Plasma catecholamine and ascorbic acid levels in smokers and nonsmokers as a function of stress

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PLASMA CATECHOLAMINE AND ASCORBIC ACID LEVELS IN SMOKERS AND NONSMOKERS AS A FUNCTION OF STRESS

A Thesis

Presented to the

Department of Psychology

and the

Faculty of the Graduate College

University of Nebraska

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

University of Nebraska at Omaha

by

Audrey A. Wickiser

May 1984
Accepted for the faculty of the Graduate College, University of Nebraska, in partial fulfillment of the requirements for the degree Master of Arts, University of Nebraska at Omaha.

Committee

<table>
<thead>
<tr>
<th>Name</th>
<th>Department</th>
</tr>
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<tbody>
<tr>
<td>A. James Thi</td>
<td>Psychiatry</td>
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<tr>
<td>Jorge Rodriguez</td>
<td>Psychology</td>
</tr>
<tr>
<td>Jack L. Smith</td>
<td>Biochemistry</td>
</tr>
</tbody>
</table>

Chairman

April 27, 1984
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF THE LITERATURE</td>
<td>2</td>
</tr>
<tr>
<td>Stress and Catecholamine Levels</td>
<td>2</td>
</tr>
<tr>
<td>TABLE I. REPORTED BASAL CATECHOLAMINE LEVELS (pg/ml)</td>
<td>3</td>
</tr>
<tr>
<td>Stress and hypertension</td>
<td>4</td>
</tr>
<tr>
<td>Electric shock as a stressor</td>
<td>5</td>
</tr>
<tr>
<td>Physical activity as a stressor</td>
<td>7</td>
</tr>
<tr>
<td>Psychological stress</td>
<td>8</td>
</tr>
<tr>
<td>Nicotine as a stressor</td>
<td>11</td>
</tr>
<tr>
<td>Nicotine and ascorbic acid levels</td>
<td>12</td>
</tr>
<tr>
<td>HYPOTHESES</td>
<td>17</td>
</tr>
<tr>
<td>METHODS</td>
<td>19</td>
</tr>
<tr>
<td>Materials</td>
<td>19</td>
</tr>
<tr>
<td>Design</td>
<td>21</td>
</tr>
<tr>
<td>Procedures</td>
<td>22</td>
</tr>
<tr>
<td>Statistical Analyses</td>
<td>23</td>
</tr>
<tr>
<td>BIOCHEMICAL ASSAYS</td>
<td>24</td>
</tr>
<tr>
<td>Radioenzymatic Assay for Catecholamines</td>
<td>24</td>
</tr>
<tr>
<td>Oxidation Spectrophotometric Assay for Plasma Ascorbic Acid (DNPH Method)</td>
<td>31</td>
</tr>
<tr>
<td>RESULTS</td>
<td>33</td>
</tr>
<tr>
<td>Plasma Ascorbic Acid</td>
<td>33</td>
</tr>
<tr>
<td>TABLE II. MEAN(±SD) PLASMA ASCORBIC ACID LEVELS (mg/dl)</td>
<td>34</td>
</tr>
<tr>
<td>IN SMOKERS AND NONSMOKERS BY TREATMENT OVER TIME</td>
<td>34</td>
</tr>
</tbody>
</table>
Plasma Catecholamine Levels

TABLE III. MEAN (±SD) PLASMA CATECHOLAMINE LEVELS (pg/ml) FOR SMOKERS AND NONSMOKERS DURING THREE CONDITIONS

POMS

DISCUSSION

BIBLIOGRAPHY
I. INTRODUCTION

Research on the relationship between the role of stress and blood catecholamine levels began in the early 60's. Studies since that time have shown that both physical and psychological stressors can cause an increase in the circulating levels of catecholamines. Epinephrine and norepinephrine are the catecholamines most implicated as being affected by stress. The results have not been clear as to whether the two catecholamines respond differentially to physical and psychological stress. Epinephrine secretion appears to increase in response to anxiety while norepinephrine may be related to aggression.

Nicotine is a chemical stressor which causes an increase in epinephrine secretion and has produced adrenal hypertrophy in exposed animals. Nicotine is also responsible for the significantly lower ascorbic acid levels in smokers as compared to nonsmokers. L-ascorbic acid might be involved in the regulation of normal or increased levels of tissue catecholamines by its role as a cofactor in enzymic biosynthesis (1-3). Other evidence points to L-ascorbic acid as a regulatory agent against both inactivation and excessive build-up of circulating catecholamines. It might act as an inhibitor of catecholamine inactivation by competing for the methylation enzymes, catechol-0-methyl transferase and s-adenosyl methionine (4) and prevent high levels of catecholamines by protecting the activity of monoamine oxidase, an enzyme responsible for the transformation of catecholamines to their inactive state (5,6). Plasma ascorbic acid has been shown
to decrease significantly following an intravenous administration of epinephrine to humans (7).

II. REVIEW OF THE LITERATURE

A. Stress and Catecholamine Levels

The two catecholamines of interest in this study, epinephrine and norepinephrine, are released in response to numerous stimuli. Almost all of the circulating epinephrine is secreted by adrenergic chromaffin cells in the adrenal medulla, however, some evidence exists for central epinephrine-producing neurons located in brain stem areas (8-10). Norepinephrine enters the circulatory system as a neurotransmitter released by the sympathetic nervous system with a minor amount secreted by noradrenergic chromaffin cells in the adrenal medulla.

It is difficult to find published normal ranges, but basal plasma catecholamine levels have been established on fasting, supine normotensive subjects. Before the development of assays that were sensitive to the lower ranges of catecholamines, investigators assumed the basal levels to be zero or less than one. Even after the advent of the more sensitive and specific methods, reported basal levels show a great deal of inter and intra-study variability, especially those using flurometric or double isotope techniques. As this study will use a single isotope assay, only basal data
obtained by a comparable method will be discussed. Basal levels from various sources are shown in Table I.

**TABLE I**

**REPORTED BASAL CATECHOLAMINE LEVELS (pg/ml)**

<table>
<thead>
<tr>
<th>Study</th>
<th>**X(±SEM)**NE</th>
<th>**X(±SEM)**E</th>
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<tbody>
<tr>
<td>Cryer, et al (11)</td>
<td>227(±23)</td>
<td>44(±4)</td>
</tr>
<tr>
<td>Peuler and Johnson (12)</td>
<td>213(±21)</td>
<td>28(±6)</td>
</tr>
<tr>
<td>Cryer and Weiss (13)</td>
<td>182(±21)</td>
<td>44(±11)</td>
</tr>
<tr>
<td>Weise and Kopin (14)</td>
<td>208(±17)</td>
<td>67(±9)</td>
</tr>
<tr>
<td>Johnson, et al (15)</td>
<td>165(±12)</td>
<td>16(±2)</td>
</tr>
</tbody>
</table>

The lower levels in the last study might be due to the sophistication of the subjects, who had participated previously in similar experiments. In all of the studies discussed below, baseline, as opposed to basal levels, were established during the pre-treatment phase of the experiments and used for comparison purposes.

The level of circulating catecholamines found at any given time is the result of physical, psychological and chemical influences. Gender, body position, physical and mental activity, and mood can alter catecholamine secretion rates. Caffeine, nicotine, and prescription or non-prescription drugs can produce fluctuating levels (16). An increase in the secretion and excretion rate of catecholamines is found after exposure to a stressor. Catecholamine secretion is measured as either total or separated
(epinephrine, norepinephrine and dopamine) plasma catecholamines, while the excretion rate is usually determined on 24-hour urine samples. Total plasma catecholamine values provide no information as to the relative levels of epinephrine and norepinephrine, although it is commonly assumed that, all other factors being equal, norepinephrine comprises 80% of the total plasma catecholamines.

B. Stress and hypertension

The synergistic effect of stress and catecholamine secretion has been implicated in the etiology of hypertension. The data are equivocal as to whether E and NE act together or separately to produce and/or exacerbate hypertension. Genetic hypertensive rats (SHR's) show significantly increased E and NE levels as compared to their normotensive controls (WKY's) following cage transfer, footshock, and forced immobilization (17-19). An accelerated maturation of epinephrine secretion at five weeks of age in the Lyon hypertensive strain has been reported with no apparent difference in secretion at twenty-one weeks of age (20).

Maudsley reactive rats had lower basal plasma norepinephrine levels than their normotensive controls, but no difference in basal epinephrine levels (21). Norepinephrine levels were negatively correlated with blood pressure and heart rate, a finding explained as sympathetic compensation for "other" influences.
The results obtained from studies of human hypertensives also are not definitive. Elevated plasma catecholamine levels have been reported in human hypertensives and experimentally induced hypertensive rats (22). When separated catecholamines were assayed, higher baseline plasma epinephrine levels were present in human hypertensives as compared to normotensives, but norepinephrine levels were equivalent (23).

The stress of annoying disturbances during a monotonous task produced an increased epinephrine excretion in both hypertensives and normotensives (24). An increase in NE was noted only in normotensive females. There was no evidence that hypertensives have an exaggerated catecholamine response either at rest or under stress.

C. Electric shock as a stressor

The administration of various intensities of electric shock has proven extremely effective as a stressor. Total plasma catecholamines were measured in Sprague-Dawly rats before, during and at intervals for five hours following a protracted, high level of footshock stress (25). This level of shock resulted in fatalities for eleven of the nineteen experimental animals and produced plasma catecholamine levels significantly elevated over baseline in the survivors and the lethals, with higher levels present in the lethals. The survivors' CA levels reached 150% over baseline after 30 minutes of stress and remained there throughout the post-stress period. At this same point, CA levels in the rats that
did not survive had reached 280% over baseline and continued to rise until death occurred. The ability to survive stress appears to be related not only to plasma CA levels, but also to the rate of increase and the time needed to return to baseline. This study would have provided more valuable information had epinephrine and norepinephrine been measured separately.

Studies that investigated separated catecholamines reported an increase in both epinephrine and norepinephrine following footshock stress and transfer to the shock chamber (17,18). These results might have been confounded by obtaining blood samples immediately after transfer, as catecholamine levels are known to be affected by the physical activity produced by transfer from the home cage. The high NE levels might reflect physical stress rather than fear or anxiety. Gentle handling of Sprague-Dawly rats produced a rise only in epinephrine levels, but immobilization resulted in an increase of both epinephrine and norepinephrine while decapitation produced an 80-fold increase in E levels and a 10-fold increase in NE levels (26). Dopamine levels were undetectable under any condition. These results were confirmed in two later studies (19,27).

Combined footshock and brain stimulation had an additive effect on norepinephrine levels but their effect on epinephrine secretion was not greater than that caused by either stressor alone (28).
Increased epinephrine excretion was found following strong, but harmless levels of shock in humans (29) and, in a subsequent study, anticipation of shock produced a significant rise in epinephrine excretion over levels obtained during a relaxation period (30). Norepinephrine excretion was not affected in either study.

D. Physical activity as a stressor

Physical activity has been used as a stressor in human studies. An early study measured the effect of muscular work, ranging from moderate to heavy, on norepinephrine and epinephrine excretion rates (31). An increase in NE excretion over levels obtained during relaxation was noted, but epinephrine levels did not change from baseline.

A 6-fold increase in norepinephrine excretion between pre and post-game levels occurred in professional hockey players (32). Only a moderate increase in epinephrine excretion was noted. No differences were found among amateur boxers, whose epinephrine levels were elevated in the pre-match period and remained high after the match. The authors concluded that high norepinephrine levels are associated with states of active aggression, while epinephrine levels increase under the condition of passive anxiety experienced by the amateur boxers who spent a great deal of time waiting to go on for three minutes of actual fight time.

A three-minute handgrip was used as stressor with blood samples taken before and during the handgrip (33). No
differences were found in plasma norepinephrine levels but epinephrine levels increased significantly in the exercising arm. In subjects who had continuously climbed stairs for a total of four minutes, both plasma epinephrine and norepinephrine levels increased over baseline (34). These contradictory findings might reflect the fact that norepinephrine does not appear at peak levels in the bloodstream until four minutes after release, while epinephrine peaks at three minutes.

Some support for the relationship of norepinephrine and physical stress was provided by a study that measured the urinary excretion of norepinephrine and epinephrine in thirteen members of an amateur softball team during the last seven games of the season (35). Results showed no significant differences in epinephrine excretion but a significant group effect was found for norepinephrine, with the less skilled players excreting a higher amount. The studies that attempt to look at the effects of physical stress cannot control for the confounding effects of whatever psychological stress might be present.

E. Psychological stress

Real life situations that involve considerable psychological stress have provided a convenient model to investigate the effect of stress on catecholamine levels. Urinary excretion rates of norepinephrine and epinephrine were measured in paratroop trainees during night rest, ground
training and during 2-3 hour jump sessions (36). Epinephrine excretion was significantly increased during the jumping period over the ground activity level. Norepinephrine levels were elevated only during the sixth and eighth jump. Both E and NE levels were increased significantly over baseline during surface attack training in A-10 pilots (37). The increase in NE might be due to the physical activity involved in flight maneuvers, while the previously cited study controlled for this by taking usual ground activity as the baseline measure.

Plasma levels of epinephrine and norepinephrine were assayed before and after students took the State Examination for internal medicine at a medical school in Russia (38). Both plasma epinephrine and norepinephrine were elevated after the examination when compared to pre-examination values.

In similar studies, a compulsory, 6-hour matriculation examination was used as the stress condition (39,40). Epinephrine excretion increased for both males and females under the stress condition, but norepinephrine increased only in males. None of the studies reported whether the students remained seated or were allowed to move around.

Epinephrine and norepinephrine excretion levels were examined as a function of mental stress which ranged from working under distracting conditions to mild punishment (41-43). All of the studies reported a significantly higher level of epinephrine excretion over baseline under the mental stress
conditions. Changes in norepinephrine excretion levels were less marked.

An increase in epinephrine excretion was found in female subjects after watching a disturbing movie when compared to excretion rates measured after viewing a boring travel film (44). No change in norepinephrine levels occurred. Both NE and E levels increased significantly after watching a comedy film and a particularly gruesome ghost story.

Two studies used public speaking as the stress condition. One study obtained subjects who were scheduled to speak in a variety of situations (45) and the other used young physicians who were presenting at medical conferences (34). Both studies looked at plasma epinephrine and norepinephrine levels with a baseline value taken directly prior to speaking, however one study collected the final sample after the speech was completed (45), while the other obtained the stress sample within the first three minutes of speaking (34). A significant increase was reported only in plasma norepinephrine levels after speaking when sampling time was variable. Opposite results were found when samples were obtained after three minutes. A significantly higher level of epinephrine was found, with no change noted for norepinephrine levels. A follow-up study measured catecholamine response during the initial moments of public speaking and again at 15 minutes into the speech (46). Epinephrine levels were significantly elevated over baseline at three minutes but had
approached baseline at 15 minutes. Norepinephrine increased at three minutes and remained high at the second sampling. Both catecholamines respond to stress but epinephrine shows a dramatic increase in secretion that disappears rapidly from the circulation; norepinephrine appears to remain in the blood stream for a longer period.

F. Nicotine as a stressor

The evidence that nicotine directly stimulates the adrenal medulla has been well documented. Epinephrine excretion increased following a period of heavy smoking but no comparable rise was seen for norepinephrine excretion (47). These results were confirmed by two different assay techniques. The intravenous administration of nicotine produces a high level of blood epinephrine, exceeded only by the intravenous administration of epinephrine (48,49).

The effect of nicotine on epinephrine secretion was demonstrated in dogs who had inhaled cigarette smoke for an average of 3.5 minutes (50). Inhalation of cornsilk cigarette smoke was used as control and no significant change over baseline was noted. Epinephrine levels in blood obtained from the adrenal vein, vena cava and femoral artery all increased after inhalation of cigarette smoke.

Epinephrine excretion in smokers was significantly higher than nonsmokers, but no differences were found in norepinephrine excretion levels (51). These results were replicated in a later study (52).
Significant increases in both plasma epinephrine and norepinephrine levels occurred after subjects had smoked two non-filtered cigarettes (53). Contradictory results were reported by a study that found a significant decrease in norepinephrine excretion when subjects stopped smoking but no corresponding decrease in epinephrine excretion (54).

Ascorbic acid levels and adrenal alteration were investigated in guinea pigs who had inhaled cigarette smoke for a period of up to twenty-one days (55). Ascorbic acid concentration in the adrenals was significantly lower in the animals receiving the smoke and, after 18 days, marked adrenal hypertrophy was noted.

G. Nicotine and ascorbic acid levels

A variety of factors other than dietary intake can influence ascorbic acid status in man. These influences are reflected in changing ascorbic acid levels in plasma, whole blood, serum or leukocyte samples. Some factors which can affect ascorbic acid levels are sex, age and smoking habits. Evidence will be presented below which discusses the influence of each of these variables, but the emphasis will be on studies which investigated the effect of nicotine on ascorbic acid levels.

An early investigation of the effects of vitamin C on toxic substances reported that 25 mg of the body's ascorbic acid was used to neutralize the amount of nicotine contained in one cigarette (56), and it has been shown that cigarette
smoke destroys vitamin C in solution (57). A comparison of plasma and leucocyte ascorbic acid levels revealed that smokers had lower ascorbic acid levels than nonsmokers in both measures and those smokers who smoked more than 14 cigarettes per day had lower plasma levels than those who smoked less than 14 per day (57).

Ascorbic acid status was measured in 4600 subjects who had previously participated in the Nutrition Canada National Survey (58). Median serum ascorbic acid levels were found to be 40% lower for individuals who smoked more than 20 cigarettes per day. Intake of dietary vitamin C was controlled for by grouping subjects according to information obtained from a 24-hour dietary recall. Median serum ascorbic acid levels increased as dietary intake increased, but smokers were consistently lower at each range of intake. Median serum levels were higher in females than males and female smokers had consistently lower values than female nonsmokers.

A comparison of male smokers and nonsmokers found significantly lower baseline whole blood and plasma levels of ascorbic acid in smokers (59). During the entire experiment, all subjects were on a vitamin C restricted diet. After saturation with 2 g ascorbic acid for five days, plasma and whole blood ascorbic acid levels were identical in both groups. Measurement of urinary excretion of ascorbic acid prior to and after saturation revealed that smokers excreted half the amount excreted by nonsmokers.
A replication of this experiment (60) produced similar results. No significant differences were noted for age, weight, consumption of coffee, tea or alcohol, and dietary intake. Baseline whole blood ascorbic acid levels were 30% lower in smokers. Following a 1.1 g ascorbic acid load, urinary excretion after eight hours was significantly less for the smokers, indicating a greater retention. Subjects were saturated by receiving 1.1 g ascorbic acid for five days and by removal of all dietary restrictions. Desaturation was accomplished by resuming intake restrictions and discontinuing the supplements for five days. Finally, two loading doses of 1.1 g ascorbic acid were given on the last day of the experiment. After saturation and desaturation, there was no significant difference in whole blood ascorbic acid levels between the smokers and nonsmokers. Comparison of urinary excretion after the load on Day 1 and on the last day of the experiment indicated that the smokers retained significantly more ascorbic acid. The author speculates that less ascorbic acid is available for use by smokers or that smokers utilize ascorbic acid differently than nonsmokers.

Plasma and leukocyte ascorbic acid levels were measured in subjects stratified by age, sex and number of cigarettes smoked (61). Among nonsmokers, there was a decrease in plasma ascorbic acid concentration with increasing age and a higher concentration in women of all ages. Plasma ascorbic acid levels were lower in smokers than nonsmokers for both males
and females. Because of the age related decrease in nonsmokers, the plasma ascorbic acid values for smokers and nonsmokers are similar at age 60 for both males and females. There was no change in leukocyte ascorbic acid concentration with age, but women had higher concentrations than men. Both male and female smokers had reduced values as compared to non-smokers. The authors state that heavy smoking has the same effect on plasma ascorbic acid levels as the addition of 40 chronological years.

Similar results were reported for a population of 300 individuals older than 65 (62). There was also a significant male-female difference, with females showing consistently higher values. Older smokers had lower levels than nonsmokers of the same age but this difference was not significant when the amount of fresh fruit consumed was considered. The correlation between ascorbic acid levels and fruit intake was greater than the relationship between blood ascorbic acid levels and smoking, perhaps indicating that intake can compensate for the deleterious effect of smoking on ascorbic acid levels. Other studies have reported similar results for age and sex (63-67).

Two related studies used a kinetic approach to investigate the distribution rate, metabolism, body pool size and excretion of ascorbic acid in male nonsmokers and smokers who used more than 20 cigarettes per day (66,67). To achieve a steady state condition, subjects followed an ascorbic acid
deficient diet during a three week equilibration period and during the two week experimental period while taking an oral dose of ascorbic acid which ranged from 30 to 180 mg/day. At the beginning of the two week experimental period, one dose of I $^{14}$C ascorbic acid was administered. Washout of the remaining radioactivity was accomplished by ingesting megadoses of ascorbate (8 x 500 mg/day) for eight days. Steady state concentration of plasma ascorbic acid, total turnover (mg/day), metabolic turnover (mg/day) and body pool (mg/kg body weight) were calculated for both groups. Results indicate that smokers require significantly higher total turnovers to achieve plasma levels and body pools comparable to nonsmokers. Smokers have a higher metabolic turnover than nonsmokers at corresponding steady state concentrations (70-90 mg/day versus 40-50 mg/day). Based on these findings, the authors recommend that non smokers require an intake of 100 mg/day and smokers 140 mg/day.

A pilot study (Smith, 1981) conducted in this laboratory has confirmed that smokers have significantly lower ascorbic acid levels than nonsmokers of comparable age and dietary intake. Biochemical determinations were done on whole blood, serum and leukocyte samples. The results showed that smokers have significantly lower ascorbic acid levels irrespective of the method used.
III. HYPOTHESES

It has been well established that an increase in catecholamine secretion occurs concomitantly with stress. The conflicting results as to which catecholamine is affected by psychological as opposed to physical stress are most probably due to experimental designs that confound these variables and that do not control for time of appearance and the short half-lives of catecholamines in the circulatory system. The most accurate information on the effect of psychological stress would appear to be that obtained from fasting subjects who had been sitting quietly for over 30 minutes, with blood samples drawn three to four minutes after the onset of psychological stress. Public speaking has been shown to be an effective psychological stressor that involves a minimum of physical activity.

It has also been well documented that nicotine is a chemical stressor that directly stimulates the adrenal medulla and depletes plasma ascorbic acid, either directly or as a result of increased catecholamine levels. There is some evidence that other types of stress will cause a decrease in plasma ascorbic acid levels.

Because of the strong evidence relating psychological stress and nicotine to catecholamine levels and the weaker evidence implicating a depletion of blood ascorbic acid and a simultaneous increase in catecholamine levels as a result of stress, this study has the following hypotheses:
1. Smokers will have higher initial circulating catecholamine levels and lower plasma ascorbic acid levels than nonsmokers.

2. Smokers taking an ascorbic acid supplement will have lower catecholamine levels during a pre-stress measurement than smokers taking placebo.

3. Both smokers and nonsmokers will show an increase in circulating catecholamines following the stress period.

4. The catecholamine most affected by psychological stress will be epinephrine.
IV. METHODS

A. Materials

Subjects The subjects consisted of 30 smokers and 13 non-smokers who met the following criteria:

1. Because of age and sex differences in ascorbic acid levels and catecholamine response, subjects were limited to healthy males between the ages of 18-45.

2. Subjects passed a physical exam designed to detect clinical signs of vitamin C deficiency.

3. Nonsmokers had never smoked or had abstained from smoking for at least one year.

4. Smokers had consumed at least 20 cigarettes/day for the last year.

5. Only smokers who had no desire to stop smoking or to decrease their present rate of consumption at the time of the study were accepted.

6. Subjects were not taking any prescription medications.

7. Users of drugs which have an effect on the secretion rate and/or serum level of ascorbic acid and catecholamines were excluded from the study.

8. Vitamin C supplements had not been used on a regular basis for at least three months previous to the study.

The subjects were recruited by advertisement from the University of Nebraska Medical Center, the University of
Nebraska at Omaha, and, when response from these sources was not adequate, from the general public.

Smokers were paid $200.00 for their participation in the study and nonsmokers received $150.00. All subjects had to complete the entire experiment to be eligible for payment.

**Dietary and smoking information** All subjects were provided with a dietary record form which covered two randomly selected days of each week. In addition, smokers were required to complete a smoking record form for the same two randomly selected days.

The dietary record form provided information on the adequacy of the subjects' diets and detected any dramatic changes in the consumption of foods containing high levels of ascorbic acid.

**Stress** Psychological stress and the resulting anxiety were produced during a three-hour session where the subjects randomly chose three jokes which they were instructed to memorize. Prior to the stress test, the jokes were collected. Subjects then delivered their jokes during a three-minute period and the other subjects rated their presentation on a scale of 0 to 20. After all subjects had participated, the subject with the highest score was awarded a $25.00 prize.

Baseline and pre-stress plasma catecholamine levels were measured after all subjects had been seated quietly for at least 30 minutes and before being told the details of the stress test. The stress sample was obtained at the end of the
three-minute presentation period as the subject was finishing with his third joke.

**Profile of Mood States (POMS)** An additional measure of anxiety was provided by the administration of the tension-anxiety (T) scale from the POMS test.

The T-scale is composed of a 5-point adjective rating scale with which the subject indicates how closely they describe his feelings. A zero indicates 'not at all' while a 4 indicates 'extremely.' The T-scale is self-administered and can be completed in five minutes.

Studies with patient normative samples show the POMS to be internally consistent and to have a satisfactory test-retest reliability. Content, predictive and concurrent validity were established on normal college students, psychiatric outpatients and male VA outpatients. Tension-anxiety scores correlated .80 with the Taylor Manifest Anxiety Scale, decreased in patients after treatment with Librium when compared to placebo and showed a concurrent increase with palmar sweating during a stress condition (68).

The POMS was given during the pre-treatment baseline period and again during the three-hour stress session.

**B. Design**

1. **Groups** Smokers were randomly assigned to either the treatment or control group. The treatment group ingested 500 mg of vitamin C once each day while the control group was given an identical placebo. Nonsmokers received
placebos only. All subjects reported once each week on a
preassigned day to turn in their smoking and/or dietary
record forms and to pick up their next week's supply of
forms and supplements.

2. Experimental schedule The experiment covered a total of
two weeks. This was broken down as follows:
a. A one-week pre-treatment period. Subjects followed
their regular diets and took no supplements.
b. A four-week treatment period where subjects ingested
their supplements or placebos.

C. Procedures
The procedures for the five-week experimental period
were as outlined below:

1. One-week pre-treatment period All subjects reported at
the end of the first week for a three-hour session
(beginning at 7:00 A.M.).
a. POMS (Mood Scale) was administered.
b. After sitting quietly for 30 minutes, ten ml of blood
was drawn for baseline plasma ascorbic acid and plasma
catecholamine levels.
c. Subjects were given a smoking and/or dietary record
for the following week. The first completed records were
collected.
d. Each subject was given a week's supply of supplement
or placebo.
2. **Four-week treatment period** The procedure for the first three weeks was as follows:
   a. Subjects reported once a week for blood draw to determine ascorbic acid levels (smokers only).
   b. Subjects turned in their smoking and/or dietary records and received next week's forms.
   c. Supplement boxes were returned and new supplements distributed.
   d. The number of tablets remaining were counted as an estimation of compliance.

3. **Fourth-week** At the end of the fourth week, the following measures were repeated at a three-hour stress session:
   a. POMS
   b. Blood for plasma catecholamine and plasma ascorbic acid levels (2 X, once before speaking and during speaking).
   c. Number of cigarettes smoked during the session.
   d. Smoking and/or dietary records and supplements were returned.
   e. Stress test

D. **Statistical Analyses**

All data were analyzed with SPSS procedures available through the University of Nebraska Medical Center's interactive time-sharing system. Probability level for rejecting the null hypotheses was established at \( p < .05 \) (two-tailed).
Baseline (pre-treatment) plasma epinephrine and norepinephrine and plasma ascorbic acid levels of smokers and nonsmokers were compared by Student's t-test for differences between groups to verify the hypothesized initial differences.

Plasma ascorbic acid levels of smokers taking vitamin C supplements and smokers taking placebo were analyzed by repeated measures beginning one week after starting treatment to confirm that smokers on vitamin C supplements had reached saturation levels and that plasma C levels of smokers on placebo remained low.

The effects of stress and treatment on epinephrine and norepinephrine levels were analyzed by a two-way ANOVA, NE and EX condition (baseline, pre-stress, during stress) X group (smokers on C, smokers on placebo, nonsmokers).

V. BIOCHEMICAL ASSAYS

A. Radioenzymatic Assay for Catecholamines

The radioenzymatic assay for the determination of plasma catecholamines is based on the single isotope method developed by Passon and Peuler (69). The enzyme, catechol-O-methyl-transferase (COMT), is used to catalyze the transfer of a $^3$H-methyl group from S-adenosyl-L-methionine, ($^3$H-methyl)($^3$H-SAM) to norepinephrine and epinephrine. The resulting products, $^3$H-normetanephrine and $^3$H-metanephrine are extracted and isolated by thin layer chromatography and counted by liquid scintillation.
Thin layer chromatography (TLC) The separation of the labeled norepinephrine and epinephrine by TLC depends on two phases. The stationary phase is formed by the adherence of a thin layer of silica gel sprayed onto a glass plate. After the samples have been applied as spots, the plate is placed vertically in a chamber containing the mobile phase (solvent). The solvent ascends through the layer by capillary migration. Sample substances are detected by viewing under a UV light after the mobile phase has been removed and the plate dried. The dried samples are scraped from the plate into the appropriate scintillation vials. TLC was developed in 1938 by Izmailon and Schraiber (70).

Liquid scintillation counting Liquid scintillation derives its name from the liquid mixture of sample, solvent and fluor. The $^3$H labeled epinephrine and norepinephrine emit low levels of beta radiation that cannot be measured by solid scintillation counting. For this study, toluene was used as solvent and Liquifluor as the fluor. Fluors are complex heterocyclic, organic compounds which, when excited, emit photons in the near ultraviolet range. The sample emissions first excite the solvent molecules, which in turn excite the fluor electrons to emit photons as they return to the ground state. Paired photomultiplier tubes are utilized to detect the photons and result in electrical pulses which are passed through a coincidence circuit and electronically summed (71).
Sample preparation
- Blood samples must be collected in special evacuated tubes containing EGTA and glutathione, which have been added to the tubes in a solution (pH 6.0-7.4) containing 90 mg/ml EGTA and 60 mg/ml glutathione. Twenty microliters of the solution was needed for each ml of blood.
- After collection, the tube is inverted several times, but not shaken, to mix the blood with preservatives.
- Samples must be placed immediately in an ice bath and centrifuged in a refrigerated centrifuge as soon as possible.
- After separation of the plasma, samples can be frozen and are stable for at least three months when stored in a tightly closed container at temperatures below -20°C.

Procedure
1. Thawed samples must be centrifuged if the plasma is not clear.
2. The following 13 x 100 mm tubes are labeled and placed in an ice bath: Blank (X 2), control (X 2), control+standard (X 2), sample unknown (X 2), sample+standard (X 2). Each sample is analyzed in duplicate.
3. 50 µl of each sample is added to the sample tube and the sample+std tube.
4. 50 µl of each control is added to the control and control+std tubes.
5. 50 µl of the sample vehicle is added to the blank tubes.  
   (If samples are analyzed in different vehicles, a separate 
   blank must be run for each).

6. 10 µl of diluted stabilizing solution (1 to 10,000 with 
   distilled H₂O) is added to the control, sample and blank 
   tubes.

7. 10 µl of diluted catecholamine standard solution (1 to 
   10,000 with distilled H₂O) is added to the control+std and 
   sample+std tubes in a concentration equal to 100 pg/sample 
   tube.

8. The reagent mixture is prepared in a disposable tube in 
   the following proportions:
   
   Distilled water         10 µl
   Buffer solution         10 µl
   ³H-SAM                  10 µl
   COMT enzyme solution    10 µl

   The mixture is vortexed lightly before and after addition 
   of COMT. Because SAM is very acidic, the enzyme should 
   not be layered on the acid as this causes denaturation.

9. 40 µl of the reagent mixture is added to each tube.

10. The tubes are vortexed gently and centrifuged briefly at 
    300 X g for 30 seconds (1500 rpm for Beckman J21B).

11. All tubes are incubated at 37°C for 60 minutes in a 
    shaking water bath. The tubes are then placed in an ice 
    bath.
12. 50 μl of stopping solution is added and the tubes are vortexed vigorously.

13. A second set of 13 X 100 mm glass tubes is labeled.

14. 0.1 ml of 0.1 M acetic acid is added to each.

15. 2 ml of toluene/amyl alcohol (3:2, v/v) is added to the incubation tubes and vortexed vigorously. After centrifuging at 800 X g for 2 minutes, each tube is quick frozen (15 seconds) in a dry ice alcohol bath.

16. The tubes are removed from the bath and blotted to remove alcohol. The organic phase is decanted into the second set of tubes and the lower aqueous phase is discarded.

17. After vortexing vigorously, the tubes are centrifuged at 800 X g for 2 minutes and quick frozen in a dry ice alcohol bath. The upper layer is aspirated off and discarded.

18. Stopping point: stopper tubes and store at -20°C.

19. 0.1 to 0.15 ml absolute ethanol is added to the thawed acetic acid to facilitate evaporation. Vortex until a clear solution results and centrifuge at 800 X g for 1 minute.

20. Label channels on thin layer chromatography plate, omitting the outer lanes. The acetic acid/ethanol mixture is applied as a single spot 2 cm from bottom of plate. A warm air stream will aid evaporation.

21. The plates are placed in a paper lined developing tank. The solvent should be no less than 1 cm below the spots.
Develop with tertiary amyl alcohol/benzene/methylamine (6:2:3, v/v/v). Solvent prepared immediately before use produces the best separation. The solvent is permitted to migrate to the top of the plate.

22. The plates are removed from the tank and air dried. The developing solvent is discarded. The plates are placed under a 254 nm light and the zones are marked as follows:
   - Top - 3 methoxytyramine - dopamine
   - Middle - Metanephrine - epinephrine
   - Bottom - Normetanephrine - norepinephrine

The zones are outlined with a sharp object WITHOUT TOUCHING THE ZONES.

23. Stopping point - if longer than overnight place the plates in a dessicator.

24. One scintillation vial is labeled for each catecholamine.

25. TLC plates are separated into sections at the prescored lines. The silica gel is carefully scraped from each zone into the appropriate vial.

26. Stopping point (if desired).

27. 1 ml of 0.05 ammonium hydroxide is added to the scintillation vials containing the silica gel from the Middle zone (A-epinephrine) and the Bottom zone (B-norepinephrine) and vortexed to extract the amine from the silica.
28. Periodate Oxidation Step - 50 μl of sodium metaperiodate solution is added at timed intervals to both A and B vials and mixed vigorously.

29. After 5 minutes, 50 μl of glycerol solution is added to each vial (A and B). Maintain time interval.

30. 1 ml of 0.1 M acetic acid is added to each vial (A and B) and vortexed to neutralize the reaction.

31. 10 ml of toluene/Liquifluor (1000:50, v/v) is added to each vial, using adequate ventilation.

32. The vials are capped and shaken vigorously. The vials are dark controlled and counted in a Liquid Scintillation Counter.


Precautions

Standards - The assay is linear to 3000 pg/sample for NE and E. The amount of standard added to the sample may be adjusted to be compatible to the quantity found in the sample. Further dilutions with stabilizing solution or distilled water is more acceptable than increasing the standards. Samples can be diluted with stabilizing solution if the concentration is greater than 3000 pg.

Radioactive counting

- Instrument background is included in the assay blank and is automatically subtracted by the liquid scintillation counter.
Inhibition of O-methylation

- Some plasma samples may inhibit O-methylation of catecholamines. The amount of radioactivity attributed to added standard is not as high as expected.

B. Oxidation Spectrophotometric Assay for Plasma Ascorbic Acid (DNPH Method).

There has been a reliable and precise assay for plasma ascorbic acid levels available since 1945 (72) and the present method is essentially unchanged.

Plasma ascorbic acid is oxidized to dehydroascorbic acid in the presence of copper ion. The addition of 2,4 dinitrophenyl hydrazine (DNPH) forms bis 2,4-dinitrophenylhydrozone. This derivative, when dissolved in sulpheric acid, forms a red-orange product that absorbs at 520 nm and can be measured spectrophotometrically.

Sample collection and handling.
- Blood samples are collected with EDTA as anticoagulant.
- Proteins are precipitated from the sample by the addition of trichloroacetic acid (10 g/100 ml).
- After centrifuging, the protein-free filtrate is decanted into clean tubes and can be frozen at -20°C. The samples are stable at this temperature for one month.

Procedure

1. Oxidation step

The color reagent, consisting of 2.2% 2,4 dinitrophenylhydrazine, 5% thiourea and 0.6% CuSO₄, is
added to each tube. Thiourea prevents the further oxidation of dehydroascorbic acid.

The tubes are incubated at 60°C for one hour.

2. **Color development**

   After incubation, the tubes are chilled in an ice bath and slowly, by drops, 2.5 ml of cold 65% sulfuric acid is added to each tube and mixed well.

   The tubes must be kept well chilled or destruction of the sample will result.

   Sample tubes are removed from the ice bath and allowed to stand for 20 minutes at room temperature. Other color products which can be read at the same wavelength are produced by this reaction. These interfering products are short lived and will degrade during the 20 minute standing period.

   The tubes are read at 515 nm in a spectrophotometer.

**Controls**

No commercial controls are available as the ascorbic acid is destroyed by the production processes. A serum pool, with samples obtained monthly from normal subjects, is used as control and are run with each assay. A standard curve also is run with each assay.
VI. RESULTS

A. Plasma Ascorbic Acid

As shown in Table II, mean plasma ascorbic acid (PAA) concentration in the smokers was significantly lower than the nonsmokers at the baseline measure ($F(1,41)=16.04; p<.01$). Smokers assigned to the treatment group attained a significantly higher than baseline level after one week on vitamin C supplements and maintained these levels throughout the remainder of the treatment period (Repeated measures: $F(5,13)=15.34; p<.01$). Mean PAA levels of smokers in the placebo group did not change over time from baseline (Repeated measures: $F(5,15)=2.12; p>.05$) and were significantly lower than nonsmokers at all measures (Student's $t$ values $>4.00; p<.01$).

Group comparisons between treatment and placebo smokers showed that mean PAA was significantly higher in the treatment group after one week and remained higher at the four successive sampling times (all $t$ values $>6.00; p<.01$). Comparison with the two subsequent measures in the nonsmoking group indicates that mean PAA levels in smokers assigned to the treatment group surpassed the nonsmokers at both times ($t$ values $>2.2; p<.05$).

Mean PAA in nonsmokers did not change over time from baseline (Repeated measures: $F(2,26)=2.00; p>.15$).
<table>
<thead>
<tr>
<th></th>
<th>BASELINE</th>
<th>WEEK 1</th>
<th>WEEK 2</th>
<th>WEEK 3</th>
<th>PRE-STRESS</th>
<th>STRESS</th>
<th>REPEATED MEASURES</th>
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</thead>
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<tr>
<td><strong>SMOKERS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TREATMENT (14)</td>
<td>0.74(±0.35)†</td>
<td>1.75(±0.52)*</td>
<td>1.81(±0.44)*</td>
<td>1.65(±0.23)*</td>
<td>1.63(±0.48)*†</td>
<td>1.68(±0.59)*†</td>
<td>p=0.0001</td>
</tr>
<tr>
<td>PLACEBO (16)</td>
<td>0.74(±0.35)†</td>
<td>0.67(±0.40)</td>
<td>0.63(±0.34)</td>
<td>0.51(±0.17)</td>
<td>0.53(±0.32)*†</td>
<td>0.54(±0.32)*†</td>
<td>NS</td>
</tr>
<tr>
<td><strong>NONSMOKERS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLACEBO (13)</td>
<td>1.12(±0.24)</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>1.30(±0.36)</td>
<td>1.15(±0.32)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Significant (p<0.01) treatment/placebo differences within smokers.
†Significant (p<0.01) smoker/non-smoker differences.
B. Plasma Catecholamine Levels

Analysis of variance indicated that vitamin C supplements had no effect on either E (F(1,123)=.31; p>.50) or NE (F(1,123)=1.89; p>.10). Pearson correlations between PAA and both E and NE values were not significant at any condition (r's <.32; p>.05). Because PAA was not related to catecholamine levels, treatment and placebo smokers were combined for analysis of stress effects.

Mean plasma E and NE values for smokers and nonsmokers are shown in Table III. An analysis of variance of catecholamine levels by group (smoker or nonsmoker) with repeated measures on conditions (baseline, pre-stress, and stress) showed that there were no differences between groups for either plasma E (F(1,41)=.14; p>.50) or NE (F(1,41)=1.19; p>.25). A significant condition effect was present for both E (F(2,41)=31.6; p<.01) and NE (F(2,41)=5.3; p<.01). Within group repeated measures indicated that mean plasma E levels increased significantly during the stress condition in both smokers (F(2,58)=23.8; p<.01) and nonsmokers (F(2,24)=10.6; p<.01). Mean plasma NE levels in smokers at pre-stress and stress conditions did not change from baseline (F(2,58)=1.59; p>.20). A significant increase in mean plasma NE occurred in the nonsmokers during stress (F(2,24)=11.0; p<.01).
### TABLE III

**MEAN (±SD) PLASMA CATECHOLAMINE LEVELS (pg/ml)**

**FOR SMOKERS AND NONSMOKERS DURING THREE CONDITIONS**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Baseline Epinephrine</th>
<th>Pre-Stress Epinephrine</th>
<th>Stress Epinephrine</th>
<th>P Level*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smokers</td>
<td>57.9(±43.6)</td>
<td>48.6(±32.2)</td>
<td>122.7(±76.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>49.7(±45.5)</td>
<td>52.8(±37.4)</td>
<td>144.7(±91.8)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>Norepinephrine</th>
<th>Norepinephrine</th>
<th>Norepinephrine</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Smokers</td>
<td>330.6(±182.7)</td>
<td>329.8(±200.2)</td>
<td>397.2(±214.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>275.2(±88.6)</td>
<td>243.6(±87.3)</td>
<td>399.0(±134.9)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Repeated Measures
C. **POMS**

Pearson correlations between anxiety scores and plasma E and NE levels were not significant (all r's < .40; p > .05). The smoker's mean anxiety score at baseline was significantly higher than the nonsmokers (9.76±5.5 versus 6.3±3.12: t=2.46; p<.05). This difference was not present during the stress session (8.4±4.9 versus 6.4±2.4: t=1.62; p>.10).
VII. DISCUSSION

Ascorbic acid, by its role in their production and metabolism, appears to be involved in the regulation of catecholamine levels (1-6). It was predicted that PAA levels would be related to plasma E and NE measures. This would be particularly evident in smokers who have lower PAA levels due to the destructive effect of nicotine. This hypothesis was not substantiated. Ingestion of vitamin C supplements had no effect on E or NE levels and correlations indicate that there was no relationship between PAA and NE or E values. Previous studies have shown alterations of tissue catecholamine levels related to ascorbic acid supplementation or deficiency (73,74). More recent investigations in rodent and human subjects failed to support these results. Dietary megadoses of ascorbic acid had no effect on serum or tissue amine levels in rats (75), and urinary excretion values in man did not change over a three-month period while ingesting 3.0 g/day ascorbic acid (76). However, an induced deficiency in guinea pigs produced decreased NE levels in cerebrum, cerebellum and midbrain attributed to depressed activity of dopamine-β-hydroxylase (77). If catecholamine levels are affected only under scorbutic conditions, this would account for the negative findings in the present study. Physical examination of the subjects revealed no clinical signs of ascorbic acid deficiency and PAA levels in smokers, while below those found in nonsmokers, were above the lower limits of normal. The study that reported changes in E and NE levels with supplementation
administered ascorbate by injection (73). Both the form of the supplement and the route of administration differ from the present study and the investigators did not measure plasma catecholamine levels (73,77). In the present study, oral vitamin C supplementation of 500 mg/day had no effect on plasma catecholamine levels.

The POMS anxiety scores indicate that, as a group, smokers are more anxious than nonsmokers. This difference was present only at the initial measure. Mean anxiety scores obtained during the stress session show higher, but not significantly different, values in the smoking group. The decrease in the mean anxiety score in smokers at the time of the second measure might be due to a learning effect from the first test or a habituation to the experimental setting.

The present finding that smokers and nonsmokers have comparable plasma E and NE levels is in disagreement with results from previous studies (48,51-53). While the consensus regarding the effect of smoking on NE is far from unanimous, most investigators have reported appreciably higher E levels in smokers following a period of heavy smoking. They attribute this increase to adrenomedullary stimulation by nicotine. Five studies examined urinary (47,48,51), serum (52) and plasma (53) E and NE levels in a series of smoking versus nonsmoking periods. All of the studies found significantly higher E levels during the smoking as compared to the nonsmoking segments. Only one of the studies reported a concomitant increase in NE levels following smoking (53). Three
studies measured urinary E and NE levels in groups of smokers and nonsmokers (47,52,78). Two of the studies found no difference in E or NE levels between smokers and nonsmokers (47,78) and one reported a higher E level in the smoking group (51). Elevated catecholamine levels under the artificial conditions of controlled smoking and nonsmoking periods might be more representative of acute nicotine stress effects than an accurate portrayal of the chronic effects experienced by the habitual smoker. Results obtained from manipulated smoking behavior do not appear to be valid in real-life application. The present study shows that smokers and nonsmokers have comparable plasma E and NE levels, indicating an adaptation in smokers to continual nicotine exposure. Adaptation has been shown experimentally to occur in rats after 14 days of chronic nicotine administration (79). Significant increases in the activity of enzymes responsible for catecholamine metabolism, particularly catechol-0-methyltransferase (COMT), were concomitant with the return of amine levels to normal and remained elevated during the time nicotine stress was present (79).

Significant increases in plasma E levels were found in both smokers and nonsmokers during the stress condition. Several factors point to public speaking anxiety as the causal agent in these increases. To ensure that valid baseline data were available, two separate measures were obtained. An initial baseline was established at the first session and a second, pre-stress level was determined four weeks later. Comparison with
pre-stress levels showed no change from initial E baseline for either smokers or nonsmokers. The dramatic rise in E over both the baseline and pre-stress measures appears to be due solely to the effects of the stress condition.

Sample timing has been shown to be a critical variable in the accurate assessment of environmental stress effects on circulating E concentrations (46). Epinephrine peaks and disappears rapidly from the circulation after the initial onset of environmental stress conditions. Plasma levels obtained after the first three minutes would detect E as it descends toward normal. The present study measured plasma catecholamines at three minutes after initiation of public speaking and should reflect peak values.

While the results for E are straightforward, the NE data do nothing to clarify the already confusing issue regarding its relationship to stress. Mean NE levels in smokers did not change during the course of the experiment; however, the stress condition produced a significant increase in NE in the nonsmokers. These results are complicated further by group comparisons that indicate no difference in NE levels between smokers and nonsmokers. This might be explained, in part, by the large within group variance and unequal group numbers. Although the difference was not significant, mean NE levels in smokers were consistently higher than the nonsmokers at baseline and pre-stress measures. Norepinephrine values in smokers did not show an additional increase under the stress condition, but remained at their
previous high level. Mean NE values in nonsmokers increased during stress until it reached a level almost identical to that found in the smokers. The elevated mean NE in nonsmokers during stress also was similar to that measured in smokers under nonstress conditions. The physiological implications of this are important. Smokers exhibit NE concentrations in nonstress conditions that are comparable to those found in nonsmoking subjects during an environmental stress situation. It is as if smokers were experiencing the effects of a continually applied environmental stress. Their NE levels are high and perhaps cannot increase even with an additional stressor.

In spite of the confusion surrounding the NE data, certain conclusions can be drawn from the results of the present study. Smokers appear to be more anxious than nonsmokers as measured by their initial scores on the POMS. It is a matter for speculation whether this is a result or a cause of smoking. Circulating levels of E and NE are affected by exposure to an environmental stress condition. The increase of NE appears to be masked in smokers, whose high nonstress levels are not additionally increased with stress. Adaptation to chronic nicotine ingestion does not appear to occur as successfully with NE as with E. Mean epinephrine levels in smokers measured under nonstress conditions are as low, or lower, than those found in nonsmokers. Thus some mechanism exists to regulate circulating E in the presence of continual nicotine exposure.
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