and easier to handle which was also associated with the return of both brain amines to normal levels.

In contrast, reserpinized animals subjected to chronic restraint stress became progressively more excitable and difficult to handle as the experiment proceeded. This behavior can

best be described by C.N.S. depression associated with extreme hypersensitivity to handling. This progressive change in behavior was correlated with the progressive depletion of brain NE levels, since brain 5-HT remained at relatively normal levels. The progressive increase in excitation thus appeared to be dependent on NE depletion or release.

Reserpine (1 mg/kg, I.P.) induced a progressive depletion of brain NE while it did not do so with 5-HT. This was interpreted as indicating that 5-HT synthesis was equivalent to its release. In contrast 0.5 mg/kg, I.P., of reserpine was found to produce a progressive depletion of 5-HT as well as NE. On the other hand, higher doses of reserpine inhibited the serotonin depletion effects produced by the 1 mg/kg. In fact, some animals demonstrated levels above normal following the chronic administration of reserpine (2 mg/kg, I.P.). Therefore, a possible serotonin-feedback mechanism involving the free and bound concentrations of this amine may be indicated.

Increased brain 5-HT levels twenty-four hours after the thirty-second dose of reserpine was also suggestive of an increased synthesis rate. Behavioral excitability was also found to characterize these increased serotonin levels in all experiments conducted. Norepinephrine, on the other hand, did not demonstrate any observable changes in the rate of synthesis under the influence of reserpine.

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ABBREVIATIONS[a]

ACTH	-	Adrenocorticotrophic Hormone
ADW	-	Adrenal Dry Weight (mg)
%ADW	-	% Adrenal Dry Weight $\left(\frac{\text{Dry Weight}}{\text{Wet Weight}} \times 100\right)$
A.N.S.	-	Autonomic Nervous System
C.N.S.	-	Central Nervous System
C.R.F.	-	Corticotrophic Releasing Factor
KS	-	Corticosterone
NE	-	Norepinephrine
5-HT	-	5-Hydroxytryptamine (Serotonin)
RS	-	Restraint Stress

a - These accepted abbreviations are used in the following pages of this thesis.

I. INTRODUCTION

The ability of an animal to adapt to both internal and external changes in environment has been known to be essential to life since Claude Bernard's discussions on the "Internal Milieu". Modern research in this field has amassed evidence which clearly indicates that adaptation to changes of environment or to a stressor involves an interplay between the central and autonomic nervous systems and the endocrines, especially the pituitary-adrenal axis. The fact that adaptation involves nervous activity has led to studies of the effects of various drugs acting on the central nervous system on these interrelationships.

Tranquilizers such as reserpine have been especially studied because their sedative effects indicate an inhibition of the pituitary-adrenal axis. However, much to the surprise of previous investigators, reserpine produced either depression or stimulation of the pituitary-adrenal axis in response to acute stressors. These investigators studied reserpine in animals subjected to various stressors, but due to great variation in methodology and experimental design, divergent views of the effects of reserpine resulted.

To reconcile this state of apparent confusion recent investigators have presented strong evidence indicating that reserpine stimulates pituitary-adrenocortical function and does not inhibit stress acutely. Although this work is well

documented, little consideration of the chronic effects of reserpine in stressed animals has been observed. A few isolated experiments on the effects of the chronic administration of reserpine in normal animals have not resulted in conclusive statements as to specific pituitary-adrenal interactions. Along with this, little thought has been given to the study of the chronic effects of reserpine on its proposed central nervous system mediators of tranquilization, norepinephrine (NE) and serotonin (5-HT).

In view of these inadequacies, this investigation was designed to study of chronic effects of reserpine on the pituitary-adrenal axis and its proposed C.N.S. mediators of tranquilization. It has been the prime purpose of this investigation to study the interrelationships of the pituitary-adrenal axis and the brain neurohumoral agents NE and 5-HT with the behavioral sequences observed during chronic reserpine treatment in unstressed and chronically stressed rats. The significance of this research will reside in its ability to clarify the mode of action of reserpine in view of attempting to correlate these data with human pharmacology.

II. REVIEW OF THE LITERATURE

Adaptation

The problem of animal adaptation has interested biologists for some time and has now become of interest to the pharmacologist since it has been realized that any drug could be considered a stressor to which an animal should adapt. Claude Bernard was first to recognize the necessity to maintain an internal constancy under any environmental change. Other investigators, including Fredericq, Pfluger, and Richet (Cannon, 1932) were also quick to realize that in order to carry out such equilibrations under environmental changes, an animal must possess some regulatory mechanism which could quickly and efficiently meet these provocative insults.

W. B. Cannon in attempting to solve this problem suggested that the autonomic nervous system (ANS) is the initiator of adaptive mechanisms. He also recognized the remarkable ability of an animal to maintain a varying internal environment within limitations so as to hold a relative constancy. This he termed Homeostasis. Cannon wrote:

> "Here then is a striking phenomenon. Organisms composed of material which is characterized by the utmost inconstancy and unsteadiness, have somehow learned the methods of maintaining constancy and keeping steady in the presence of corditions which reasonably might be expected to prove profoundly disturbing."

Cannon proposed that under environmental changes, A.N.S. stimulation, and especially plasma adrenalin, could initiate cellular preparadness, and therefore, permit adaptation. His main methodological approach was total or partial sympathectomy. Homeostatic responses were then observed under normal and stress conditions. To better appreciate his conclusions a passage from his monograph on Homeostasis has been cited:

> "If sympathectomized animals were set free in the outer world and had to meet its demands in struggle for food, safety and warmth, they could be found more or less defective according to the variable efficiency of their accessory stabilizing mechanisms. Even in the most favorable conditions displayed by the sympathectomized dog, however, absense of sympathetic control of corrective devices is accompanied by an inability to preserve constancy of the internal environment though the stress is only moderate."

Not being satisfied with Cannon's theories, Han Selye (1950) began a long series of experiments demonstrating that any animal presented with a noxious stressor responds in a very characteristic pattern elicited by adrenocortical hyperfunction via anterior pituitary stimulation. Because of the impact of his research on modern thinking concerning homeostatic mechanisms, his major postulates are now outlined:

I. When an animal is presented with some drastic change of either its internal or external environment, the pituitary-adrenal axis is stim-

ulated, eliciting an increase in plasma corticosteroids. These steroids are believed to be the substances enabling an animal to adapt to these changes.

II. With prolonged stress a complete adaptive syndrome can be characterized, known as the General Adaptation Syndrome (G.A.S.).

III. The adaptive trigger, the anteriory pituitary, is essentially controlled by the hypothalamus.

IV. There is no essential qualitative difference in the responses to different chronic stresses. The differences are basically quantitative.

Cannon's and Selye's theories do not conflict but tend to expose the true overall picture. Cannon worked on the adrenal medulla whereas Selye studied the adrenal cortex. There is now little doubt of the central position of the pituitary-adrenal axis in adaptation, but A.N.S. activity is assuredly not without importance. George Sayers (1950) has compared these relationships in much clearer a fashion. He states that the adrenocortical hormones play a general supportive role rather than an initiating role in bodily processes; whereas the adrenal medulla initiates cellular and metabolic changes in response to an emergency. The adrenal cortex plays a passive role and makes it possible for various regulatory systems to expend the additional effort necessary for homeostatic adjustment.

The Pituitary-Adrenal Axis

The relationship between adrenocortical activity and the pituitary was first demonstrated by Smith (1927). By extirpating the pituitary of the rat, he was able to demonstrate a rapid adrenocortical atrophy due to hypophysectomy. Since this classic investigation, numerous steroid hormones have been isolated and identified as either secretory or biosynthetic precurser substances of the adrenal cortex. The release of these hormones into the general circulation has been found to be dependent upon the elaboration of a hormone released by the pituitary, the adrenocorticotrophic hormone (ACTH) (Vogt, 1960). It has further been observed that numerous types of stimuli (stressors) including chemical, physiological and psychological, induce ACTH release, thus producing a secondary release of the adrenocortical hormones.

Recently it has also been demonstrated that in addition to a negative feedback effect of plasma corticosteroids on the pituitary, ACTH is also regulated by higher centers of the C.N.S., particularly the hypothalamus (Harris, 1955). However, another mediator substance has been postulated as being responsible for ACTH release during stress situations. This second mediator has been postulated since there are no direct nervous connections between the hypothalamus and anterior pituitary. Saffaran (1962) has accumulated evidence supporting this view through the extraction and partial

purification of a hypothalamic factor which will induce ACTH release from the pituitary in vitro.

The General Adaptive Syndrome

As mentioned previously, Hans Selye has characterized a syndrome of chronic animal adaptation (1950). Selye has conclusively demonstrated the dependence of this syndrome on the pituitary-adrenocortical function through the classical methods of endocrinology of extirpation and replacement experiments. An animal which is presented with a chronic stressor, will undergo three distinct stages of change. An initial stage, or alarm reaction, a secondary stage of adaptation and finally a stage of exhaustion.

During the first stage, which may last from one to three days, physiological changes, such as lowered blood pressure, decreased body temperature, C.N.S. depression, and antidiuresis, are observed. During the second stage the stressor will be compensated for, and these physiological responses will return to normal or even increase. This second stage will continue until the animal can no longer adapt to or compensate for the stressor and the animal will pass into the exhaustive stage and eventual death.

There are several organ weight changes which characterize each peculiar stage of this syndrome. In general, an increase in adrenal weight, a decrease of both the thymus and gonadal weights are indicated during the alarm reaction. During the latter stages of the syndrome similar changes will persist or return to normal. In terms of hormonal controls, these are

associated with increased hormonal activity whereas a decreased organ weight is associated with decreased hormonal activity.

Throughout this syndrome several biochemical patterns also change indicating specific homeostatic adjustments. Changes in the biochemistry of the adrenal gland are indicative of increased adrenal activity. Increases of adrenocorticosteroid output and decreases in adrenal cholesterol and ascorbic acid have been found to be good indexes of adrenocortical hyperactivity. Within the past four years methods for the direct analysis of plasma and adrenal corticosteroids have also been developed (Guillemin et al (1958). This development has very greatly enhanced recent endocrinological and pharmacological investigations concerning the pituitary-adrenal axis.

Thus, as one can see, adaptation involves many homeostatic mechanisms. The sequence of events from the initiation of the adaptive trigger to the final release of adrenocortical hormones into the general circulation, involves several chain reactions which are basically controlled through feedback mechanisms. Therefore, theoretically a drug could either stimulate or depress any one of these mechanisms and thus alter the end result. It should also be apparent that although adrenocortical activity may be either stimulated or inhibited by a drug, this does not mean that this drug produces its effects via the C.N.S. alone.

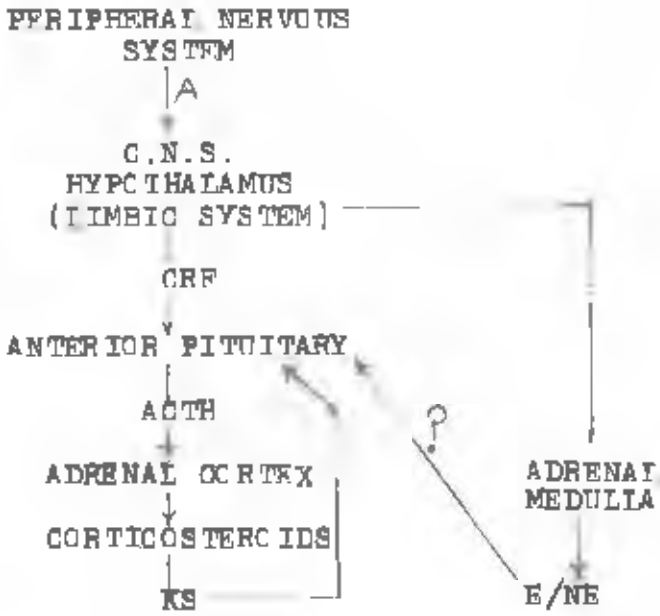

Fig. 1. A theoretical representation of the hypothalamico-adenohypophyseal-adrenocortical axis and drug action.

Possible Sites of Drug Action
(Inhibition or Stimulation)

1. Drugs can inhibit any segment of the nervous segment (A-)

2. Drugs can act on any of the hormonal secretions CRF, ACTH, and KS
   They could affect any one of the following aspects of hormonal secretions:
   1. Synthesis
   2. Release
   3. Site of action

Fig. 1 is an attempt at presenting the possibilities of where a drug could attack the pituitary adrenal axis. As one can see there are more than a dozen ways of affecting this system and still obtaining the same end result, either stimulation or inhibition of corticosteroid release. The most important point to be made here is the fact that some investigators after determining the effects of a drug on the adrenal

corticosteroid will then draw conclusions without examining drug effects on the intricate sequence of events outlined above.

The Mode of Action of Reserpine

Reserpine is a crystalline alkaloid obtained from the plant Rauwolfia serpentina (Schlittler et al, 1954). This drug has been known since the time of Arab and Greek physicians and was used in Europe for many years in the treatment of various anxiety states. Due to technical difficulties, reserpine was not isolated nor characterized until 1952 (Mueller et al), when its sedative and hypotensive effects were also first exposed. Although aggressive animals such as monkeys could easily be handled after the administration of reserpine, the animals did not go to sleep and they could respond to all external stimuli. This new type of sedative activity was defined as "tranquilization".

The mode of action of reserpine is one of the most interesting and challenging problems confronting current pharmacology. Because of its diffuse pharmacological effects, the mode of action of reserpine has escaped most investigators. This problem, however, is not peculiar to reserpine, for in dealing with drugs which affect the psyche, one has the overwhelming problem of attempting to evaluate changes in animal behavior.

1. Electrophysiological Mechanisms

The first pharmacological investigations of reserpine indicated that reserpine inhibited the hypothalamus, initiating

sympatholytic or parasympathomimetic effects (Plummer, 1954). Since these early investigations as to the effects of reserpine on brain electrophysiology, its effects have been observed to be increasingly more complex and diffuse. Depending upon the dose used, methods of electrical recording and stimulation, and on the species of animal used, one observes either facilitation or inhibition of many brain areas. Domino (1962) summarizes the effects of reserpine on various electrical systems of the brain by concluding that reserpine either stimulates or depresses most areas of the brain, except for the Limbic System which it facilitates.

In studying the behavioral pattern of reserpine, Weiskrantz (1957) observed that reserpine appears to inhibit sensory input to the brain. Jacobsen (1959) further studied reserpine under psychic stress and found that it produced very nonspecific effects. He also stated that the general stupor produced by reserpine prevented the observation of possible specific effects.

The major problem associated with the study of reserpine can be summarized best by Bein (1957). He states:

> "Reserpine, which exhibits a complex pattern of activity, differs from other known substances having central inhibiting activity, and its mode of action is of a fundamentally new type."

2. Neurochemical Mechanisms

Current interest in the effects of reserpine on brain neurohumoral agents was initiated by the observation that reserpine has the ability to reduce normal brain levels of Serotonin (5-HT), (Pletscher et al, 1956). Since reserpine could not be detected in the brain during maximal sedation, Brodie and Shore 1957

proceeded with the theory that it exerted its effects by releasing the normally bound 5-HT which would then be free to inhibit central synapses, thus producing tranquilization (1957).

Holzbauer and Vogt (1956) also found that reserpine depleted norepinephrine (NE) from its normal brain stores and concluded that reserpine acted as a sympatholytic agent. Carlsson et al (1957) demonstrated that 3:4 dihydroxyphenylalanine (DOPA), one of the precursors of NE, restored the normal activity of reserpinized animals, presumably via the restoration of depleted brain NE. When a reserpinized animal was administered the precursor of 5-HT, 5-hydroxytryptophan, no change of activity occurred. Carlsson (1958) has further demonstrated that reserpine depletes 3-hydroxytyramine (dopamine), a precursor of NE which is now believed to also possess a physiological role of its own. Therefore, Carlsson theorized that reserpine produces its effects by depriving central synapses of accessible NE and dopamine. (This is what Holzbauer and Vogt essentially proposed.)

Paasonen (1961) aided Carlsson's theory by observing that raunescine, a rauwolfia alkaloid, also produced sedation similar to reserpine, which selectively lowered NE to a greater degree than 5-HT. He, however, utilized bioassay procedures for brain NE and 5-HT determinations as opposed to Brodie's fluorometric procedures. Pletscher et al (1959) were also able to demonstrate differential effects of various benzoquinolizine derivatives on these brain amines. From these studies they showed that the compound with the greater sedative effects, also produced a greater depletion of NE than of 5-HT.

Although evidence has been presented contradicting Brodie's original postulates, he has been able to withstand these attacks with very convincing evidence that reserpine does produce its effects through 5-HT depletion. The present evidence supporting this theory is outlined as follows: (Brodie et al, 1961, Burns and Shore, 1961)

1. Reserpine causes equivalent depletion of both NE and 5-HT from their normal bound states in the brain.
2. Brain levels of reserpine cannot be detected at the onset of its sedative effects and depletion of both amines.
3. 5-hydroxytryptophan produces sedative effects similar to that of reserpine.
4. Reserpine appears to affect the binding of 5-HT rather than its synthesis.
5. Alpha-Methyl-m-Tyrosine depletes the brain of its NE stores without demonstrating sedative effects.
6. Cold stress (4°) four hours prior to the administration of reserpine prevented its sedative effects. Similarily it also inhibited the depletion of 5-HT induced by reserpine, but not that of NE.

In a more recent paper, Revizin et al (1961) studying the effects of alpha-Methyl-m-Tyrosine, tetrabenazine and reserpine, demonstrated excellent correlation between CNS depression, evoked Limbic potentials (reserpine usually causes facilitation, Domino, 1962), and lowered 5-HT levels. They could show no relationship between NE depletion and evoked potentials. This last investigation is extremely interesting and important

in view of the fact that it is one of the few attempts at correlating CNS behavior with electrical and chemical events.

It should also be pointed out that most of this work involves whole brains with little regard as to what occurs at the intracellular level. In an effort to overcome this deficiency Giarman and Schanberg (1950) and Weil-Malherbe et al (1961) studied centrifuged brain fractions from reserpinized animals. Giarman working with 5-HT and Weil-Malherbe working with NE found that reserpine increases the FREE/BOUND ratio of the concentrations of both amines. This ratio refers to the concentrations of amines of the cytoplasmic fraction to the concentrations of the amines in the particulate fraction. The significance of these previous investigations points to the ability of reserpine to increase the concentrations of NE and 5-HT in the "Free" form. It is assumed that the "Free" form of either amine is that quantity accessable to central synaptic sites.

Effects of Reserpine on the Pituitary-Adrenal Axis

Past research on the mechanism of the action of reserpine has emphasized the brain neurohumoral agents, 5-HT and NE. However, within the past two years, renewed interest in the effects of reserpine on the adrenal cortex has been noted.

The endocrine aspects of reserpine (0.5 - 1.0 mg/kg) were first studied by Guant et al (195') in normal animals. From organ weight data, they concluded that reserpine was a mild stimulant of the adrenal cortex, while also possessing the ability to depress thymus and gonadal weights of male and fe-

male albino rats. Accordingly, reserpine acted as a mild stressor to which the organism was adapting.

Christian (1956) studied the chronic effects (three weeks) of reserpine (6.67 mg/ml of drinking water) in mice living in isolation or crowding. Reserpine was found to inhibit the adrenal growth associated with each stress and was found to decrease fighting amongst mice subjected to chronic crowding. Some animals after two weeks of isolation were suddenly placed in crowded quarters. Reserpine was found to have little effect on the environmental stress in mice induced by the preceeding change. It was concluded that reserpine was able to inhibit the sociological conflicts with no apparent effect on changes in environment.

In contrast Wells et al (1956) found reserpine to be a potent inhibitor of ACTH release in rats. Reserpine was administered (2.5 mg/kg, I.P.) for five days in order to adapt the animals to the drug. On the last day the animals were subjected to either histamine or ether stress. These workers utilizing the depletion of adrenal ascorbic acid (AAA) as an index of adrenocortical hyperfunction, demonstrated that reserpine was an initial stimulator of stress, but after five days, inhibited these superimposed stresses.

Guillemin (1957), while attempting to determine whether any of the proposed neurohumoral agents (Serotonin, Norepinephrine, Acetycholine and Histamine) were identical to the Corticotrophic Releasing Factor (CRF), did not agree with previous findings as to the ability of tranquilizers to inhibit acute stressors. In

fact, he found that some of these drugs were themselves potent stressors (chlorpromazine and reserpine) although animals in this investigation were preinjected with the drugs 7 days prior to the initiation of the stress. Guillemin's experiment differed from previous research in that it utilized a psychological stressor, forced restraint stress (holding an animal on its back for 90 min.). He observed that while reserpinized rats did not resist being restrained, the animal still displayed adrenocortical hyperfunction as indicated by the depletion of AAA.

The ability of reserpine to inhibit environmental stressors was again demonstrated by Mahouz and Ezz (1958). Utilizing continuous ether anesthesia with exsanguination, heat stress, and cold stress, they found that reserpine inhibited the usual depletion of AAA associated with these stressors. Since reserpine was not able to inhibit the usual depletion of AAA by various doses of ACTH, and reserpine did inhibit the above mentioned stressors, these workers concluded that reserpine inhibited the pituitary-adrenal axis by inhibiting some central regulatory mechanism, possibly the hypothalamus.

A novel theory of reserpine inhibition of ACTH release was reported by Kitay et al (1959). They contend that reserpine could cause depletion of ACTH, such that a stress following its administration would be unable to induce ACTH release, because of exhausted ACTH stores. Thus, a drug could appear to inhibit the pituitary, but in essence, be actually stimulating it. This research has been extremely controversial, but

might help explain previous divergent results concerning drug effects on the pituitary-adrenal axis.

Saffaran and Vogt, (1960), found that 2.5 mg/kg of reserpine (I.P.) caused ACTH release equivalent to that produced by the reserpine vehicle used in this experiment (the identical vehicle used in this investigation). These workers studied the effects of reserpine on the pituitary directly by estimating the ACTH content of the pituitary, by in vitro methods. They further demonstrated that the depletion of ACTH by reserpine lasted only five days. Thus, they also concluded that at best reserpine affected the pituitary-adrenal axis as a non-specific stressor.

Recently Montanari and Stockham (1962), have clarified to some degree previous conflicting reports. They have demonstrated that a single 2.5 mg/kg dose of reserpine produces an increase in the plasma corticosterone levels. After the administration of this dose for four days, ether still produced adrenocortical hyperfunction (an increase of the plasma corticosterone levels). This finding, therefore, appears to contradict the predictions of Kitay et al (1959) and Maickel et al (1961).

In a series of interesting papers, Maickel, Westermann and Brodie (1962) have demonstrated excellent correlation between ACTH release and decreased brain serotonin levels. Maickel et al (1961) showed that reserpine was able to stimulate the pituitary-adrenal axis as evidenced by increased plasma corticosterone levels. By studying these changes it was concluded that reserpine (5 mg/kg, I.V.) produced a persistent

stimulation of ACTH for at least 20 hours. An equivalent effect was also observed during the exposure of rats to 20 hrs. of a cold stressor (4°C.). A similar relationship between cold stress and reserpine was also demonstrated by their ability to deplete the pituitary of its normal stores of ACTH by at least 75%. Westermann et al (1962) in their most recent publication again demonstrated the ability of reserpine to induce ACTH release, but with selective NE and 5-HT depleters (alpha-MMT and various benzoquinolizine derivatives) have presented excellent evidence indicating the dependence of ACTH release on brain serotonin. With the combined efforts of these workers and others (Revizin et al, 1961) they have presented a good case indicating the relation between Limbic stimulation via reserpine and ACTH release.

The Present Investigation

In reviewing the research concerning the mode of action of reserpine, the lack of correlation between different investigations at first may not be obvious unless one critically reads the papers cited here. The major difficulty in this research has been the inability of different investigators to agree on approaches and methods in which to study the mode of action of reserpine. This is especially true of investigations involving reserpine and its effects on the pituitary-adrenal axis.

It has been found very frustrating to observe different workers criticizing each other while at the same time they are studying reserpine in different systems. A good example of this is Montanari's (1962) statement that reserpine does not

exhaust the pituitary of its ACTH stores as a means of pituitary inhibition of further stresses as proposed by Maickel. Maickel studied ACTH directly in the pituitary, whereas Montanari based his conclusions on adrenal and plasma corticosterone levels. He apparently overlooked the possibility that reserpine may also be acting directly on the adrenal cortex. A second example of this criticism is the fact that different workers utilize different indexes for determining pituitary-adrenal function when criticizing each other as to whether reserpine stimulates or inhibits the pituitary adrenal axis, (adrenal ascorbic acid and plasma corticosterone). This point is of even more importance since Montanari and Stockham (1962) have shown that AAA and plasma corticosterone do not always parellel each other in response to pituitary-adrenal stimulation.

A second criticism of previous research in this area is the apparent disregard of basic pharmacological principles in studying the mechanism of action of reserpine. Throughout pharmacology's short history, much has been learned by studying the effects of chronic doses of drugs, especially in relation to the modes of action and side effects of drugs. In spite of this, present investigators refuse to study the effects of drugs chronically in attempting to fortify their own theories as to mechanisms of action. In relation to this, few investigators have taken the time out to study the chronic effects of reserpine on brain neurohumeral levels or the pituitary-adrenal axis.

It would seem logical to conclude that if reserpine acts by depleting normal brain 5-HT levels after one dose, it should also do so after thirty-two or even one hundred doses. Reserpine stimulation or inhibition of the pituitary-adrenal axis should also fall under the same analogy. If the thirty-second or one hundredth dose does not induce similar effects as the first dose, then either the drug does not act via this mechanism or the mechanism is being obscured by a secondary cause. Very few workers have utilized such an approach and because of this dismaying fact, the present investigation was initiated.

In view of these statements the following hypothesis concerning the relationship between chronic stress and reserpine sedation will be presented:

Hypothesis I

If reserpine is either a specific stimulant or depressent of pituitary-adrenocortical hyperfunction in response to stress then these effects should be maintained throughout the duration of the present experiment.

Hypothesis II

If reserpine produces its sedative effects by releasing either brain NE or 5-HT, then these effects should persist throughout the duration of the experiment.

Hypothesis III

If either of the above statements cannot be shown to be true, then either reserpine does not act by these mechanisms, or a secondary effect is obscuring them.

Since the first pages of this dessertation constant reference has been made to animal adaptation to environmental changes and the possible effects of drugs on various segments of these homeostatic mechanisms (Fig. 1). The above hypotheses make little mention of these ideas but were certainly instrumental in their formulation.

Unfortunately many drugs which affect the C.N.S. are usually only studied in normal animals. This may appear irrelevant to this investigation, but when one considers that drugs are usually used in the treatment of diseased states rather than normal activity, this fact does have importance. It would also be important to know how drugs affect animal responses to environmental stressors, since many drugs are used for long periods of time in human therapeutics. This is especially true for reserpine.

Thus, aside from attempting to learn more about the mode of action of reserpine, the importance of studying drug effects in chronically stressed animals from the view point of human pharmacology was also emphasized.

III EXPERIMENTAL

General

Male albino rats (150-220 g) of the Sprague Dawley strain were used throughout this investigation. Reserpine[1] was dissolved in a solution consisting of: 10% polyethylene glycol, 1% benzyl alcohol and 0.25% citric acid.

The following general procedures were conducted throughout this investigation except where otherwise stated:

1. Solutions of reserpine were prepared such that each dose was equivalent to 1 ml/kg.
2. All animals serving as controls received 1 ml/kg, I.P., of the reserpine vehicle.
3. In each individual experiment all animals were administered either reserpine or its vehicle at 8:00 A.M.
4. Except where otherwise stated, all animals were sacrificed by decapitation 8 hours following the administration of reserpine or its vehicle.
5. All animals used in chronic experiments initially weighed between 145 and 160 g. All other animals used weighed vetween 190 and 220 g.

In order to determine the degree of sedation produced by reserpine three characteristic symptoms were utilized. These were: sedation, bleopharospasm or ptosis (eye closure) and

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[1] Kindly supplied by Dr. R. Gaunt, Ciba Pharmaceuticals, Inc., Summit, New Jersey

diarrhea (Montanari and Stockham, 1962). In normal animals this syndrome was always observable indicating the inability of animals to develop tolerance to reserpine. Ptosis, however, was found to be of little quantitative value due to daily subjective interpretations.

Design of Experiments

Due to the complex nature of this investigation, it was found necessary to perform three major interrelated experiments.

1. Single and Repeated Dose Recovery Experiments with Reserpine

This experiment was designed to determine the recovery of serum corticosterone (KS) and brain serotonin (5-HT) and norepinephrine (NE) following one and thirty-two doses of reserpine (1 mg/kg, I.P.) respectively. Thirty-two animals receiving a single dose of reserpine were sacrificed at either 4, 8, 12, or 24 hours following its administration. Twenty-four animals serving as controls were also sacrificed at equivalent times.

Animals were preinjected with the reserpine vehicle (1 ml/kg, I.P.) every other day for 14 days to permit adaptation to both vehicle and injection procedure. Experiments were conducted on the 15th day.

Similar experiments were conducted following 32 doses of reserpine. Twenty-eight animals receiving reserpine (1 mg/kg, I.P.) daily were sacrificed at either 8, 24, 80, or 154 hours following the last dose of reserpine. An equal number of animals serving as controls were also sacrificed. In this experiment animals were allowed food ad libitum.

2. Effects of Chronic Restraint Stress in Normal and Reserpinized Male Albino Rats

This experiment was designed to test the previous hypotheses made. It was hoped that this experiment would ultimately determine whether reserpine possessed any specific effects on the pituitary-adrenal axis.

One hundred and eighty animals initially weighing 145-160 g were used in the experiment. Animals in each experiment were divided into four experimental groups.

1 - CONTROL (These animals received (1 ml/kg, I.P.) the reserpine vehicle daily)

2 - STRESS (Control animals + a daily forced restraint stress)

3 - CONTROL + RESERPINE (These animals received 1 mg/kg, I.P. of reserpine daily)

4 - STRESS + RESERPINE (These animals received 1 mg/kg, I.P. of reserpine daily + restraint stress daily)

Individual experiments of 1, 6, 12, 18, and 32 days were conducted. Stressed animals were subjected daily to a forced restraint stressor for a period of three hours by tying these animals on their backs to specially constructed boards (Renaud, 1959). Stress was initiated 5 hours after reserpine or its vehicle was administered to respective experimental groups. Animals were sacrificed immediately following release from restraint at the termination of the above mentioned experiments.

The animals of both stress groups were housed singly in standard metabolism cages constructed to isolate them from light stimuli while not under restraint. Control animals (unstressed) were housed in similar metabolism cages, two animals

per cage; these animals were not isolated from light stimuli.

Urine volume, water and food intake were determined daily for all experimental groups in each experiment. Each animal was allowed 18 g of purina rat chow daily. This quantity of food was based on the average food intake for rats receiving chronic reserpine treatment (1 mg/kg, I.P.).

3. Effects of Single and Repeated Graded Doses of Reserpine in Normal Male Albino Rats

This experiment was a second attempt at determining whether reserpine possesses any specific effects on the pituitary-adrenal axis. It was also utilized to clarify the interrelationships between reserpine sedation and brain 5-HT and NE levels.

Single graded doses of reserpine, 0.5, 1.0, 2.5, and 5.0 mg/kg, I.P., were administered to male albino rats (8 animals/dose). Eight hours following administration all animals were sacrificed. These animals were also preinjected with the reserpine vehicle as in the first experiment (every other day for 14 days).

A repeated graded dose experiment was also conducted using 64 male rats. These animals were divided into four groups. Three groups received different doses of reserpine (0.5, 1.0, and 2.0 mg/kg, I.P.); the last group serving as control. All animals were sacrificed 8 hours after the 32nd dose of reserpine. Doses of reserpine and its vehicle were administered each day at 8:00 A.M.

In an attempt to separate rats which would be sensitive or resistant to reserpine, a special feeding and housing schedule

was utilized. This schedule was based on the premise that sensitive rats would eat less and thus, present a decreased rate of growth. This is a logical statement since the degree of reserpine-induced inanition is dependent on its dose (Gaunt et al, 1954). In relation to sensitive and normal reserpinized rats, resistant rats would be expected to eat more and present a greater rate of growth.

The schedule used is as follows:

1. Animals were allowed 18 g of food/animal for 10 days and were housed 20 animals/cage.
2. Animals were then subdivided by weight groups and placed in cages in groups of 10 animals/cage. Food was allowed ad libitum.
3. After 20 days of the experiment animals were again subdivided into three weight groups of 6-8 animals per group.

Sacrifice, Removal and Preservation of Tissue

Sacrifice was accomplished by decapitation with a manual guillotine[1]. Blood was collected in 12 ml centrifuge tubes and allowed to stand for 8 to 16 hrs. in a cold room (12°C). Blood samples were then centrifuged at 2,000 R.P.M., serum removed by decantation and stored at -10°C until assay.

Brains were removed within two minutes after sacrifice and frozen immediately in test tubes immersed in a freezing mixture of solid carbon dioxide and trichlorethylene. The brains were later removed from the tubes and preserved in tin (Al) foil at -10° until assay.

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[1] Harvard Instrument Co.

Organ Weight Data

Following the removal of brains, rat carcasses were placed in a cold room (12°) and were autopsied no later than 24 hrs after sacrifice. At autopsy, the adrenals, pituitaries, thymus and testes of each animal were removed, freed of fatty tissue, blotted and weighed to the nearest 0.1 mg on a micro tissue balance.

After weighing, the adrenal glands were dried to a constant weight in an oven at 105°C. In some instances pituitaries were air dried in a desiccator and weighed (due to the small weights involved mean weights were determined by pooling groups of pituitaries).

Chemical Assays

1. Serum Corticosterone (KS)

In rats, the major adrenal corticosteroid has been determined to be corticosterone (Zenker and Bernstein, 1958). KS levels were determined according to the spectrofluorometric method of Guillemin et al (1959) in which corticosterone develops a fluorescent product in 84% H_2SO_4. Fluorescence was read on an Aminco-Bowman Spectrofluorometer[1] at an activating wave length of 470 mu and a fluorescent wave length of 510 mu (photomultiplier tube = IP21). Standard curves (external standards) were prepared by running known amounts of corticosterone[2] (0.2-1.0 ug/ml) through the assay procedure. Recoveries from serum were 98±5% (± S.D., N=6). Values were reported as ug/100 ml. of serum.

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[1] American Instrument Co. In all experiments utilizing this instrument assays were performed in quartz cuvettes utilizing an Xenon light source with a No. 5 slit arrangement.

[2] Kindly supplied by Dr. G. C. Strayer, Schering Co., Bloomfield, N. J.

2. Brain Serotonin (5-HT) and Norepinephrine (NE)

Brains were homogenized with 0.1N HCl in a Teflon Homogenizer and a 3-6 ml aliquot of the homogenate (300-400 mg of brain/ml) was taken for assay. Assays were usually performed on pooled samples (2-5 brains/pool) except where indicated. Both amines were extracted with an NE butanol extraction procedure which has also been used to extract 5-HT (Mead and Finger, 1961). Serotonin was assayed by its natural fluorescence at an activating wave length of 292 mu and a fluorescent wave length of 342 mu (photomultiplier tube = IP28) in an Aminco-Bowman Spectrofluorometer. Brain NE was assayed by the development of fluorescence with potassium ferricyanide (Maynert and Klingman, 1962). Its wave lengths for activation and fluorescence were respectively 475 mu and 520 mu. It was also measured in an Aminco-Bowman Spectrofluorometer (photomultiplier tube = IP21).

Standard curves (external standards) were prepared by running known concentrations of both NE[1] and 5-HT[1] (0.5-5.0 ug/ml) through the assay procedure. Recoveries from grain homogenates were 95±10% (± S.D., N=6) and 76±8% (± S.D., N=12) respectively for NE and 5-HT. Values were reported as uug of free base/g of brain tissue. Duplicates for NE determinations were run with each assay. Duplication between homogenates for each amine was of the order of 97% (S.D.=±3.3%). Maynert and Klingman, (1962) reported a S.D. of ±2.7%.

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[1] Both amines were obtained from Nutritional Biochemical Corp., Cleveland, Ohio. NE was used as the free base, whereas 5-HT was used as the Creatinine sulfate.

Control Data

Before the results obtained from these experiments is presented, the validity and variability of the control data should be discussed (TABLES I-XV). During the chronic restraint experiment, controls were separated from experimental animals by one closed door. Although no animals were restrained in the presence of control animals, they were released from restraint in their presence. Aside from the presence of other species in the animal room, several other persons conducting research were also present at various times. All of these factors, therefore, appeared to exert an influence as observed from the presentation of control data. Therefore, all animals (normal and reserpinized) were subjected to an inconsistent type of stress. If each control value is compared against the mean value, this variable is demonstratable. Furthermore, the control animals present during this experiment also exhibited a slight G.A.S. of their own.

The recovery experiments following 1 mg/kg reserpine also demonstrate the daily variability of control data (TABLES I & II).

The results presented and discussed in this investigation have all been interpreted in light of this great variability, and were compared in some manner with a control value at the appropriate day or time of day.

Although the data presented demonstrate great variation (control and experimental) as demonstrated by the high standard deviations, the data presented are considered accurate and valid, due to the consistency of the values.

It should further be added that the organ weight relationships expressed in the following tables and figures, were not

expressed as relative weights (weight/100 g of animal), but as absolute weights. This preference was utilized because of the large variation in the growth rates of different groups of animals (TABLE III).

Finally, all values reported were analyzed statistically utilizing the student "t" test (Burn, 1952). The level of significance used throughout this research was at the 0.05 level.

IV. RESULTS AND DISCUSSION

Single and Repeated Dose Recovery Experiments with Reserpine

TABLE I

THE RECOVERY OF PITUITARY-ADRENAL FUNCTION AND BRAIN NOREPINEPHRINE AND SEROTONIN LEVELS FOLLOWING A SINGLE DOSE OF RESERPINE IN MALE ALBINO RATS[a]

HOUR OF DAY	SERUM[b] CORTICOSTERONE (KS)	BRAIN SEROTONIN (5-HT)	BRAIN NOREPINEPHRINE (NE)
	ug/100 ml	uug/g	uug/g
12:00[c] A.M.	39.0±15.0[s](8) 10.6±3.0(8)	431±26 586±87	286±179 481±61
4:00 P.M.	27.0±11.8(8)[s] 16.0±4.0(6)	458±23 578±51	123±42 250±79
8:00 P.M.	17.0±10.0(8) 21.7±8.0(6)	357±28 486±64	112±12 343±160
8:00 A.M.	10.3±3.0(8) 9.0±5.0(6)	443±33 529±90	215±112 623±34

a - The dose of reserpine was 1 mg/kg, I.P.

b - The values in this column represent the number of animals studied. For brain assays pools of two brains each were used.

s - Significant from control values. All values are means ± S.D.

c - All animals were administered either drug or vehicle at 8:00 A.M. The lower values at each hour (horizontal) are control values.

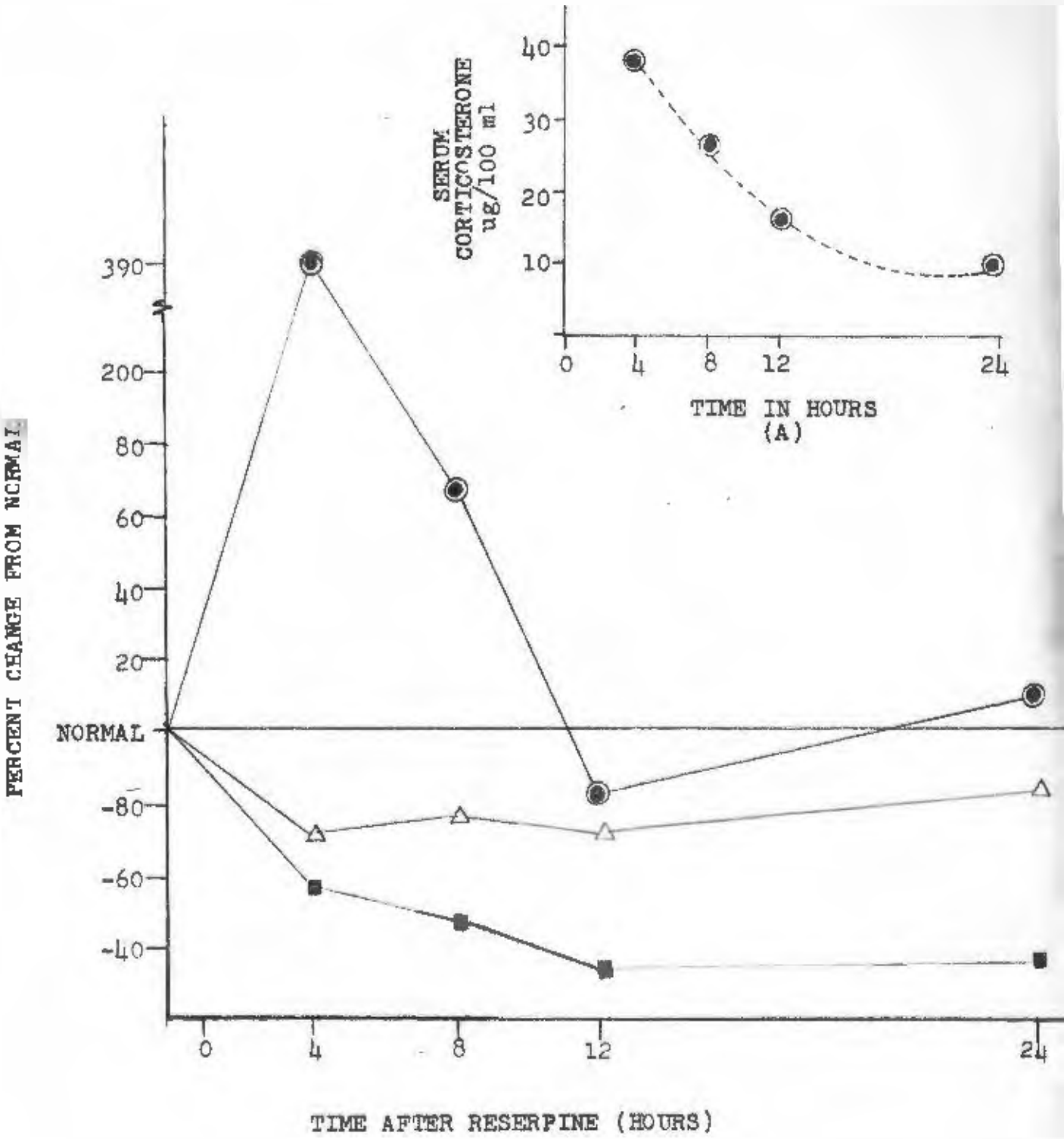

Fig. 2. The relationship between pituitary-adrenocortical responses and brain neurohumoral changes following a single dose of reserpine (1 mg/kg, I.P.)

Levels of serum corticosterone (●—●), brain serotonin (△—△) and brain norepinephrine (■—■) were given. Each point represents the mean of either 6 to 8 animals or 3 to 4 brain pools (2 brains/pool)

(A) This curve represents the actual serum corticosterone changes following a single dose of reserpine.

TABLE II

THE RECOVERY OF PITUITARY-ADRENAL FUNCTION AND BRAIN NOREPINEPHRINE AND SEROTONIN LEVELS FOLLOWING REPEATED DAILY DOSES OF RESERPINE (32 Days)[a]

TIME[b]	SERUM[c] CORTICOSTERONE (KS)	BRAIN SEROTONIN (5-HT)	BRAIN NOREPINEPHRINE (NE)
hr	uug/100 ml	uug/g	uug/g
8	27.9±10.5(10) 25.0±12.0(8)	422±83 605±77	165[d] 498±191
24	9.5±4.0(6) 15.8±6.0(6)	973±546[s] 534±29	163±23[s] 623±29
80	16.0±8.0(8) 27.3±9.0(6)	620±200 563±69	106±23[s] 645±168
152	47.0±18.5(4) 34.0±17.0(8)	495±92 697±123	247[e] 399±92

a - The dose of reserpine was 1 mg/kg, I.P.

b - Time refers to time after the last dose of reserpine.

c - The values in this column represent the number of animals studied. For brain assays pools of two brains each were used.

d - Two of 3 brains assayed exhibited no NE.

e - Represents a pooled value (4 brains).

s - Significant from control values. All values are means ± S.D.

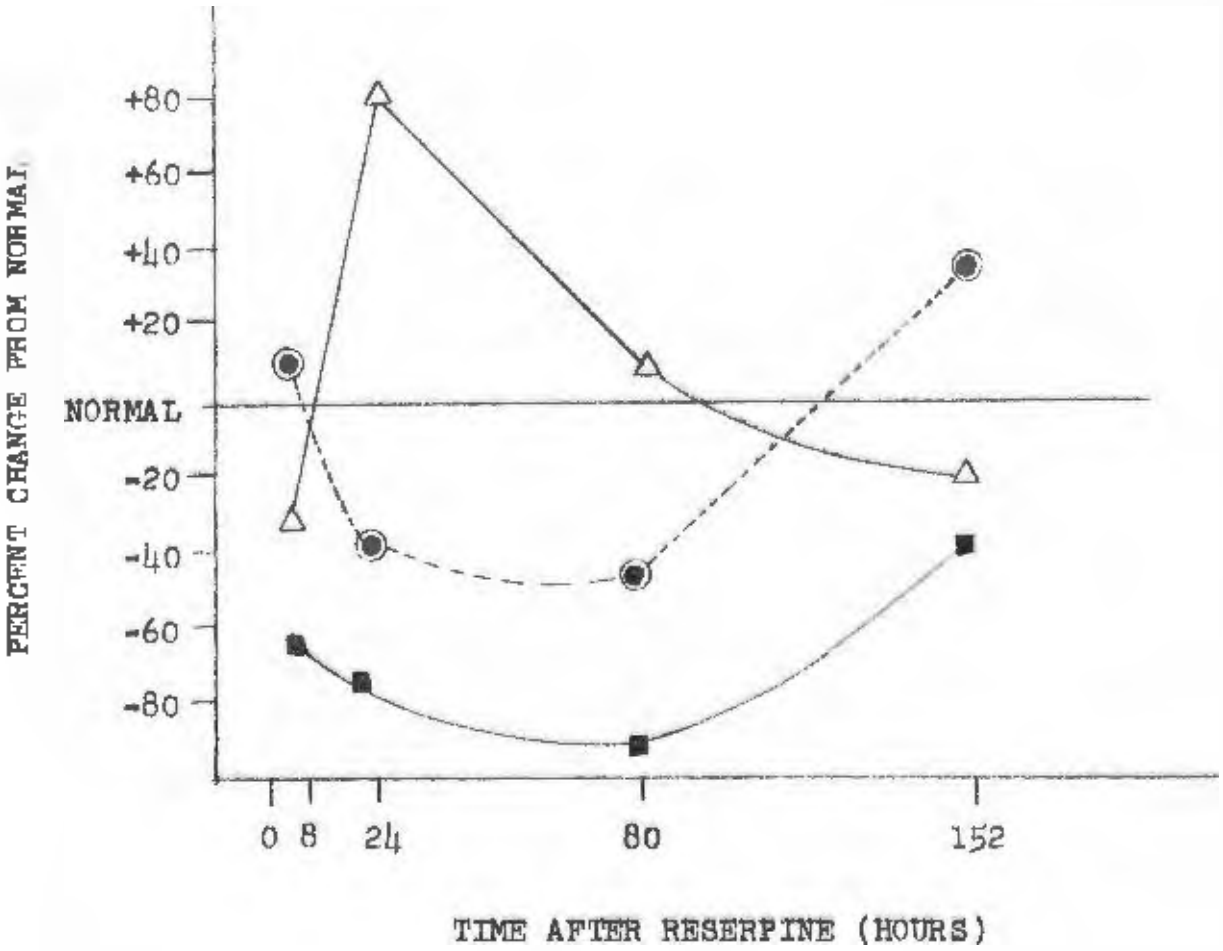

Fig. 3. Pituitary-adrenocortical and brain neurohumoral recovery following thirty-two doses of reserpine (1 mg/kg, I.P.) Levels of serum corticosterone (●—●), brain norepinephrine (■—■) and brain serotonin (△--△) were given. Time refers to the number of hours following the last dose of reserpine. Each point represents the mean of 4 to 6 animals or 2 to 4 pools of brains (2 brains/pool)

DISCUSSION

Reserpine (1 mg/kg, I.P.) was observed to produce acute pituitary-adrenocortical activation as indicated by the increase in serum KS levels four hours following its administration (TABLE I, Fig. 2). This effect was not observed to be continuous with reserpine-induce sedation since at the time of maximal sedation, 8-12 hrs following its administration, serum KS levels were either decreasing or below normal levels. A similar lack of correlation was also observed with brain NE and 5-HT depletion. Therefore, pituitary-adrenocortical activation did not appear to be dependent upon brain NE or 5-HT levels nor upon reserpine-induced sedation. These results are in direct conflict with the work of Westermann et al (1962) who have shown a relationship between reserpine-depletion of brain 5-HT and increased serum KS levels. Since these investigators did not preinject their animals prior to drug administration, they may not have observed the desired specific effect necessary for such a conclusion.

Reserpine also appeared to inhibit the diurnal variation of serum KS levels at 4:00 P.M. (TABLE I). Whether this effect was induced by sedation or via pituitary exhaustion is not clear. The significance of this finding, however, is evident after reading the papers of Westermann et al (1962) and Montanari and Stockham (1962). These workers have neglected to analyze their own data in light of daily serum KS variation,

or have made no mention of its presence. Therefore, it is difficult to imagine how these workers could conclude that reserpine-induced pituitary-adrenocortical activation was continuous with its sedative effects. As one can see, reserpine appears to possess a stimulating effect when considering actual KS levels (TABLE I, Fig. 2A)), whereas when compared to control levels, inhibition is evident (at 4:00 P.M.).

Recovery from chronic reserpine administration (1 mg/kg, I.P.), however, appears to produce an inhibition of the pituitary-adrenocortical axis variation 24 hrs following its administration (TABLE II, Fig. 3). The decreased serum KS values 80 hours following the last dose of reserpine suggests pitituitary-adrenocortical inhibition by reserpine. This fact is further verified by the apparent adrenocortical compensation demonstrated by increased serum KS levels 152 hours following the 32nd dose of reserpine. Since serum levels were slightly above normal 8 hrs following reserpine administration on the 32nd day, subsequent pituitary-adrenocortical inhibition may have resulted from exhaustion of this system.

As observed throughout this experiment, the onset of reserpine sedation appeared about 4 hrs following its administration with maximal sedation appearing 8-12 hrs later. Recovery from sedation was evident by 24 hrs. In contrast, throughout the latter half of the chronic recovery experiment several animals were abnormally excitable 24 hrs following a previous dose of reserpine. These animals were extremely excitable and very sensitive to handling. They also frequently fought amongst themselves.

The development of acute sedation correlated well with decreased NE levels, whereas brain 5-HT depletion reached a maximum four hours following reserpine administration and was maintained at this level throughout the 12th hr (TABLE I, Fig. 2). Twenty-four hours following a single dose of reserpine brain 5-HT appears to be returning to normal in contrast to brain NE. Since sedation was also minimal at this time, brain 5-HT appears to be a better candidate as mediator of reserpine-induced sedation. Although these results are in conflict with the work of Brodie et al (1960) concerning equivalent depletion of both amines by reserpine, they do support his theory that reserpine mediates its effects through 5-HT depletion rather than NE depletion.

The recovery of brain 5-HT levels following thirty-two doses of reserpine took on a different character. Brain 5-HT levels were 80% above normal 24 hrs after the last dose of reserpine (TABLE II, Fig. 3). This increased level corresponded well with the behavioral excitability observed.

Since the appearance of maximal sedation was equally present 8 hrs following the administration of reserpine throughout the chronic experiment, this time was used to compare all data. When comparing the effects of reserpine on brain neurohumoral levels after one and thirty-two doses of reserpine, equivalent progressive depletion of both amines was apparent (TABLES I and II). However, in subsequent experiments reserpine appeared to induce a greater progressive effect on brain NE than on 5-HT. The following table has been prepared to summarize this and subsequent experiments in order to clarify the preceding statements.

TABLE IIA

A COMPARISON OF THE EFFECTS OF RESERPINE ON BRAIN NE AND 5-HT LEVELS OBTAINED FROM DIFFERENT EXPERIMENTS

	% DEPLETION[a]		
	DAY 1	DAY 32	PROGRESSIVE DEPLETION DAY 1/DAY 32
5-HT	26%	31%	16%
NE	37%	73%	51%

a - The values presented are the means of experiments conducted in subsequent experiments; TABLES I, II, XI, XII, and XV. % DEPLETION refers to the percent of control values at that time.

Therefore, reserpine does appear to have a greater chronic effect on brain NE levels than on brain 5-HT levels when comparing the progressive depletion of both amines (TABLE IIA). It should be added that confirmation has since been obtained in this laboratory which re-emphasizes the results of TABLE IIA. These observations coupled with the high brain 5-HT levels observed with chronic recovery experiments (TABLE II), could be explained on the basis of an increased rate of 5-HT synthesis.

In relation to the hypotheses formulated initially, the following observations can be made.

1 - As indicated by the serum KS levels after one and thirty-two doses, reserpine stimulates the pituitary-adrenocortical axis acutely, but apparently loses

its effect during chronic administration. However, whether the lack of a chronic effect was due to pituitary exhaustion it is not clear.

2 - The comparative effect of reserpine on brain NE levels was greater than on 5-HT levels after single and thirty-two doses of reserpine. The differences in brain levels could be due to an increased rate of 5-HT synthesis during reserpine treatment.

IV. RESULTS AND DISCUSSION

Effects of Reserpine in Animals Subjected to Daily Forced Restraint Stress

TABLE III

EFFECTS OF CHRONIC RESTRAINT STRESS ON THE FOOD AND WATER BALANCE AND MORTALITY RATE OF NORMAL AND RESERPINIZED MALE ALBINO RATS (32 days)[a]

	n	WATER INTAKE	URINE EXCRETION	MEAN WEIGHT GAIN/DAY	FOOD INTAKE	M.R.[b] %
		ml	ml	g	g	%
CONTROL	8	38.7±4.1	7.5±4.0	3.88	18	0
STRESS	8	41.6±4.3[s]	7.8±2.1	2.84	18	0
CONTROL + RESERPINE	8	29.1±4.8[x]	3.5±1.7[x]	1.99	15.7	6.6
STRESS + RESERPINE	12	24.8±6.7[z]	3.5±1.3[x]	0.50	12.6	50.0

a - All values are means of 32 days/animal/day. Where indicated values are means ± S.D. Dose of reserpine was 1 mg/kg, I.P.

b - Abbreviations - M.R. = Mortality Rate (%Dead/Alive)
n = Number of animals studied.

s - Significant from control group.

x - Significant from both the control and stress groups.

z - Significant from all previous groups.
All animals initially weighed about 160 g.

TABLE IV

EFFECTS OF CHRONIC RESTRAINT ON THE THYMUS WEIGHTS OF NORMAL AND RESERPINIZED MALE ALBINO RATS[a]

DAY	CONTROL	STRESS	CONTROL + RESERPINE	STRESS + RESERPINE
	mg	mg	mg	mg
1	399±104 (8)	300±44^{s} (8)	329±62 (8)	369±61^{x} (8)
6	344±86 (6)	244±32^{s} (8)	313±149 (8)	231±77^{s} (8)
18	432±97 (8)	324±77^{s} (8)	366±81 (10)	167±64^{sx} (10)
32	385±63 (8)	324±64 (8)	298±69^{s} (8)	118±64^{sx} (12)

a - All values are means ± S.D. Values in parenthesis represent the number of animals used. Dose of reserpine was 1 mg/kg, I.P.

s - Significant from control group.

x - Significant from stress group.

TABLE V

EFFECTS OF CHRONIC RESTRAINT STRESS ON THE TESTES WEIGHTS OF NORMAL AND RESERPINIZED MALE ALBINO RATS[a]

DAY	CONTROL	STRESS	CONTROL + RESERPINE	STRESS + RESERPINE
	mg	mg	mg	mg
1	1.88±0.28	1.94±0.92	1.79±0.30	1.77±0.33
6	2.42±0.67	2.14±0.52	2.16±0.92	2.11±0.40
18	2.96±0.50	2.90±0.64	2.74±0.45	2.53±0.38
32	2.80±0.45	3.11±0.54	2.05±0.67	2.20±0.87

a - All values are means ± S.D. (None of the values presented here were significant from any other group). The number of animals studied appear in TABLE IV. Dose of reserpine was 1 mg/kg, I.P.

TABLE VI

EFFECTS OF CHRONIC RESTRAINT ON THE WET ADRENAL WEIGHTS OF NORMAL AND RESERPINIZED MALE ALBINO RATS[a]

DAY	CONTROL	STRESS	CONTROL + RESERPINE	STRESS + RESERPINE
	mg	mg	mg	mg
1	27.3±4.6	23.2±1.6[s]	27.5±1.9	25.8±2.6[x]
6	35.8±6.0	34.2±5.5	33.5±6.0	35.1±3.3
18	31.0±7.6	39.7±5.0[s]	38.7±5.6	39.0±4.3[s]
32	37.8±4.5	49.1±7.6[s]	42.7±4.7	48.2±8.5[s]

a - All values are means ± S.D. The number of animals used in this experiment appear in TABLE IV. Dose of reserpine was 1 mg/kg, I.P.

s - Significant from control group.

x - Significant from stress group

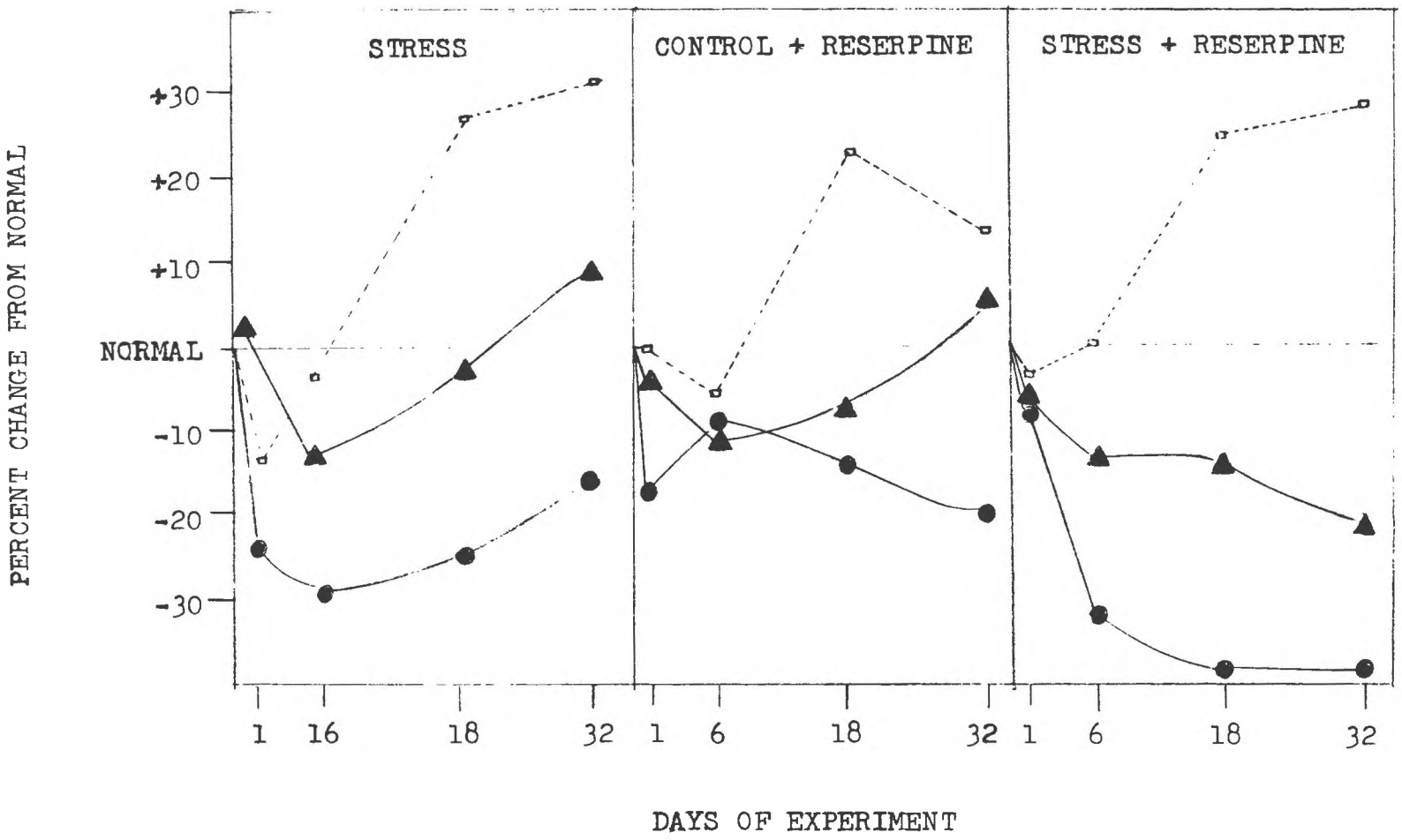

Fig. 4. A comparison of the effects of chronic restraint stress on various organ weights of normal and reserpinized male albino rats.

The wet weights of adrenals (▫——▫), thymus (● ●) and testes (▲ ▲) are given. Each point represents the means of 6 to 12 animals. Dose of reserpine was 1 mg/kg, I.P.

TABLE VII

EFFECTS OF CHRONIC RESTRAINT STRESS ON THE DRY ADRENAL WEIGHTS OF NORMAL AND RESERPINIZED MALE ALBINO RATS[a]

DAY	CONTROL	STRESS	CONTROL + RESERPINE	STRESS + RESERPINE
	mg	mg	mg	mg
1	6.2 ± 0.2	5.6 ± 0.2^{s}	6.8 ± 0.8	5.8 ± 0.5^{sx}
6	7.1 ± 1.6	8.5 ± 0.4^{s}	7.7 ± 1.6	8.9 ± 1.3^{s}
18	8.2 ± 1.5	10.5 ± 1.5^{s}	9.8 ± 1.8	11.5 ± 2.7^{s}
32	9.1 ± 0.4	10.0 ± 2.5	12.3 ± 2.9	12.8 ± 1.1^{sx}

a - All values are means $\pm$ S.D. The number of animals studied in this experiment appear in TABLE IV. Dose of reserpine was 1 mg/kg, I.P.

s - Significant from control group.

x - Significant from stress group.

TABLE VIII

EFFECTS OF CHRONIC RESTRAINT ON THE % ADRENAL DRY WEIGHTS (ADW) OF NORMAL AND RESERPINIZED MALE ALBINO RATS[a]

DAY	CONTROL	STRESS	CONTROL + RESERPINE	STRESS + RESERPINE
	%	%	%	%
1	23.0±4.1	24.3±2.7	24.8±1.5	22.3±2.4
6	21.7±4.2	24.0±1.5	25.3±1.3	26.5±2.6
18	24.8±4.1	26.4±1.6	25.6± .6	29.2±2.5
32	24.7±2.7	21.7±8.7	27.4±4.2	27.0±4.1

a - All values are means ± S.D. (None of the values presented here were significant from any other group). The number of animals studied appear in TABLE IV. Dose of reserpine was 1 mg/kg, I.P.

TABLE IX

EFFECTS OF CHRONIC RESTRAINT STRESS ON THE PITUITARY WEIGHTS OF NORMAL AND RESERPINIZED MALE ALBINO RATS[a]

DAY	CONTROL	STRESS	CONTROL + RESERPINE	STRESS + RESERPINE
	mg	mg	mg	mg
1	1.09	0.89	0.90	1.10
6	1.67	1.05	1.30	1.28
18	1.71	1.65	1.57	1.25
32	1.76	1.89	1.56	1.54

a - The dry pituitaries from each group were pooled (6-8 pituitaries) for accurate weighing and are expressed as mg/animal (mean value).
Dose of reserpine was 1 mg/kg, I.P.

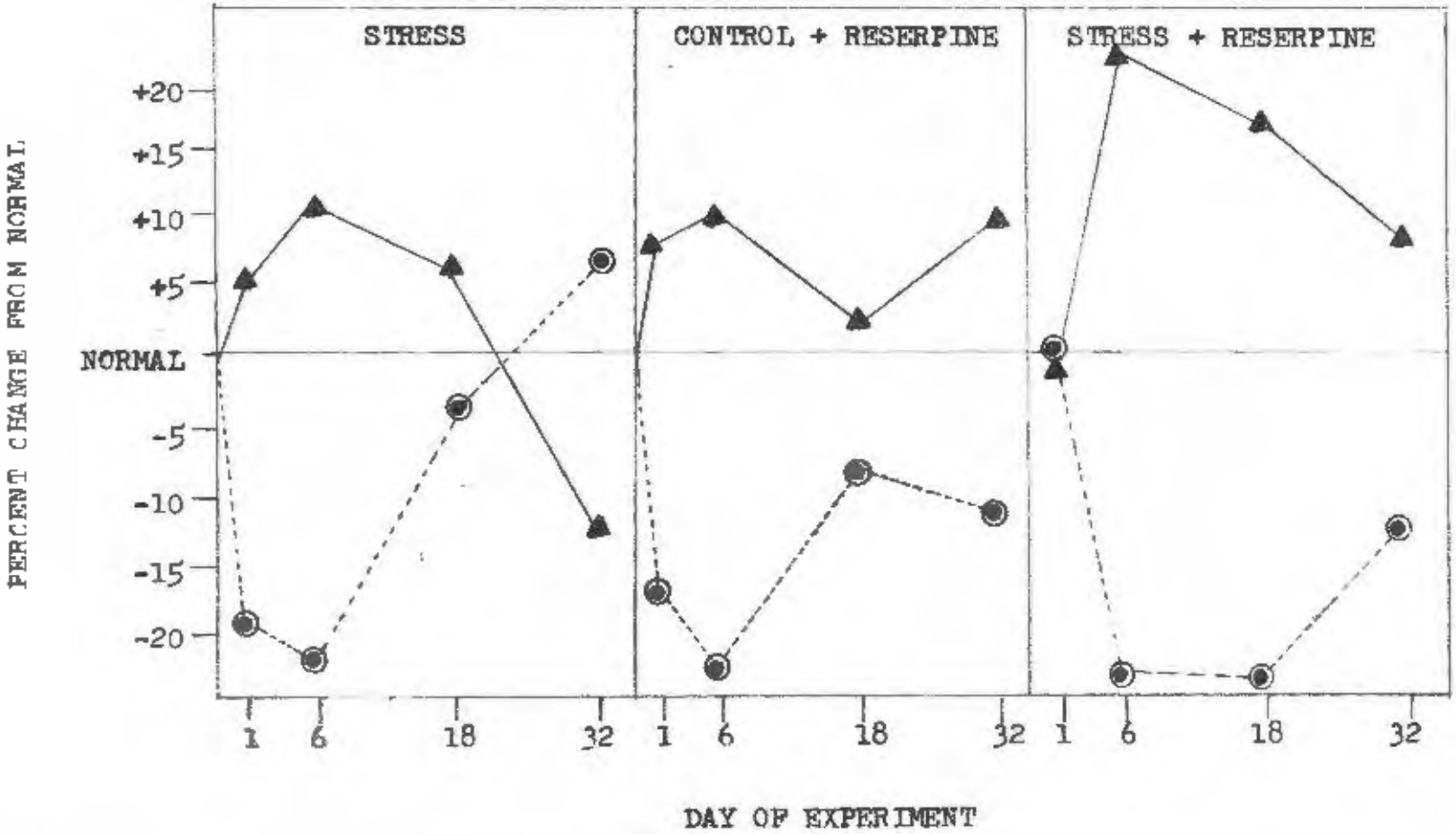

Fig. 5. A comparison of the effect of chronic restraint stress on pituitary-adrenocortical function of normal and reserpinized male albino rats.
The weights of dry pituitaries (●—●) and % adrenal dry weights (▲—▲) were given. Each point represents the mean of 6 to 12 animals. Dose of reserpine was 1 mg/kg, I.P.

TABLE X

EFFECTS OF CHRONIC RESTRAINT STRESS ON THE SERUM CORTICOSTERONE LEVELS (KS) OF NORMAL AND RESERPINIZED MALE ALBINO RATS[a]

DAY	CONTROL	STRESS	CONTROL + RESERPINE	STRESS + RESERPINE
	ug/100 ml	ug/100 ml	ug/100 ml	ug/100 ml
1	24.7±16.0	71.0±28.1[s]	51.5±16.2[s]	77.9±29.0[sx]
6	43.0±24.6	82.1±36.0[s]	42.6±17.2	64.0±35.9
12	36.1±22.7 (6)	59.5±22.2 (6)	44.3±14.0 (10)	84.7±15.7[sx] (8)
18	43.8±27.7	69.5±25.2[s]	38.2±21.4	50.3±30.6
32	33.5±16.9	59.1±22.1[s]	42.4±28.8	58.7±27.4[s]

a - All values are means ± S.D. The number of animals studied in this experiment appear in TABLE IV. Dose of reserpine was 1 mg/kg, I.P.

s - Significant from the control group.

x - Significant from the stress group.

TABLE XI

EFFECTS OF CHRONIC RESTRAINT STRESS ON BRAIN SEROTONIN (5-HT) LEVELS OF NORMAL AND RESERPINIZED MALE ALBINO RATS[a]

DAY	CONTROL	STRESS	CONTROL + RESERPINE	STRESS + RESERPINE
	ug/100 ml	ug/100 ml	ug/100 ml	ug/100 ml
1	796±43 (4)	961±210 (4)	485±125 (4)	850±137 (4)
6	698±43 (3)	1426±823 (4)	567±111 (4)	960±443 (4)
12	674±14 (4)	673±63 (3)	522±187 (3)	627±64 (3)
18	737±148 (4)	525±160 (5)	482±45 (4)	605±41 (6)
32	697±123 (5)	554±123 (5)	482±167 (6)	605±49 (6)

a - All values are means ± S.D. The number of brain pools studied is represented in the parenthesis (2 brains/pool). Dose of reserpine was 1 mg/kg, I.P.

s - Significant from control group.

y - Significant from control + reserpine group.

TABLE XII

EFFECTS OF CHRONIC RESTRAINT STRESS ON THE BRAIN NOREPINEPHRINE (NE) LEVELS OF NORMAL AND RESERPINIZED MALE ALBINO RATS[a]

DAY	CONTROL	STRESS	CONTROL + RESERPINE	STRESS + RESERPINE
	uug/g	uug/g	uug/g	uug/g
1	242±68	196±61	182±123	177±82
6	340±218	137±86	182±91	199±52
12	-------	-------	161±16	231±37
18	590±188	497±190	118±12[s]	122±64[s]
24	507±74	560±38	48-88	102-305
32	399±96	318±23	128±61[s]	112±73

a - All values are means ± S.D. The number of brain pools studied appear in TABLE XI. Dose of reserpine was 1 mg/kg, I.P.

s - Significant from control group.

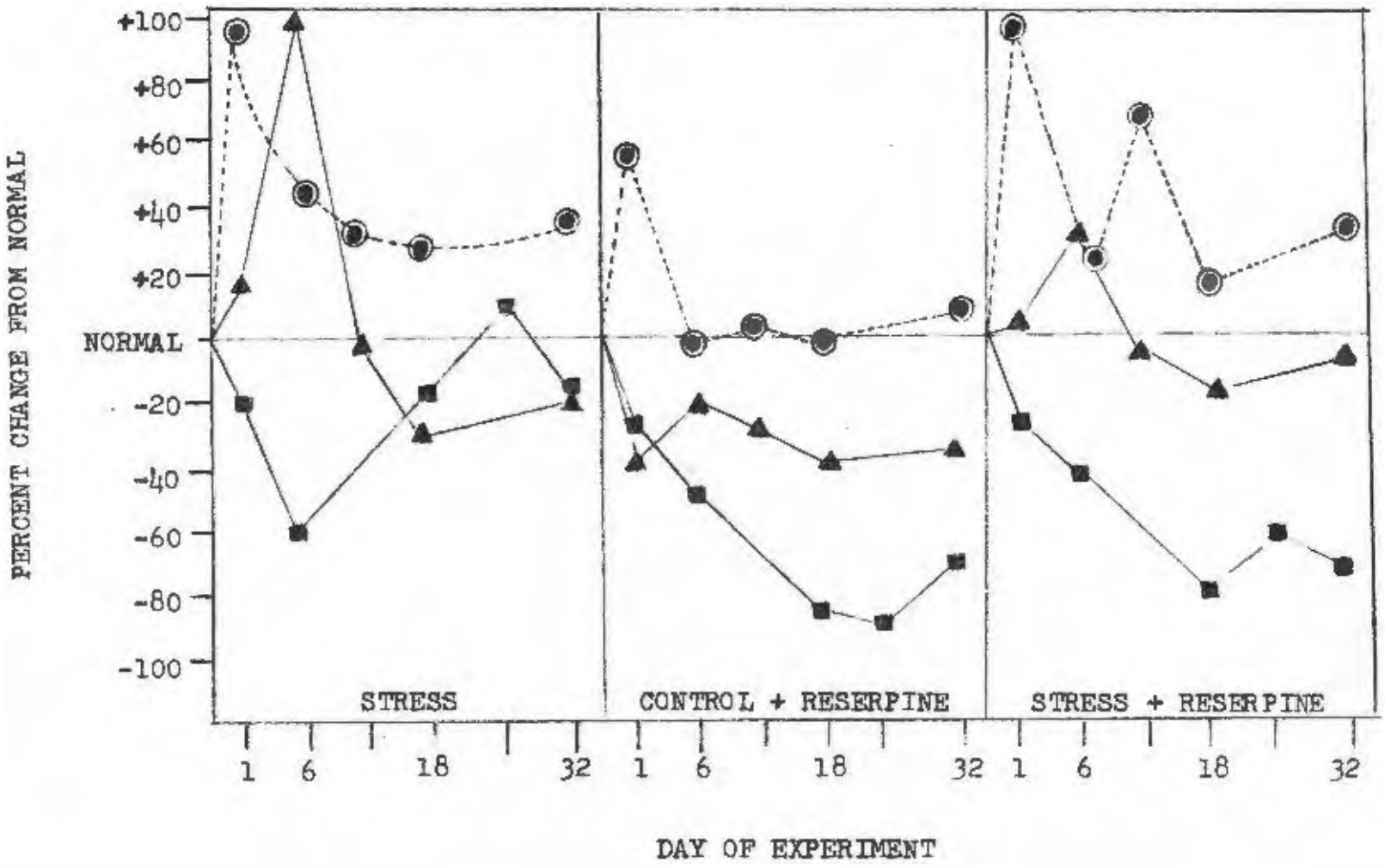

Fig. 6. A comparison of the effects of chronic restraint stress on serum corticosterone levels and brain neurohumoral levels.

Levels of corticosterone (●--●), brain norepinephrine (■—■) and brain serotonin (▲—▲) were given. Actual serum KS changes are obtained by doubling the values appearing above. Each point represents the means of either 6 to 12 animals or 3 to 6 brain pools (2 brains/pool). Dose of reserpine was 1 mg/kg, I.P.

DISCUSSION

The Effects of Chronic Restraint (RS) on the Pituitary-Adrenal Axis and Various Organ Weights of Normal Male Albino Rats

According to Selye's theories regarding chronic stress, animals respond differently to individual stressors only in a quantitative way; the responses all being qualitatively the same. Therefore, one of the first objectives of this experiment was to characterize the effects of chronic restraint stress in normal animals in terms of the G.A.S.

It was found difficult to completely characterize the alarm reaction, since experiments were designed at six day intervals (the alarm reaction usually occurs within the first three days of chrinic stress). The alarm reaction was evident to some degree by the significant decrease of the thymus weight of stressed animals on day one (TABLE IV). There was also a noted decrease in the pituitary weight which was also observed by Turner and Tinerty (1956) (TABLE IX). It is difficult to analyze glandular activity from only a weight change, but on the basis of the severity of restraint stress, the loss of weight could be due to ACTH and associated chemical depletion during extreme activation.

The significant decrease in wet and dry adrenal weights of normally stressed rats after the first day of the experiment (TABLES VI VII) was extremely interesting and unexpected. In contrast to this effect, Selye (1950) has observed an increase

of adrenal weight during the alarm reaction indicating adrenal hyperfunction. This effect could also be interpreted as indicating adrenal exhaustion caused by this stress. This fact, however, seems highly unlikely.

Another unexpected finding was the initial lack of effect of RS on the testes weight, which according to Selye (1950), should decrease following an acute stress. The present finding was confirmed by Albert (1942) who observed a delay in decrease of gonadal weights following chronic formalin stress (0.2 ml, I.M.). This was also observed in this experiment (TABLE V).

The ability of normal male albino rats to adapt to chronic restraint stress is evident after studying the effects of this stress on the thymus, testes, adrenal and pituitary weights (Figs. 4 & 5). Following the initial alarm reaction the convergence of all changes towards original or normal levels is an excellent example of animal adaptation to external environmental changes.

The inverse relationship which exists between the pituitary dry weight and %ADW of stressed rats appears to be one of the clues which may aid in explaining pituitary-adrenal adaptation (TABLES VIII-IX: Fig. 5). This relationship appears to confirm the earlier observation that a decrease in pituitary weight could be interpreted as indicating a state of hyperfunction. Thus, as an animal adapts to a stressor, the pituitary becomes less active as indicated by the progressive increase of dry pituitary (Fig. 3). The progressive decrease in the %ADW also indicates a similar decrease in adrenal function.

Although an apparent decrease in pituitary and adrenal function prevails during adaptation to stress, the serum corticosterone (KS) levels exhibit no relative adaptation and appear to be maintained at high levels throughout the experiment (TABLE X, Fig. 6). If the adrenal weights are an indication of corticosteroid synthesis and release, then the concurrent maintenance of both serum KS and ADW would indicate an increased adrenal efficiency during adaptation to stress (TABLES VIII and X).

The Effects of Chronic Restraint (RS) on the Pituitary-Adrenal Axis and Various Organ Weights of Reserpinized Male Albino Rats

The mortality rate of reserpinized rats subjected to RS was extremely high (50%, TABLE III), and clearly indicates the inability of reserpinized animals to adapt to chronic forced restraint stress. The reserpinized animals subjected to stress appear to die from starvation effects which seemed to stem from their inability to adapt to the stress since normal reserpinized animals demonstrated a mortality rate one-fifth that of the stressed animals. The decreased water and food balance caused by reserpine, attests to this nonadaptation.

The most striking effect of reserpine on the first day was its apparent inhibition of the effects of RS on the organ weights (thymus, pituitary and adrenal) of normal animals (TABLES IV, VI-IX, Figs. 4 and 5). It inhibited the weight changes by approximately 50%. The effects of reserpine on the testes of stressed animals is also interesting since it appeared to act independently of the stress by producing a decrease in weight (TABLE V).

Although reserpine apparently inhibits stress on the first day, thereafter, nonadaptation prevails. As observed in Figs. 4 and 5, organ weight changes are divergent in contrast to untreated animals under stress. There were some signs of adaptation as indicated by the increased pituitary weight and decreased %ADW on the thirty-second day (Fig. 5).

The serum corticosterone levels indicate that reserpine does not prevent animals from achieving adrenal adaptation since these levels are equivalent to those of untreated stressed animals (TABLE X, Fig. 6).

That this may not be the case is indicated by the apparent nonadaptation of the pituitary-adrenocortical axis (TABLES VI-IX). In all cases, the progressive increase of adrenal wet weights and ADW's, would indicate increased adrenal function. The apparent decrease in dry pituitary weight (TABLE IX), would also tend to confirm continued stimulation. Since no other experimental evidence has been presented, these divergent results cannot be explained at present. However, the great difference between the growth rates of stressed and reserpinized stressed animals, may offer some future explanation. The fact that starvation may inhibit the usual detoxication of corticosterone in the liver of rats (Herbst et al, 1960), may also aid in explaining this discrepancy.

Reserpine appeared to produce a significant effect of its own on the pituitary-adrenal axis. At times, especially at the onset of the experiment, the effects of reserpine on organ weights of normal animals were almost mirror images of the effects of RS in normal animals (TABLES IV, V, IX, & X). Another inter-

esting effect appears on the pituitary gland itself (Fig. 5). The initial decrease of pituitary weight which occurred on day one could be caused by nonspecific effects, and thus, may reflect a release of secretory substances. However, on the eighteenth day this effect could also be interpreted as complete pituitary exhaustion, as predicted by Kitay et al (1959) and Maickel et al (1961). It is interesting to note that reserpine produces an equivalent effect on the pituitary on day one as does RS in normal animals. On the other hand, reserpine plus stress produces an inhibition of the decreased pituitary weight as caused by stress alone. It thus appears that these effects on the pituitary may indicate different mechanisms which are antagonistic. Similar differences were also observed on the thymus and adrenal weights.

In a final analysis, reserpine appears to prevent adaptation to RS as evidenced by its divergent effects on various organ weight changes.

The Effects of Chronic Restraint (RS) Stress on the Behavior and Brain Serotonin (5-HT) and Norepinephrine (NE) Levels of Normal and Reserpinized Animals

Normal animals subjected to RS demonstrated an extreme degree of excitability during the first six days of the experiment. This behavioral effect decreased progressively with the duration of the experiment indicating behavioral adaptation.

On the other hand, restrained reserpinized animals were less excitable at the onset of the experiment but became progressively more excitable and difficult to handle as the experiment proceeded. During the latter half of the experiment reserpinized

animals appeared vicious as indicated by their attacks upon the investigator. To be more specific, reserpinized animals were quite easily tied down throughout the experiment, but became increasingly vicious upon release from restraint.

Generally, most of the stressed reserpinized animals displayed some degree of ptosis while under restraint throughout most of the experiment. After the sixth day of the experiment, the feeble state of the animals (animals weighed about 140 g) prevented an accurate analysis of the degree of ptosis and sedation. On about the twelfth day the reserpinized animals also developed watery secretions around the eyes which made it even more difficult to determine the degree of ptosis. Very infrequently normally stressed animals developed similar secretions.

To summarize, reserpinized animals became progressively excitable upon release from restraint, although the stress was initiated with greater ease than with the control animals. Thus, there were no indications that reserpinized animals evidenced any sort of behavioral adaptation to chronic RS.

There also appeared to be neurohumoral adaptation occurring concurrently with behavioral adaptation. The problem in analyzing these results is the fact that the 5-HT and NE brain levels of normally stressed animals were not significantly different from control data (TABLES XI-XII). As seen in Fig. 6, there is an increase in brain 5-HT levels and a decrease in brain NE levels which correspond very well with the general activity of the stressed animals during the first six days of the experiment. As was pointed out above, these animals were extremely excited during the first six days of the experiment.

Although there is little statistical significance to defend the findings presented, the importance of a possible correlation between behavioral adaptation, and brain 5-HT and NE levels must not be denied.

One of the most important observations made in this experiment was the fact that chronic stress (RS) will inhibit the usual 5-HT releasing effects produced by chronic reserpine administration (TABLE XI, Fig. 7). The effects of RS on brain NE were lacking as was previously demonstrated acutely by Brodie et al (1960). The significance of these findings will be discussed later. To determine whether chronic stress inhibited reserpine sedation as well as brain 5-HT was very difficult. In relation to the correlation between behavioral and neurohumoral adaptation in reserpinized animals subjected to chronic stress, the fact that stress had no effect on brain NE levels appears to associate this amine with the progressive increased behavioral activity associated with this group of animals.

One point, however, was very obvious. Reserpine did not induce a progressive depletion of brain 5-HT but it did to brain NE (Fig. 4). On the other hand, Brodie and Shore (1957) have postulated that reserpine should produce a progressive depletion of both brain amines since they do so acutely. The above observations were also present in the first experiment (TABLES I and II).

Summary

In view of the hypotheses presented earlier, this experiment has certainly produced effects which were not expected.

Therefore, the initial hypothesis will now be discussed in relation to this experiment.

Hypothesis I - Reserpine has not been found to inhibit chronic restraint stress to any degree. In fact reserpine appears to be an acute stimulator of the pituitary-adrenocortical axis and appears to prevent animals from adapting to this stress. How reserpine prevents adaptation cannot be determined at present.

Hypothesis II - Reserpine does appear to deplete brain 5-HT equally throughout this experiment. The fact that brain NE was depleted progressively throughout this experiment in contrast to brain 5-HT, makes it impossible to conclude as to which neurohormone is responsible for reserpine-induced-sedation. The fact that it was not possible to positively state whether chronic stress inhibits reserpine sedation as it inhibited 5-HT release, gives added support to the preceeding statement.

Hypothesis III - Since there are no clear cut results which would either prove or disprove the stated hypotheses, there does appear to be secondary mechanisms which may be responsible for the observed effects.

The data obtained by chronically stressing reserpinized rats has made it necessary to formulate two ancillary hypotheses.

Hypothesis IA - If reserpine is specifically stimulating the pituitary-adrenal axis, then single and chronic graded doses of reserpine should produce graded effects on this system.

Hypothesis IIA - Since reserpine did not induce a progressive depletion of 5-HT, but only of NE, and maintained an equivalent

degree of sedation throughout the experiment, it is postulated that reserpine is inducing a rapid synthesis of 5-HT.

To test these hypotheses, a chronic graded dose experiment involving reserpine was also conducted. The above hypotheses will be discussed in view of these three major experiments.

IV. RESULTS AND DISCUSSION

Effects of Single and Repeated Graded Doses Reserpine in Normal Male Albino Rats

TABLE XIII

EFFECTS OF SINGLE GRADED DOSES OF RESERPINE ON PITUITARY-ADRENAL FUNCTION IN NORMAL ALBINO RATS

DOSE	ADRENAL[z] DRY WEIGHT (ADW)	SERUM CORTICOSTERONE (KS)	WET PITUITARY WEIGHT
mg/kg	mg/100g	ug/100 ml	mg
0 (4)*	4.1±0.4**	16.0±4.0	-------
0[y] (6)	4.6±0.7	21.6±14.0	4.6±0.7
0.5 (8)	3.4±0.5	13.8±11.6	4.2±0.9
1.0 (8)	4.4±0.1	27.0±12.0[a]	3.8±0.6
2.5 (7)	4.6±0.5	34.0±7.9[a]	4.4±0.8
5.0 (8)	4.0±0.3	38.3±12.0[a]	5.3±0.5

* - Numbers in parenthesis represent the number of animals studied.

** - All values are means ± S.D.

a - Significant from the control group (0).

y - These animals were administered 2 ml/kg of a 25% solution of the reserpine vehicle.

z - The organ weights presented are mg/100 g of animal weight.

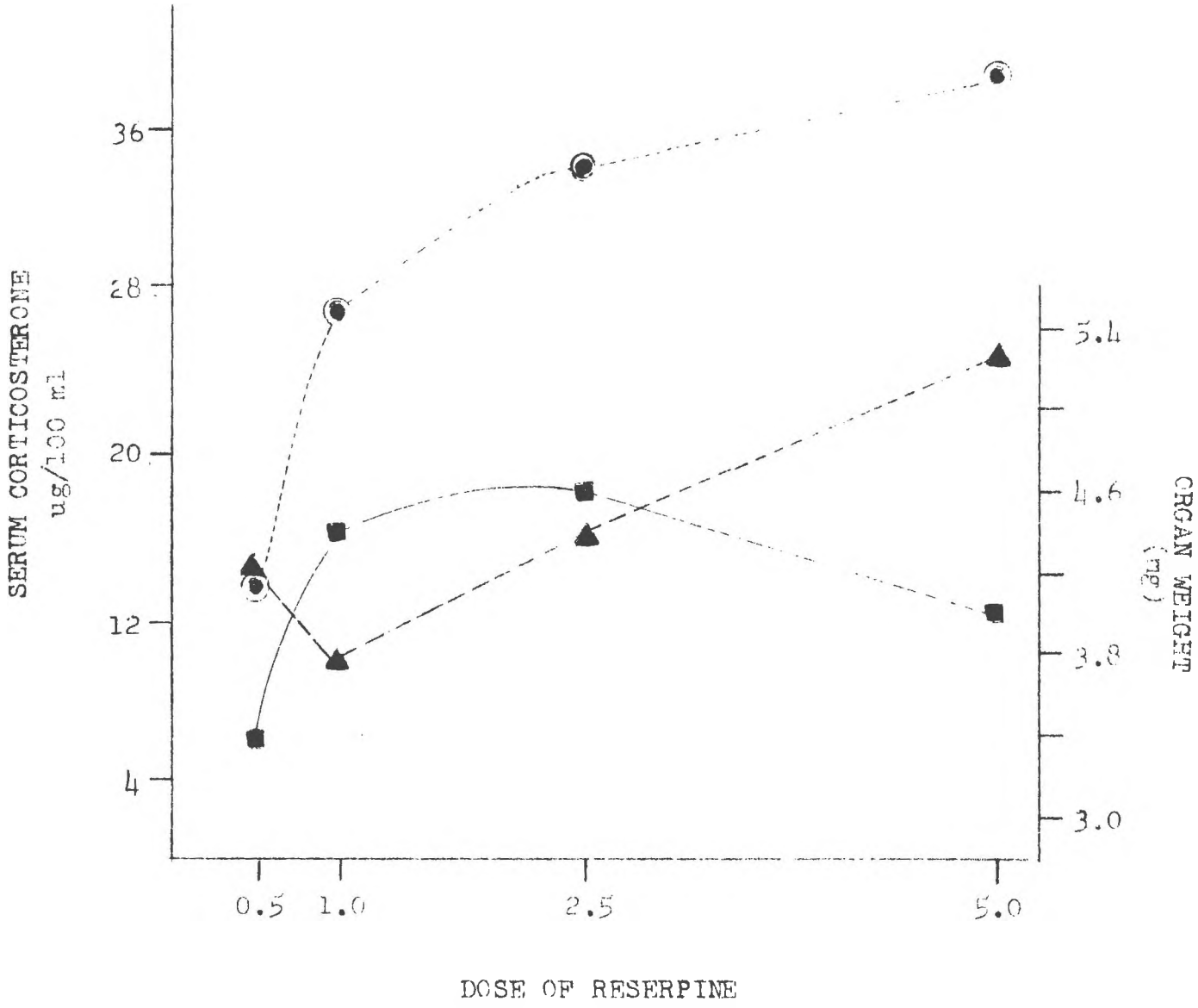

Fig. 7. Effects of single graded doses on pituitary-adrenocortical function in normal male albino rats.

Serum corticosterone (●—●), pituitary weights (▲—▲), and adrenal weights (■ ■) are given. Each point represents the mean of 6-8 animals.

TABLE XIV

EFFECTS OF REPEATED GRADED DOSES OF RESERPINE (32 days) ON PITUITARY-ADRENAL FUNCTION IN NORMAL MALE ALBINO RATS

DOSE	ADRENAL DRY WEIGHT (ADW)	SERUM CORTICOSTERONE (KS)	DRY PITUITARY WEIGHT
mg/kg	mg	ug/100 ml	mg
0 (8)[z]	13.5±2.2**	25.0±12.0	9.8±0.8
0[y] (6)	10.1±1.4	27.3±0.0	7.1±2.4
0.5 (16)	8.9±2.1	25.8±10.6	8.3±1.1
1.0 (10)	9.97±2.6	27.9±105	8.6±2.2
2.0[III] (18)	10.05±2.8	27.4±13.6	7.9±1.4
2.0 (6)	10.0	18	-------

** - All values are means ± S.D.

z - Values in parenthesis represent the number of animals studied.

y - All animals in this experiment were given food ad libitum except for this group; they received 18 g/animal. The final weights of animals in this experiment were as follows: (0) 339 g, (0^y) 271, (0.5) 240 g, (1) 220 g, and (2.0) 204 g.

TABLE XV

EFFECTS OF SINGLE AND REPEATED GRADED DOSES OF RESERPINE ON BRAIN SEROTONIN AND NOREPINEPHRINE LEVELS OF NORMAL MALE ALBINO RATS

SINGLE DOSES			REPEATED DOSES (32 Days)		
DOSE	5-HT	NE	DOSE	5-HT	NE
mg/kg	uug/g	uug/g	mg/kg	uug/g	uug/g
.0 (4)*	577±51**	245±77	0 (7)	605±77	498±191
0.5 (4)	516±99	129±84	0.5 (5)	426±27	76±58[a] (2)
1.0 (4)	458±28	123±39	1.0 (10)	427±87 563±69	72±51 645±168 (3)
2.5 (4)	410±36	191±36	2.0 (4)	461±65	94±57 (2)
5.0 (4)	365±56	148±39	2.0[III] (3)	673±79 789***	107±51

* - Values in parenthesis represent the number of pooled assays (2-4 brains/pool).

** - All values are means ± S.D.

*** - These values are significantly different from the values above at 2 mg/kg. The values below represent a pool of three assays.

III - Extremely excitable group.

a - The values in parenthesis in this column represent the number of assays in which NE could not be determined.

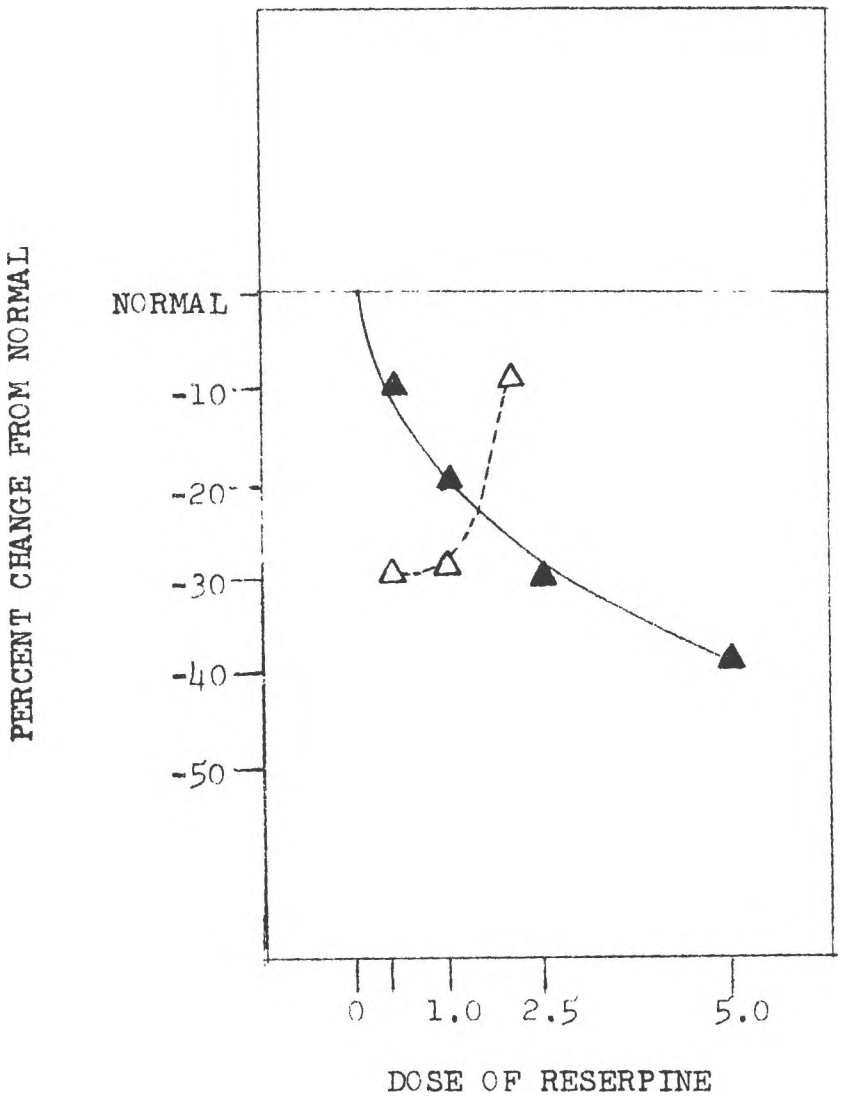

Fig. 8. Effects of single and chronic graded doses of reserpine on brain serotonin (5-HT) levels of normal male albino rats.

Single doses (▲—▲) and chronic doses (△···△) are given. Each point represents the mean of 3-7 brain pools (2-4 brains/pool). The chronic experiment was conducted for 32 days.

DISCUSSION

Single graded doses of reserpine resulted in a dose-response effect on the pituitary-adrenal axis (TABLE XIII, Fig. 7). The sigmoid character of serum KS levels does indicate a specific stimulatory effect by reserpine. The attempt to permit animal adaptation to the vehicle and injection by two weeks of preinjection re-emphasizes the specific effects of reserpine on this system. However, the vehicle was also administered in a greater dose (0^a, TABLE XIII). As indicated, this concentration also had an effect on the pituitary-adrenal axis, but was not as potent a stressor as reserpine.

The effects of graded doses on adrenal weights are extremely unusual and do not present the same dose-response effect as observed with reserpine and serum KS (TABLE XIII). The character of the pituitary weights vs. dose of reserpine indicates a biphasic action of reserpine, first stimulation, then inhibition of the pituitary-adrenal axis (Fig. 7). The complementary nature of the relationship between the ADW and pituitary weight re-emphasizes this possible biphasic effect of reserpine on the pituitary-adrenal axis. In contrast to the biphasic effect on this system, serum KS indicates only a monophasic stimulating effect by reserpine on the pituitary-adrenocortical axis. These conclusions are valid if one considers an increase in adrenal weight in contrast to a decrease in pituitary weight, as an indication of pituitary-adrenocorti-

cal axis hyperfunction. The %ADW and dry pituitary weight relationships in normally stressed rats would appear to confirm such a conslusion (Fig. 6).

This effect could be explained if reserpine possessed both central and peripheral effects. In support of such a possibility, Verdesca et al (1961) has shown that 5-HT stimulates adrenal function directly, and increases corticosteroid secretion. Since reserpine also induces peripheral release of 5-HT (Erspmer, 1961), a sedative dose could be envisaged as producing both an inhibition of the pituitary-adrenal axis centrally, and a stimulatory effect peripherally on the adrenal cortex directly. Such a mechanism could explain the apparent biphasic effects of single graded doses of reserpine.

The reserpine control animals present during the restraint stress experiment, exhibited unusually high adrenal dry weights (TABLE VII). This effect was not duplicated in this experiment. In several other experiments conducted in this laboratory, the adrenal dry weights of animals receiving 1 mg/kg of reserpine (32 days) were 10.0 mg per animal. This apparent discrepancy may be due to possible undue stress provided during the restraint stress experiment. The high serum KS levels of this experiment, as compared to the first experiment (TABLE X) also indicates that such increased stress conditions did exist.

After thirty-two days of daily administration of reserpine, no effective stimulation of the pituitary-adrenal axis was apparent as compared with the effects of single graded doses (TABLES XII and XIII). What occurs between the first and thirty-second day might be explained by three possibilities:

1. Reserpine acts as a nonspecific stressor to which the animal eventually adapts (TABLE X).
2. Reserpine causes inhibition by continued depletion of pituitary stores of ACTH (Kitay et al, 1959).
3. Reserpine produces a direct stimulatory effect on the adrenal gland via peripheral serotonin (5-HT) depletion, to which the adrenal either adapts or eventually is itself exhausted by chronic stimulation.

Single graded doses of reserpine produced a progressive depletion of brain 5-HT indicating a causal relationship between dose and effect (TABLE XV, Fig. 8). No such relationship was established for brain NE which is in contradiction to Brodie and Shore (1959) who have established equivalent depletion of both amines following a single dose of reserpine.

Since reserpine-induced sedation appears to correlate better with brain 5-HT as was observed in previous experiments, (TABLE I) it is suggested that reserpine-induced sedation may ultimately be caused by brain 5-HT release.

Chronic administration of graded doses of reserpine indicated somewhat of a reversal of 5-HT depletion while NE levels were lower than the first day. In fact, in almost half of the assays conducted on animals under chronic administration of reserpine, no NE could be detected (TABLE XV).

As indicated in the first experiment (TABLE XI), 1 mg/kg of reserpine did not induce progressive depletion of 5-HT with time. The 0.5 mg/kg dose of reserpine in this experiment, however, did appear to produce progressive depletion of 5-HT

in contrast to the apparent inhibition of 5-HT depletion produced by 2 mg/kg (TABLE XV, Fig. 8).

The behavioral sequences involved during this experiment clearly indicate the ability of reserpine to produce some excitatory effects. It was observed that at about half way through the experiment, animals at all dose levels became extremely excitable 24 hours following a dose of reserpine. - (This was also indicated previously, TABLE I.). There was also evidence of extreme excitability in a group of animals receiving 2 mg/kg of reserpine. It is interesting to note that these animals evidenced high brain 5-HT levels and a low mean KS level of 18 ug/100 ml of serum. As indicated in TABLE IV, group III, although extremely excitable, they demonstrated KS levels below normal. Thus, stress could not be implicated in producing such high 5-HT levels (Fig. 7).

One of the initial objectives of this experiment was to attempt to separate rats resistant or sensitive to reserpine in order to obtain added information as to the mechanisms of reserpine tranquilization. However, due to the lack of sufficient data, these objectives were not satisfied.

In relation to the ancillary hypotheses presented in the previous experiment, there are no clear indications that chronic reserpine treatment produces chronic stimulatory effects on the pituitary-adrenocortical axis.

The previous hypothesis that reserpine is inducing an increase in brain 5-HT synthesis has also gained further support. The reversal of the degree of 5-HT depletion with increasing doses of reserpine does lend support to a possible feedback mechanism.

V. GENERAL DISCUSSION

The basic assumption in this work has been that animals subjected to chronic environmental stressors will respond differently to the actions of drugs and thus, alter their usual effects. This investigation has demonstrated the utility of studying the effects of drugs in animals under the influence of chronic stress, especially those which affect the central nervous system.

At the onset of this investigation, several hypotheses were formulated which were then tested by subjecting reserpinized rats to chronic restraint stress. As has been noted, by necessity, two ancillary hypotheses were formulated and tested in order to elucidate the chronic effects of reserpine on the brain NE and 5-HT and serum KS. In relation to these hypotheses, the following discussions will be utilized in relating the results obtained to the mechanism of action of reserpine.

Neurochemical adaptation which appears to parallel behavioral adaptation has been characterized in normal and reserpinized rats. The increased behavioral activity associated with the initiation of restraint stress closely follows the increased brain 5-HT levels and decreased brain NE levels, both values returning to normal with behavioral adaptation (Fig. 6). Serum corticosterone levels aside from being an index of adrenocortical activity, also appear to be an

excellent index for behavioral adaptation.

The results from previous investigations indicate the validity of these observations. Thus Garattini et al (1960) found that electroshock produced an increase in brain 5-HT levels in rats, whereas Maynert and Clingman (1962) demonstrated depletion of brain NE in rats following a cold stress (4°C).

The significance of these findings can be observed assuming that depletion of either neurohumoral substance be interpreted as a decrease of neurotransmitter release associated with increased neuronal activity. If this assumption is correct, then during the initial phases of chronic stress, the activation of an NE dependent excitatory system in contrast to the inhibition of a 5-HT dependent system, could account for the behavioral activity observed.

Whether these neurochemicals are actual neurotransmitters released from centrally located axons is still controversial. Grundfest (1957), in summarizing research from previous years, concludes that central nervous transmission is accomplished via neurochemicals. The evidence presented in favor of this arose from his inability to demonstrate electrically excitable central dendrites. Rothballer (1959) and Brodie and Shore (1957) also contend that changes in brain NE represent fluctuations of excitatory activity initiated by an adrenergic system. Brodie further postulates that 5-HT is released from a cholinergic mechanism during depressive activity.

In relation to these previous investigations, Whittaker (1961) and DeRobertis (1962) have isolated both NE and 5-HT from a centrifuged brain fraction which contained nerve endings.

Although these findings are not conclusive proof of the physiological roles of 5-HT and NE in the CNS, they do give encouragement to the interpretations presented in this investigation.

So far, these interpretations have been based on release mechanisms associated with increased neuronal activity. However, when one consideres drug effects, different interpretations can arise. The effects of drugs on neuronal activity can be looked at in several ways. First, a drug could stimulate a nerve which would then release a transmitter substance. Secondly, a drug could act by releasing the transmitter without stimulating the nerve directly. The end result in both cases would be the same. The second possibility would assume that the transmitter is acting as though its source was stimulated. However, this does not have to be, since many workers have shown that reserpine inhibits peripheral nervous transmission by exhausting NE from the ANS (Trendelenburg, 1961).

Bonnycastle et al (1962) observed an increase in brain 5-HT levels in rats receiving sedatives such as phenobarbital and dilantin. On the other hand, Pletscher et al (1958) observed a similar increase with Iproniazid, a CNS stimulant. Thus, once again these interpretations arrive at neurohumoral crossroads. Some light may be shed on this problem through a recent paper by Schanberg and Giarman (1962). These workers found that reserpine increased the ratio of "Free/Bound" 5-HT in the brain, whereas Iproniazid decreased this ratio. They concluded that the increased brain 5-HT levels produced by Iproniazid was an increase in the bound fraction, thus

decreasing the total amount of 5-HT accessible to postsynaptic sites. Here again, if we assume that the free amount of the neurohumoral agent is associated with increased neuronal activity, 5-HT does appear to be causally related to a depressant mechanism.

Brodie and his co-workers (1961) have provided good evidence that reserpine produces sedation by releasing serontonin from its normally inactive sites. They postulate that after release, 5-HT then induces CNS depression at some postsynaptic site. They have also demonstrated that cold stress will prevent reserpine sedation concurrently with an inhibition of the normal 5-HT depletion. Since NE remains depleted, they conclude that reserpine acts via 5-HT and not NE.

Chronic restraint stress was also found to prevent 5-HT depletion by reserpine. However, in these experiments reserpinized stressed animals exhibited increased behavioral excitability concurrently with depleted brain NE, while brain 5-HT levels remained approximately normal (Fig. 6). NE is thus associated again, with increased CNS excitation.

It should be pointed out again that stressed animals receiving reserpine demonstrate neurotic behavior after release from restraint, though they appeared to be somewhat sedated while under restraint. This observation is extremely important for it cannot be concluded that this stress definitely inhibited reserpine-induced sedation. In other words, although these animals could have been under sedation, their reactions to change were violent.

Reserpine, therefore, appears to be stimulating both an inhibitory and excitatory brain mechanism since RS causes only NE depletion which is apparently associated with an excitatory mechanism. This postulate is correct if the assumptions made previously are valid. Trendelenburg (1961) in a recent review article concerning the ANS, points out that reserpine produces its peripheral effects by complete exhaustion of peripheral NE stores. Thus, reserpine produces a chemical sympathectomy. Insofar as brain NE is concerned, it could be detected in only half of the assays after 32 days of reserpine administration (total NE depletion was about 90%). Reserpine, therefore, may be producing exhausive depletion of the central sites of NE as well, thus producing a central hypersensitity similar to nerve postsynaptic hypersensitivity following denervation experiments. Such an apparent hypersensitity may be responsible for the behavioral excitation observed in reserpinized rats subjected to chronic restraint stress.

Past investigators have also demonstrated that reserpine causes a general release of several amines even including histamine in some species of animal (Burns and Shore, 1961). Reserpine, therefore appears to be producing a specific effect, sedation, through a nonspecific mechanism. Thus, reserpine should also induce depletion of brain 5-HT to eventual complete exhaustion if its action is a simple matter of preventing various amines from being bound. Reserpine, however, appears to induce an increased rate of 5-HT synthesis through a possible feedback mechanism. Such a mechanism was also suspected in

relation to MAO inhibitors by Costa et al (1961).

Reserpine (1 mg/kg) appears to deplete brain 5-HT levels equally throughout this investigation (Fig. 6). Since the degree of sedation was also unchanged in normal animals throughout the 32 days, it would be logical to assume that the rate of 5-HT synthesis and release were equivalent. This assumption can be made since the % depletion of 5-HT and sedation are causally related to the dose of reserpine (Fig. 8).

It was observed that 0.5 mg/kg of reserpine produced a progressive depletion of 5-HT over 32 days (Fig. 8). In contrast, 2 mg/kg produced an inhibition of 5-HT depletion; some animals exhibited levels above normal (TABLE XV). A possible 5-HT feedback mechanism involving the free to bound ratio of this amine may therefore be indicated. Thus, the rate of synthesis of 5-HT increased in some proportion to % depletion. As indicated at the 2 mg/kg dose of reserpine, the rate of 5-HT synthesis could be greater than its release.

A second proof of such a mechanism was evident in studying the recovery of brain 5-HT following one and thirty doses of reserpine (1 mg/kg), (Fig. 3). The increased brain 5-HT levels, 24 hours following the last dose of reserpine, could be explained on the basis of an increased rate of synthesis, since the 5-HT levels were below normal 8 hours after administration of the drug. The rate of NE synthesis did not appear to change in any experiment conducted. The obvious accumulation of brain 5-HT could be explained on the basis of its inability to cross the blood-brain barrier (Erspamer, 1961).

The behavioral sequences observed along with these increased brain 5-HT levels were also interesting. Throughout the latter half of this experiment, animals at all doses appeared extremely excitable 24 hours following a previous dose of reserpine. The animals appeared to be hypersensitive to all external stimuli. These behavioral changes could very easily be explained if the brain 5-HT is not bound to an intracellular site. If this increased level of 5-HT represents a "Free" form, then it could be blocking its own inhibiting effects in a manner similar to several cholinergic mechanisms (Brodie and Shore, 1957).

The interpretations of these results indicate that reserpine may be producing its sedative chronic effects by inducing a continuous release of brain 5-HT ("Free"), which is maintained by an increased rate of synthesis.

The fact that progressive brain NE depletion was not paralleled by an increased degree of sedation supports the contention that reserpine is producing its effects via an increase in "Free" brain 5-HT levels.

The ability of reserpine to prevent adaptation was demonstrated beyond any reasonable doubt. The means by which it accomplished this, however, is a very debatable subject. From all of the demonstrated effects of reserpine, adaptation to chronic RS is prevented by apparent starvation effects. It was observed (Tables IV-IX) that none of the organ weight changes associated with adaptation in normal animals were observable when an animal receiving reserpine was also subjected to RS. The pituitary-adrenal axis, however, appears

to be somewhat operable since high KS levels are maintained even to the 32nd day, (TABLE X), (Fig. 6).

One interesting observation was made by Rosenkrantz and Laferte (1960, and Verdesca et al (1961). These investigators have demonstrated that serotonin stimulates the adrenal cortex directly, inducing a release of corticosteroids. Since reserpine also depletes peripheral 5-HT in the rat (Erspamer, 1961), it is very tempting to speculate that the increased dry weights of reserpinized animals (Fig. 4) were due to a direct action of 5-HT on the adrenal. This fact might also explain the conflicting results of single graded doses of reserpine on adrenal dry weights (Fig. 7) and pituitary weights.

Although all of this information has been accumulated and scrutinized, we still cannot answer why a reserpinized animal does not adapt to this stressor. Of the three biochemical mechanisms assayed, (brain 5-HT and NE; serum KS), the only one which appears to be exhausted or below normal in reserpinized animals subjected to RS, is brain NE (Fig. 6). Since one would associate non-adaptation with the lack of some physiological mechanism, it would be interesting to speculate a relationship between the apparent exhaustion of NE with non-adaptation. This reflection becomes even more interesting when one compares Cannon's work (1932) with true sympathectomy, with Trendelenburg's remarks (1961) concerning a drug sympathectomy as produced by reserpine.

Necina and Krejci (1961), added to this relationship by demonstrating that the usual peptic ulcers produced by reserpinized animals subjected to cold stress (6 hours), could be

completely inhibited by administering dihydroxyphenylalanine (DOPA), a precursor of NE. They also observed that the usual 50% mortality in reserpinized animals subjected to cold stress could also be prevented by DOPA.

In relation to this investigation, on the 32nd day, brain NE was barely detectable. This, of course, is no indication of what occurs peripherally. However, after 32 doses of reserpine, the usual spinal reflexes associated with sacrifice were completely absent. This very dramatic effect may indicate the loss of all ANS reflex ability.

In view of this data concerning NE and adaptation to stress, it is suggested that NE may play an important role at the tissue level, in enabling animals to adapt to all stresses.

Initially it was mentioned that drugs potentially possess the ability to affect several segments of the pituitary adrenocortical axis (Fig. 1). Chronic restraint stress by its very nature stimulates the peripheral side, activating the complete chain of command illiciting corticosterone release into the general circulation at the opposite side. Reserpine on the other hand, acutely induced corticosterone release. Where reserpine acts, however, is not so clear.

The % depletion of brain 5-HT appears to be somewhat causally related to increased serum KS levels. Westermann et al (1962) postulated that reserpine induces serum KS release by increasing the "Free" brain 5-HT levels in the Limbic System of the brain, which in turn induces ACTH release, possibly via the CRF of the hypothalamus. If their proposal is correct, then reserpine should either induce a similar increase

in serum KS levels after 32 doses as after a single dose, or should produce inhibition of the pituitary-adrenocortical axis by exhaustion.

In contrast, neither of the above possibilities were clearly present in this investigation. Serum KS levels were neither increased nor decreased following chronic administration of reserpine. Since chronic brain 5-HT depletion was not causually related to increased serum KS levels, the hypothesis that ACTH is dependent on "Free" brain 5-HT levels, was not supported in this investigation.

That the divergent effects produced by reserpine on pituitary-adrenocortical function (serum KS levels vs. pituitary-adrenal weights) could be due to a possible direct effect of peripheral 5-HT on the adrenal cortex, is certainly immaginative. If such an effect were proved, much of the contradictory results obtained with reserpine could be rectified.

Thus, where reserpine acts in producing its effects on the pituitary-adrenocortical axis, still remains a mystery.

Concerning these two hypotheses, the following final statements are presented:

IA. Reserpine does not appear to produce chronic pituitary-adrenocortical stimulation as indicated by serum KS levels. However, indirect data does indicate that reserpine may possess a bibasic effect on this system which could be eclipsing the true picture.

IIA. Reserpine-induced brain 5-HT release appears to correlate better with sedation than does NE.

Proof that reserpine is inducing 5-HT synthesis has been presented. Whether such a mechanism can explain the mechanism of action of reserpine awaits more persuasive evidence correlating reserpine sedation and brain 5-HT levels.

VI. SUMMARY AND CONCLUSIONS

The study of the influence of chronic stress in animals receiving a drug, especially one which modifies CNS activity, was proven to be a very worth while venture. Aside from adding to the information concerning the action of a drug, the influence of the drug on chronic stress can also aid in the elucidation of basic endocrine mechanisms concerning the stress. In view of the experiments conducted in this investigation and their interpretations, the conclusions of these efforts are now presented.

1. Animals subjected to a chronic forced restraint stress adapted well in terms of Selye's General Adaptive Syndrome. Pituitary-adrenocortical adaptation may involve an increase in the efficiency of this system.
2. Behavioral adaptation associated with restraint stress was observed to be related to brain neurohumoral levels. Thus, hyperexcitability was associated with increased brain 5-HT levels and decreased brain NE levels.
3. Reserpine (1 mg/kg) prevented animals from adapting to the chronic stressor of forced restraint.
4. It was postulated that reserpine presented adaptation by producing a chemical sympathectomy. These results suggest the possible significance of the sympathetic division of the ANS and adrenal catechol amines in enabling animals to adapt to chronic stress.

5. Reserpinized animals reversed the behavioral adaptation demonstrated by normal animals. The progressive increase in behavioral hypersensitivity observed was associated with the progressive depletion of brain NE levels whereas brain 5-HT levels remained normal throughout this period of activity.
6. Evidence was presented that reserpine induced an increased rate of 5-HT synthesis with no similar effect on brain NE. The supranormal levels of brain 5-HT at higher chronic doses of reserpine and observed in brain 5-HT recovery studies, indicates the existence of a possible serotinin feedback mechanism involving the bound and free concentrations of this amine. These high serotonin levels were also associated with behavioral hyperactivity.

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VITA

John Adam Rosecrans was born July 30, 1935, in Brooklyn, New York. He received his B. S. degree in Pharmacy from St. John's University in June, 1957, and proceeded to the University of Rhode Island as a graduate assistant in September, 1957. He received his M. S. degree in Pharmacology in June, 1960, while conducting research in pharmacology with several plant drugs.

In 1961 while continuing study for the Ph. D. degree, he was appointed as Instructor in Pharmacology for one year after which he received a predoctoral fellowship from the National Institutes of Mental Health. He completed the requirements for the Ph. D. in Pharmacology in February, 1963.

He has two publications concerning the pharmacology of various plant extracts. He is also a member of the Rho Chi and Sigma Xi honor societies.

He married Norma J. Pareti in 1958 and has two children: a daughter, Jane, and a son, John.