EFFECT OF ASCORBIC ACID DEPLETION ON NOREPINEPHRINE CONCENTRATION OF GUINEA PIG HYPOTHALAMUS

by

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A THESIS

IN

FOOD AND NUTRITION

Submitted to the Graduate Faculty
of Texas Tech University in
Partial Fulfillment of
the Requirements for
the Degree of

MASTER OF SCIENCE

IN

HOME ECONOMICS

Approved

Accepted

May, 1973

ACKNOWLEDGMENTS

I am deeply indebted to Dr. Margaret E. Kassouny for her direction of this thesis, and to the other members of my committee, Dr. James M. Solar and Dr. Richard Carlson, for their helpful criticism.

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CHAPTER I

INTRODUCTION

An important chemical function of ascorbic acid in metabolism is the reversible ascorbic acid-dehydroascorbic acid oxidation-reduction reaction (Beyer 1942, Friedman and Kaufman 1965, Chalopin et al. 1966, Nakashima 1970). The presence of ascorbic acid in the sympathetic nervous system and in the brain suggests the possibility that it may function in this oxidative-reductive capacity in the sympathetic and central nervous systems (Wortis et al. 1938, Beyer 1942, Sulkin and Kuntz 1948, Levin et al. 1960, Friedman and Kaufman 1965).

In human ascorbic acid deficiency, Kinsman and Hood (1971) measured progressive changes in behavior related to scores in the "neurotic triad" of the Minnesota Multiphasic Personality Inventory (hypochondriasis, depression, and hysteria). There is some evidence that depressions, indistinguishable from some types occurring spontaneously, can be induced by drugs in humans (Axelrod 1971, McGeer 1971, Schildkraut 1969b, Coppen 1967). These drugs may exert their mood altering effect by suppression of the biogenic amines such as norepinephrine and serotonin in the central nervous system.

Jones (1972) compared guinea pigs depleted of ascorbic

acid for nine days with control animals and found that the deficient animals needed 40% more time to learn a visual discrimination test. She used avoidance of shock as the motive. After a review of ascorbic acid requirements in stress, Baker (1967) concluded there definitely is an increased ascorbic acid requirement in stress. Electroconvulsive shock in rats and electric shock applied to the feet of rats was found to decrease levels of endogenous brain norepinephrine (Maynert and Levi 1964, Kety et al. 1967) and to decrease its turnover in rats (Thierry et al. 1968).

If depression can be caused by a drug-induced lack of biogenic amines, can the depression noted in human ascorbic acid deficiency (Kinsman and Hood 1971) be related to a lack of biogenic amines? The purpose of this research was to measure the levels of norepinephrine in the nine-day deficient guinea pig with and without the added stress of behavioral testing to measure visual discrimination learning. The motive used was avoidance of electric footshock.

CHAPTER II

REVIEW OF LITERATURE

Ascorbic Acid and the Central Nervous System

Wortis et al. (1938) compared ascorbic acid values for brain, adrenal, spleen, kidney and heart in normal and scorbutic guinea pigs and concluded that the relatively high levels of ascorbic acid in the scorbutic brains acted as a reservoir of ascorbic acid for brain function. They hypothesized a specific role for ascorbic acid in the functional activity of the brain. The decrease in tissue concentration of ascorbic acid during ascorbic acid depletion of guinea pigs is well documented, as is the fact that the brain concentration remains relatively high (Nakashima et al. 1970, Bush 1971, Kassouny and Rivers 1972). Sulkin and Kuntz (1948) reported a similar decrease in the ascorbic acid content of guinea pig autonomic ganglia and in guinea pig central nervous tissue. They observed quantitative changes in the sympathetic ganglion cells during neural activity with electrical stimulation and during paralysis following interruption of the preganglionic connections. Sulkin and Kuntz (1948) postulated a role for ascorbic acid in the metabolic activity of the neuron and also suggested a functional relationship to a substance they referred to as adrenin, subsequently

considered to be norepinephrine (Iverson 1967).

Norepinephrine and the Central Nervous System

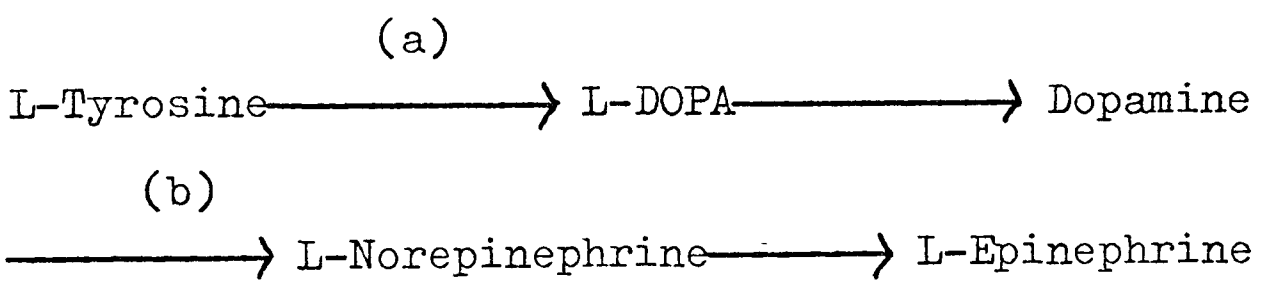
Schildkraut and Kety (1967), in their review of biogenic amines, reported that norepinephrine is stored within the nerve in intraneuronal granules, observed by electron microscopy to occur at presynaptic nerve endings. The contents are then released in response to nerve impulses. High densities of monoamine-containing neurons in the limbic system and especially in the hypothalamus are reported by Hillarp et al. (1966). Schildkraut and Kety (1967) and others (Schildkraut 1969, Iverson 1967) state that in both peripheral and central nervous systems (brain), tyrosine is converted by the same enzymatic pathway to norepinephrine and stored in central neurons in an inactive form. A review by Kety (1963) supports Hillarp et al. (1966) who found high concentrations of norepinephrine in the hypothalamus.

The evidence in favor of norepinephrine playing a role in neurotransmission in the central nervous system is further strengthened by the anatomical similarities between central norepinephrine-containing neurons and sympathetic post-ganglionic neurons in which this amine has been positively identified as the neurotransmitter (Iverson 1967).

Norepinephrine and Ascorbic Acid Relationships

Beyer (1942) first proposed the mechanism by which ascorbic acid functioned in nervous tissue. Results of his *in vitro* studies suggested that ascorbic acid functioned in the synthesis of the ortho-dihydroxy form from the para-hydroxy adrenaline (epinephrine) precursors. He also reported the *in vitro* deamination of certain sympathomimetic amines by ascorbic acid.

The following is the metabolic pathway involved in the biosynthesis of norepinephrine and epinephrine (Nagatsu *et al*. 1964, Glowinski and Baldessarini 1966, Iverson 1967, Coppen 1967, Schildkraut 1969b, Axelrod 1971):



(a) tyrosine hydroxylase (the rate limiting step)
(b) dopamine-beta-hydroxylase

Udenfriend (1956) found that phenylalanine, tyrosine, and DOPA (dihydroxyphenylalanine) can all act as precursors of adrenal epinephrine and norepinephrine in the rat. Spector *et al*. (1963) used tyrosine-^{14}C in isolated perfused guinea pig hearts and found that whereas epinephrine is synthesized only in the adrenal, norepinephrine is synthesized locally. Guinea pig heart (Spector *et al*.

1963), brain (Udenfriend 1963), and sympathetic nervous tissue (Beyer 1942) contain the catalysts required for converting tyrosine to norepinephrine.

Dopamine-beta-hydroxylase is the enzyme required for converting dopamine (3,4-dihydroxyphenylethylamine) to L-norepinephrine. Levin et al. (1960) indicated that in the in vitro reaction catalyzed by this enzyme in beef adrenal, one mole of ascorbic acid is oxidized to dehydroascorbic acid for every mole of dopamine converted to norepinephrine. He also demonstrated a dopamine-dependent oxidation of ascorbic acid. Friedman and Kaufman (1965) found dopamine-beta-hydroxylase to be an inactive form requiring interaction with ascorbic acid before it was capable of hydroxylating the substrate.

Udenfriend and Creveling (1959) found that dopamine-beta-hydroxylase was present in the brain in amounts approximately correlated with the catecholamine content of the hypothalamus and various other brain regions. Udenfriend (1963) showed that catecholamine synthesis in the brain of guinea pigs represented a major portion of the total catecholamine synthesis. Nakashima et al. (1970) compared tyrosine hydroxylase activity in brains of guinea pigs deprived of ascorbic acid for 18 days with controls deprived of ascorbic acid for 14 days and repleted for 3 days. He found lower tyrosine hydroxylase activity in the depleted guinea pig brains.

Glowinski and Baldessarini (1966) suggest that norepinephrine is stored mainly in a form with a rapid turnover and to a lesser extent in a more tightly bound form with a slow turnover. Thoa et al. (1966) found a deficient binding mechanism for norepinephrine in scorbutic guinea pig hearts. He demonstrated a significant decrease in norepinephrine concentration of heart tissue of deficient guinea pigs.

Sjostrand (1970) injected both dehydroascorbic and ascorbic acid into mice and found an increased norepinephrine turnover in heart and brain tissue only with dehydroascorbic acid. This indicated an action at both peripheral and central sites, with a more pronounced action centrally. He hypothesized a role for ascorbic acid and dehydroascorbic acid in the binding (storage) mechanism of norepinephrine in granules.

Ascorbic Acid and Norepinephrine Relations to Stress

Chalopin et al. (1966) proposed that ascorbic acid deficiency weakens the subject's resistance toward stress-provoking agents. Maynert and Levi (1964) and Thierry et al. (1968) found an increase in the endogenous norepinephrine and in the turnover rate of norepinephrine in the brain of rats after repeated electric shocks to the feet.

CHAPTER III

MATERIAL AND METHODS

Animals

Male albino guinea pigs of the English Smooth Hair Strain (Hilltop Lab Animals) from 14 to 16 days old were used for the investigation. The animals were housed in individual, suspended, wire mesh cages and weighed daily. The total experimental period was nine days.

All animals upon arrival were fed an ascorbic acid-deficient diet (General Biochemicals) described by Reid and Briggs (1953), fresh cabbage, and bottled water (Ozarka) ad libitum. After a two day acclimatization period, the animals were paired by weight with one member of 9 pairs assigned at random to the deficient group (D).

On the first day of the experimental period, the cabbage was omitted from the diet of both control and deficient groups. The deficient group was offered the ascorbic acid-deficient diet and water ad libitum. The control group was pair fed the same diet, water given ad libitum, and a daily oral dose of 3 mg ascorbic acid per 100 g body weight. A daily oral dose of distilled water, identical by volume, was supplied to the deficient animals to insure consistency in handling.

The two groups were further divided into two

subgroups; DBT (deficient animals) and CBT (pair-fed controls) which were subjected to behavioral testing; D (deficient animals) and C (control animals) which were not subjected to testing. On the fifth day of the experimental period, the visual discrimination testing was begun and continued through the ninth day.

Behavioral Apparatus

A discrimination box utilizing the motive of avoidance of electric shock similar to the one described by Thompson and Bryant (1955) and Jones (1972) was employed. The discrimination box was modified with a higher electric charge to the grid section at the choice point. The charge consisted of 220 volts with 50 K ohms resistance in series with the animal. This provided an electroshock with an approximate intensity of 4 mA (milliamps).

Time duration or latency in seconds was measured automatically at two points in the maze. Initial latency (latency 1) was measured by a timer triggered by placing the animal in the start box and automatically stopped when the animal crossed a light beam at the entrance to the choice chamber. The second latency (latency 2) was triggered as the animal passed another light beam in front of the doors to the goal box. The time between trials was regulated by a tape recorder connected to the goal box floor. The correct door for each trial was opened in a

random order with no more than two consecutive trials to the same door. Criterion for the trials was established arbitrarily at 9 out of 10 correct choices or 80 trials for the Tests and 40 trials for the Retests.

Tests of Visual Discrimination

The first day of the test period (Day 5 of the experimental period) was devoted to introducing the guinea pig to the apparatus and no permanent scores were kept. A modification of Jones' (1972) procedure was the addition of 4 trials in the introduction to the apparatus in which a short curtain was used in the start box. The first of the three Tests (initial visual discrimination Test) was given on the second day of the test period. The first Retest (after 24 hours) was given on the third day of the visual discrimination testing period.

The three Tests were considered to be indicators of short term memory because the animal had to remember the correct response from preceeding trials. The three Retests were considered to be indicators of long term memory because the animal had to remember the correct response 24 hours later.

The first Test was a measure of depth perception: the animal learned to discriminate between a gray door and no door (Open-Closed door). The second Test was a choice between a black door and a white door (Black-White) which

measured ability to discriminate light intensity. The third test was a choice between a horizontally striped black and white door and a vertically striped black and white door, used as a measure of pattern discrimination (Horizontal-Vertical).

Biochemical Analyses

On the fifth day of the visual discrimination Tests which corresponded to the ninth day of the experimental period, the animals were killed by decapitation. The right adrenal was removed and analyzed for ascorbic acid by procedures previously described (Roe et al. 1954) as modified by Kassouny and Rivers (1972). Absorption was measured at 540 nanometers on a Beckman Acta III Spectrophotometer utilizing Beckman 1.0 ml silica cuvettes.

The hypothalamus was removed, blotted, weighed and the catecholamines were extracted in 0.4 N perchloric acid and immediately frozen. The extracts were analyzed for norepinephrine by procedures described by Bertler et al. (1958) and utilized the modifications suggested by von Euler and Lishajko (1961). The chromatographic columns used in this experiment had a top reservoir of 28 mm O.D. and a body of 8 mm with a tapered opening of approximately 2 mm. The percent transmittance was measured with a Beckman Acta III Spectrophotometer with a Total Fluorescence Accessory. The fluorescence was measured at two

exciting wavelengths, 410 and 455 nanometers. A Corning Glass filter was used which transmitted only fluorescence beyond 490 nanometers. With each set of samples the transmittance readout was set to a value proportional to the concentration of the epinephrine standard (DL-Epinephrine-hydrochloride). The recovery of the norepinephrine standard (DL-Norepinephrine-hydrochloride) from the chromatograph column was approximately 20%. A recovery factor was applied and the results are reported to give 100% recovery.

Unless otherwise noted, all statistical analysis was done with Student's *t* test.

CHAPTER IV

RESULTS AND DISCUSSION

General

Statistical analysis of weight change data for the control and deficient guinea pigs showed no significant difference between the two groups. The mean weight gain of the control animals was 27.6 ± 3.28 g (mean ± standard error) over the 9 day experimental period. The deficient guinea pigs had a mean weight gain of 29.3 ± 6.83 g over the same period. The deficient group showed no signs of hemorrhages or other overt signs of scurvy.

Total Adrenal Ascorbic Acid

Biochemical analysis (Table 1) of adrenal ascorbic acid of control guinea pigs (CBT = 5; C = 4) showed mean concentrations of 67.65 mg/100 g tissue compared to 16.97 mg/100 g for the deficient animals (DBT = 4; D = 3). The decrease was significant at the 0.5% level and corresponded to a 75% decrease in adrenal ascorbic acid in the 9-day deficient guinea pigs.

The mean ascorbic acid level of CBT animals (Table 1) was 82.67 mg/100 g tissue compared to 48.88 mg/100 g tissue of the C animals ($P < 0.05$). This finding does not agree with previous evidence that ascorbic acid

TABLE 1

TOTAL ADRENAL ASCORBIC ACID CONCENTRATIONS OF PAIR-FED CONTROL AND 9-DAY ASCORBIC ACID DEFICIENT GUINEA PIGS[1]

	Behaviorally Tested	Behaviorally Untested	Total Control Versus Total Deficient
Control	82.67±14.65[2] (N=5)	48.88±6.34 (N=4)	67.65±10.09[3] (N=9)
Ascorbic acid-deficient	19.19 ± 5.67 (N=4)	14.02±4.17 (N=3)	16.97 ± 3.57 (N=7)
Total Tested versus total Untested	54.46±13.77 (N=9)	33.94±7.96 (N=7)	

[1]Values represent mean ± standard error expressed in mg ascorbic acid/100 g tissue.

[2]Behaviorally Tested control group has significantly higher mean ascorbic acid than untested control group ($P < 0.05$).

[3]Total control group has significantly higher mean ascorbic acid concentration than the deficient group ($P < 0.005$).

requirements are increased in some types of stress (cold, epinephrine injection, ACTH; Baker 1967). Perhaps electric shock applied to the feet of guinea pigs maintained on an adequate amount of ascorbic acid increases adrenal storage of the available vitamin. This point needs to be confirmed with further study with guinea pigs subjected to electric foot-shock.

Visual Discrimination Learning

Visual discrimination data on latencies (Table 2 and Figure 1) and on trials to criterion (Table 3) showed no significant differences between the CBT and DBT groups (8 animals in each group). The mean trials to criterion was greater for the DBT group than for the CBT group in the Open-Closed Test-Retest and the Black-White Test-Retest, but were less in the Horizontal-Vertical Test-Retest.

The total mean latency 2 per trial (Table 2) was less in the DBT group than in the CBT group in all Tests and Retests except the Horizontal-Vertical Test. This result is in direct opposition to Jones' (1972) who found the control group consistently required less time to make the discrimination. None of the mean latencies per trial (Figure 1) in this experiment were greater than 6 seconds (latency 1 plus latency 2) while those of Jones (1972) ranged from 4-11 seconds in the two groups. It is possible that the higher intensity of electric shock used

TABLE 2

LATENCIES ON VISUAL DISCRIMINATION TESTS FOR PAIR-FED CONTROL AND 9-DAY ASCORBIC ACID DEFICIENT GUINEA PIGS[1]

	Latency 1[2]		Latency 2	
	Control	Deficient	Control	Deficient
Open-Closed Door Test	2.09±0.56	1.91±0.49	1.11±0.96	0.92±0.08
Retest	2.44±0.64	1.84±0.55	2.10±0.78	1.34±0.13
Black-White Door Test	2.04±0.41	2.63±0.56	2.35±0.76	1.60±0.17
Retest	2.82±0.57	1.96±0.50	2.97±1.4	1.85±0.13
Horizontal-Vertical Door Test	2.10±0.49	2.15±0.50	1.97±0.50	2.32±0.54
Retest	2.68±0.88	1.66±0.35	3.77±1.87	2.00±0.42

[1]Each value represents mean ± standard error of 8 animals for the latency per trial (total latency 1 or 2 divided by trials to criterion for each animal for each Test and Retest) expressed in seconds.

[2]Latency 1 is elapsed time in second until animal leaves start box; latency 2 is elapsed time at choice point until animal enters goal box.

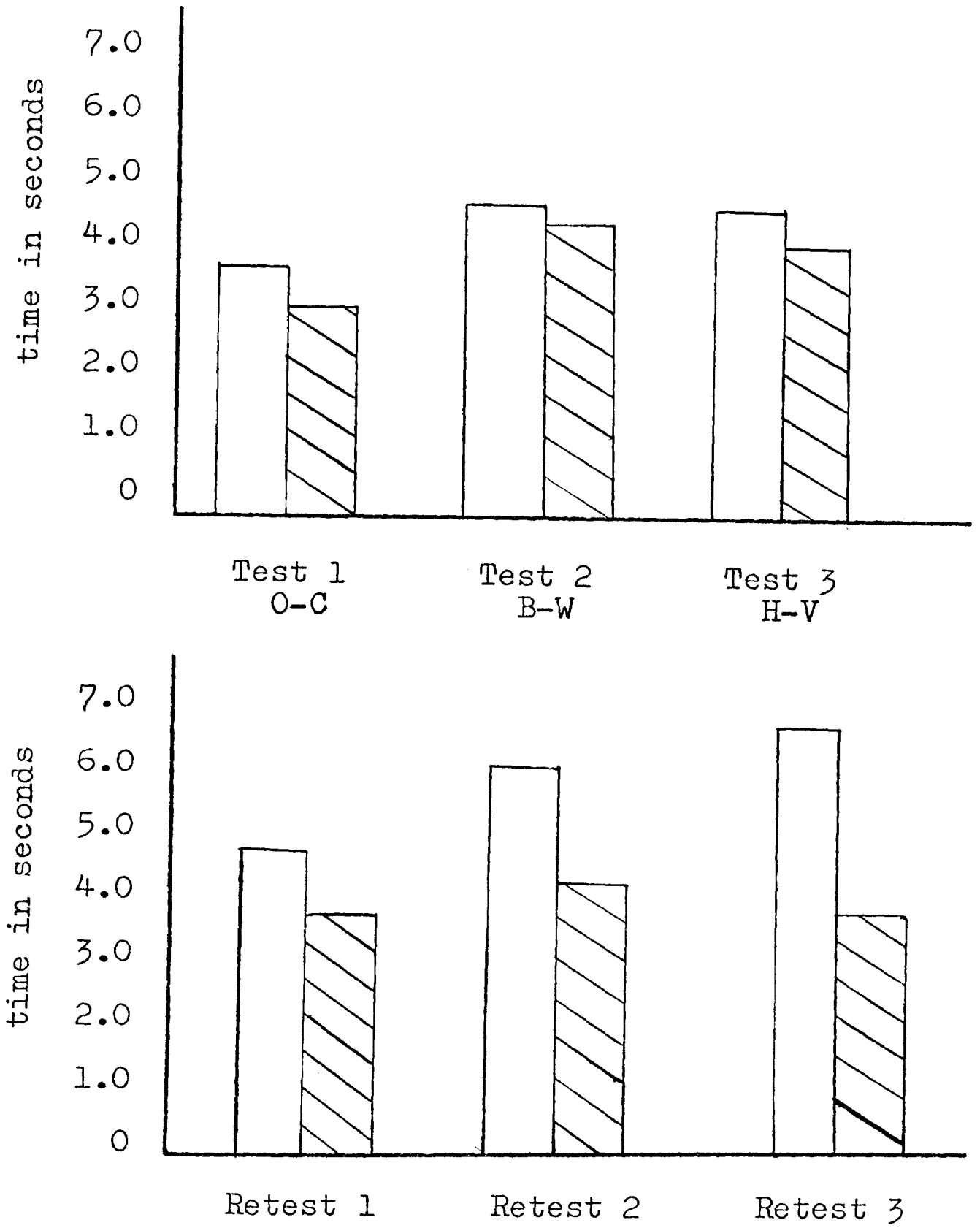


Fig. 1.--Combined latencies on visual discrimination Tests for pair-fed control (▭) and 9-day deficient (▨) guinea pigs.

Each bar represents the mean time per trial in seconds of 8 guinea pigs on three Tests and Retests. (O-C Open-Closed, B-W Black-White, and H-V Horizontal-Vertical).

TABLE 3

TRIALS TO REACH CRITERION ON THE VISUAL DISCRIMINATION TESTS AND RETESTS BY PAIR-FED AND 9-DAY ASCORBIC ACID DEFICIENT GUINEA PIGS[1]

Day of Experimental Period	Tests and Retests	Number of Trials to Criterion	
		Control (mean SE)	Deficient (mean SE)
6	Open-Closed Door Test	66.88±6.47	71.13±3.13
7	Retest	18.88±3.64	23.13±4.44
7	Black-White Door Test	74.00±3.96	77.25±1.77
8	Retest	26.63±4.28	32.38±3.99
8	Horizontal-Vertical Door Test	43.13±8.94	37.88±6.89
9	Retest	16.38±2.15	15.87±5.09

[1]Values represent mean trials ± standard error for 8 animals.

(estimated to be 4 mA at the choice point) compared to 2 mA used by Jones (1972), increased the motivational level of both groups to make the discrimination more quickly and thus decreased the latencies.

Hypothalamic Norepinephrine Concentrations

Statistical analysis of the data (Table 4) of norepinephrine levels in the guinea pig hypothalamus indicated a significant difference ($P < 0.005$) between the tested groups (CBT = 4; DBT = 2) compared to the untested groups (C = 4; D = 3). These values correspond to a 30% decrease in norepinephrine concentrations for the behaviorally tested animals.

A decrease in brain norepinephrine in guinea pigs subjected to electric foot-shock (4 mA intensity) is also reported by Paulson and Hess (1963). They found levels of 0.3 ug/g for normal guinea pigs and 0.18 ug/g tissue for foot-shocked animals. This was a 38% decrease in norepinephrine concentration in the shocked animals. Bliss and Zwanziger (1966) found a 26% decrease in the hypothalamic concentration of norepinephrine in guinea pigs subjected to foot-shock (4 mA intensity). They reported a normal norepinephrine concentration in the guinea pig hypothalamus of 1.64 ug/g which decreased to 1.22 ug/g tissue upon foot-shock. They also found that the per cent decrement was the same for all parts of the brain and postulated that

TABLE 4

HYPOTHALAMIC NOREPINEPHRINE CONCENTRATIONS OF PAIR-FED CONTROL AND 9-DAY ASCORBIC ACID DEFICIENT GUINEA PIGS[1]

	Behaviorally Tested	Behaviorally Untested	Total Control Versus Total Deficient
Control	2.46±1.37 (N=4)	7.35±1.16[2] (N=4)	4.91±1.24 (N=8)
Ascorbic acid-deficient	0.54±0.44 (N=2)	4.80±1.70 (N=3)	3.10±1.40 (N=5)
Total Tested versus total Untested	1.82±0.96 (N=6)	6.26±1.03[3] (N=7)	

[1]Values represent mean ± standard error expressed in ug/gm tissue.

[2]Separate analysis of variance with application of Duncan's New Multiple Range test showed a significant difference ($P < 0.05$) between the untested control and tested deficient groups.

[3]Combined Tested group has significantly lower mean than the combined untested group ($P < 0.005$).

the brain norepinephrine decrement was probably a neurochemical component of a general response to intense emotional stress.

Although the difference was not statistically significant, the mean norepinephrine levels of the deficient untested animals (D) showed a 35% decrease when compared to the untested controls (C). Bush (1971) reported a 35% decrease in mean concentrations of hypothalamic ascorbic acid of 9-day ascorbic acid deficient guinea pigs so it is possible that the decrease in norepinephrine parallels the decrease in ascorbic acid for nine days. No published reports of norepinephrine levels of ascorbic acid deficient guinea pigs were available; therefore comparisons with the results obtained in the present experiment cannot be made.

Analysis of variance and application of Duncan's New Multiple Range Test was done on the norepinephrine data and showed a significant difference ($P < 0.05$) between the untested control group and the tested deficient group. Since interaction possibly existed between the ascorbic acid nutriture and the animals' reaction to the stress of electric foot-shock, a factorial analysis of variance was done. The analysis (Table 5) showed no significant interaction between the stress and diet but pinpointed the significance of the effect of electric foot-shock upon the norepinephrine levels of the guinea pigs, regardless of diet. Ascorbic acid deficiency had some influence on

TABLE 5

FACTORIAL ANALYSIS OF VARIANCE ON HYPOTHALAMIC NOREPINEPHRINE CONCENTRATIONS OF PAIR-FED CONTROL AND 9-DAY ASCORBIC ACID DEFICIENT GUINEA PIGS

Source	df.	M.S.	F	P
Total	12			
Groups	3	26.56	4.25	< 0.05
Test	1	63.62	10.18	< 0.025
Diet	1	10.06	1.61	N.S.
Interaction	1	5.99	0.96	N.S.
Error	9	6.25		

norepinephrine levels, but the effect was not statistically significant ($F = 1.61$).

CHAPTER V

SUMMARY AND CONCLUSIONS

Levels of norepinephrine in the hypothalamus of guinea pigs fed an ascorbic acid deficient diet were investigated with and without the added stress of behavioral testing (DBT and D). Untested and tested pair-fed guinea pigs served as controls (CBT and C). The body weights and food intake of animals were recorded daily and adrenal ascorbic acid concentrations were determined as an indication of the degree of ascorbic acid depletion. Norepinephrine concentrations in the hypothalamus were measured to determine the effect of ascorbic acid deficiency on this catecholamine.

The added stress represented three visual discrimination Tests and three Retests with the motive of avoidance of electric foot-shock. The three Tests used were depth perception, light intensity and pattern discrimination.

The ascorbic acid analysis showed that after a 9-day depletion period, the combined D and DBT adrenals contained 25% of the combined C and CBT ascorbic acid concentration. The ascorbic acid concentration of the CBT animals was significantly higher than the C animals ($P < 0.05$) which does not agree with previous evidence that some types of stress increase the ascorbic acid requirements.

The behavioral data provided no significant results. Statistical analysis of the norepinephrine concentrations showed that norepinephrine levels of the CBT and DBT groups were significantly lower ($P < 0.005$) than the C and D animals. A significant difference ($P < 0.05$) was also found between the untested control group and the tested deficient group.

The results were interpreted to indicate that the effect of the electric foot-shock upon the animals was of such a magnitude as to overshadow any effect of dietary treatment alone. No statistically significant interaction between ascorbic acid deficiency and electric foot-shock was found.

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