THE EFFECTS OF STRESS ON LEVELS OF NICOTINE IN THE RAT

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WINDERS

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
F. EDWARD HÉBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

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Name of Candidate: Suzan E. Winders
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Jerome E. Singer — Committee Chairperson — 1 March 1990 — Date

Committee Member — 1 March 1990 — Date

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Suzan E. Winders

Suzan E. Winders
Department of Medical Psychology
Uniformed Services University
of the Health Sciences

ABSTRACT

Dissertation Title: The Effects of Stress on Levels of Nicotine in the Rat

Name of Candidate: Suzan E. Winders, Doctor of Philosophy, 1990

Dissertation directed by: Neil E. Grunberg
Associate Professor
Department of Medical Psychology

The present study was designed to determine if: a) stress reduces nicotine levels in the body; b) if reduced levels could be explained by increased rates of nicotine metabolism. Levels of nicotine in the tissues (blood, fat, muscle, and brain) of stressed and nonstressed Sprague-Dawley rats were compared in order to determine if stress would lower nicotine levels in the rat. In addition, nicotine metabolism was measured both *in vivo* and *in vitro* in order to determine if stress might exert its effects on nicotine levels directly by increasing the rate of conversion of nicotine to cotinine, nicotine's primary metabolite. Blood levels of cotinine were measured to determine nicotine metabolism *in vivo*. Livers were harvested and incubated with nicotine and the incubates were analyzed for cotinine to determine nicotine metabolism *in vitro*.

Animals were administered one of three dosages of nicotine (0, 6, and 12 mg nicotine/kg) via miniosmotic pumps implanted subcutaneously. After 14 days of continuous drug

administration, animals were subjected to one of three conditions (no stress, noise stress, and rubber ligature stress) for 2.5 hours. Immediately following this 2.5 h period, animals were sacrificed and tissue nicotine and blood and liver cotinine levels were determined.

Comparing animals receiving 12 mg nicotine/kg/day, blood nicotine levels were lower among animals in the noise and rubber ligature conditions compared to animals in the nonstress condition. There was no effect of stress condition on either measure of nicotine metabolism. These results are consistent with the explanation that smokers under stress smoke more to replace lost nicotine. In addition, the fact that reductions in nicotine levels were observed comparing animals in the 12 mg nicotine/kg/day, but not among animals in the 6 mg/kg/day conditions, suggests that drug dosage is an important factor in this relationship. And finally, these results suggest that decreased nicotine levels observed during stress are not the result of increased conversion of nicotine to cotinine.

THE EFFECTS OF STRESS
ON
LEVELS OF NICOTINE IN THE RAT

by

Suzan E. Winders

Dissertation submitted to the Faculty of the Department of
Medical Psychology
Graduate Program of the Uniformed Services University of
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1990

DEDICATION

To my parents

Robert and Bernice Winders

for their continued love and encouragement during this dissertation and throughout my life

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S.E.W.

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INTRODUCTION

Cigarette smoking is currently considered the single most important preventable environmental factor contributing to illness, disability, and death in the United States today. It is now well established that smoking is causally related to a number of disabling and fatal illnesses including a variety of cancers, cardiovascular diseases, bronchopulmonary diseases, and digestive diseases (USDHHS 1979, 1984, 1985, 1988).

The reluctance on the part of many smokers to quit smoking, particularly in light of the widely-publicized health risks, suggests that smokers have some fairly compelling reasons to smoke. Clearly, these reasons must be understood if we are ever to reduce the prevalence of smoking in our society.

One reason many smokers often give for smoking is stress. Smokers have long reported that they smoke more when under stress and that smoking reduces their stress (Barnes & Fishlinski, 1976; Frith, 1971; Ikard & Thomkins, 1973; Kleinke, Staneski, & Meeker, 1983; Linn & Stein, 1985; McKennell, 1970; Russell, Peto & Patel, 1974). Recent epidemiological and laboratory studies have confirmed that smoking rates do indeed increase under stress (Billings & Moos, 1983; Burr, 1984; Cherek, 1985; Conway, Vickers, Ward & Rahe, 1981; Hirschman, Leventhal & Glynn, 1984; Lindenthal, Myers & Pepper, 1972; Mitic, McGuire & Newman, 1985; Rose, Ananda & Jarvik, 1983; Schachter, Silverstein, Kozlowski,

Herman & Liebling, 1977; Tagliacozzo and Vaughn, 1982; Westman, Eden & Shirom, 1985; Wills, 1986; Wills & Shiffman, 1985). Unfortunately, despite the fact that the relationship between stress and smoking is well documented, the reasons for it are not.

Many of the explanations offered to account for this phenomenon involve nicotine. Nicotine is thought to be a dependence-producing pharmacological agent in tobacco smoke (Gritz, 1986; USDHHS, 1988). It is known to have a powerful range of effects on the central and peripheral nervous systems, and it has direct effects on many of the body's major organs. In addition, it shares many common properties with other substances of abuse including: ability to reinforce human and animal behavior (Pomerleau, 1986); ability to produce physical dependence (Henningfield, 1986) and tolerance (Benowitz, 1986a; Donegan, Rodin & O'Brien, 1983; Jarvik, 1979); and a well-documented withdrawal syndrome (Shiffman, 1979).

Stress is a complex phenomenon thought to be the result of both environmental demands and the coping abilities of the individual (Lazarus & Launier, 1978; Lazarus & Folkman, 1984). When environmental demands exceed the individual's coping abilities, a state of subjective stress is said to arise. This stress manifests itself psychologically, as psychological distress, and biologically, as sympathetic nervous system arousal and endocrine system changes.

There are a variety of theories which have been offered to account for the relationship between stress and nicotine. Essentially the majority of these explanations can be grouped into two types: (1) those which hold that nicotine alters the effects of stress; and (2) those which suggest that stress alters the effects of nicotine. According to the first explanation, smokers smoke more under stress because nicotine acts in some way to reduce the psychological and biological manifestations of stress. This explanation has little empirical support, but is commonly cited by smokers as a reason for smoking under stress. The second explanation holds that smokers smoke more under stress in order to maintain the effects of nicotine which are diminished in some way by stress. This second explanation was favored by Schachter and his coworkers (Schachter, 1978; Schachter, Silverstein, Kozlowski, Perlick, Herman & Liebling, 1977). These investigators demonstrated that smokers adjust their smoking rate so as to maintain their nicotine intake at a roughly constant level (Schachter, 1977, 1978). Noting that stress decreases urinary pH, which in turn increases excretion of unmetabolized nicotine, they reasoned that levels of nicotine would decrease during stress. Therefore, smokers would smoke more during stress in order to replenish nicotine loss resulting from stress. Because nicotine regulation was observed among heavy (20 or more cigarettes per day) smokers, but not light smokers (15 or less cigarettes per day), they further reasoned that

stress would increase the smoking behavior of heavy smokers, while having a lesser effect on the smoking behavior of light smokers.

Both of the theories for the relationship between stress and nicotine have received some support in the literature and deserve further research attention. The focus of the present research is limited to examination of the second explanation. The research presented in this dissertation was designed to examine the effect of stress on levels of nicotine throughout the body and to examine in particular one hypothesized mechanism. Before presenting that work in detail, the literature which has examined the relationship between stress and smoking is presented as well as a discussion of the available theories which have postulated that stress alters the effects of nicotine.

Stress and smoking among adult smokers

The literature which has examined the relationship between stress and smoking among regular smokers is comprised of a mixture of field and laboratory studies. Evidence from the field studies is relatively weak because most of these studies have employed cross-sectional designs, making causal inferences difficult. The following section first reviews the field studies and then presents laboratory studies which have examined the relationship between stress and smoking.

Field Studies

The relationship between stress and smoking was examined in two cross-sectional field studies examining the

relationship between smoking and recent life events. Lindenthal, Myers, and Pepper (1972) collected information pertaining to recently occurring life crises, smoking habits, and psychological impairment (as measured by items which have been found to discriminate between psychiatrically labeled and healthy populations) from a community sample of 938 adults in the New Haven metropolitan area. Their findings indicated that increased rates of smoking were related to a high level of negative events, particularly among psychologically-impaired individuals. The relationship between stress and smoking held after statistically controlling for differences in sex, race, age, marital status and social class.

In another study relating life events and smoking behavior, Billings and Moos (1983) surveyed a community sample of 608 adults in the San Francisco Bay area. Subjects were administered three questionnaires assessing life stressors and social resources, smoking behavior, and personal functioning. Subjects were divided into three comparison groups: nonsmokers; light smokers (1-19 cigarettes per day); heavy smokers (20 or more cigarettes per day). Pipe smokers and occasional smokers were excluded from the analysis. Heavy smokers were distinguished from nonsmokers by significantly higher levels of environmental stressors, less supportive social resources, and poorer psychological functioning. Light smokers did not differ significantly from nonsmokers on any of these factors. Comparison of light and

heavy smokers revealed some significant differences. Specifically, smoking was positively correlated with stress and negatively correlated with the number of social contacts among heavy smokers, while stress and smoking were unrelated and smoking and social contacts were positively correlated among light smokers. These results lead the authors to conclude that light smokers tend to smoke more heavily in social situations, while heavy smokers appear to smoke more in response to environmental stressors.

Three studies have supported a relationship between job-related stress and smoking. Tagliacozzo and Vaughn (1982) administered a 26-item index of job-related stress to 448 nurses involved in direct patient care at the University of Michigan hospital. The 26 items of the index were divided into two subscales in order to assess both job-related and role-related stress. Results indicated that job-related stress and role-related stress were greater among smokers compared to non-smokers on both of the subscales, although the differences were significant only on the job-related subscale. Further analysis comparing smokers and nonsmokers on a number of demographic and employment variables revealed that many of the significant differences were limited to the younger (under 29), single, more educated nurses in this sample.

Burr (1984) surveyed 505 Navy enlisted men employed on three U.S. amphibious assault ships with regard to a number of personality and situational variables. Included was a

19-item measure of perceived stress from three domains: job, organization, and family. Responses to these items were related to responses to a single item which asked "Do you smoke?" Results indicated that smokers scored significantly higher than nonsmokers on two subscales from the stress measure indexing Role Conflict and Family Strain.

Westman, Eden and Shirom (1985) examined the relationship between smoking intensity (average number of cigarettes smoked per day) and job stress among 560 male smokers recruited from 22 Israeli kibbutzim. Results indicated that smoking intensity was positively related to three subscales from the stress measure indexing hours of work, work addiction (defined as working beyond requirements and working voluntarily on holidays), and lack of influence. A fourth subscale indexing intrinsic impoverishment was negatively related to smoking intensity.

In a longitudinal field study Conway, Vickers, Ward and Rahe (1981) collected data regarding the occupational stress, cigarette consumption, coffee consumption and alcohol consumption of 34 U.S. Navy petty officers. Data were collected on 14 study days over an eight-month period during which these men were performing a job with known systematic variations in stress. On each of the study days subjects responded to a 42-item questionnaire containing eight indicators of perceived stress. In addition, current smokers were asked to indicate the number of cigarettes, cigars, and/or pipefuls they had smoked per day that week. Results

showed that among current smokers cigarette consumption was positively correlated with seven of the eight stress indicators. Furthermore, these correlations were found to be consistent across time, suggesting that adaptation to stress did not occur.

Laboratory Studies

By virtue of design, the field studies are open to alternative interpretations. However, several laboratory studies in which subjects are randomly assigned to experimental conditions and independent variables are carefully manipulated are also available in this literature. The overwhelming majority of these studies provide empirical support for the notion that increased stress is associated with increased smoking.

Schachter, Silverstein, Kozlowski, Herman, and Liebling (1977) observed the smoking behavior of 48 heavy and light smokers (28 males, 20 females) under conditions of low and high stress imposed by electric shock. Smokers in the high-stress condition smoked significantly more cigarettes and took significantly more puffs than smokers in the low stress condition. Comparing the effects of stress on heavy and light smokers, stressed heavy smokers took 65.9 percent more puffs than nonstressed heavy smokers while stressed light smokers took only 8.5 percent more puffs than their nonstressed counterparts.

Dobbs, Strickler, and Maxwell (1981) measured smoking rate and centimeters of cigarettes smoked in 32 undergraduate

male moderate to heavy smokers (20 or more cigarettes per day) subjected to anticipatory psychological stress. Increases in stress were associated with significant increases in both centimeters of cigarettes smoked and smoking rate.

Rose, Ananda, and Jarvik (1983) observed the smoking behavior of 15 moderate to heavy smokers (20 or more cigarettes per day) exposed to three conditions: stagefright anxiety, monotonous concentration, and relaxation control. Subjects were allowed to smoke one cigarette during each period and smoking topography (number of puffs and cumulative volume smoked) was continuously recorded. Subjects took significantly more puffs and inhaled a significantly greater volume of smoke during both the stagefright anxiety condition and the monotonous concentration condition. Further analysis indicated that younger people tended to increase their smoking more than older people during stagefright and females tended to smoke more than males during monotonous concentration.

Cherek (1985) examined the effects of exposure to various levels of industrial noise on the smoking behavior of seven male blue-collar smokers during performance of a simulated work task. Results indicated that as noise level increased so did smoking behavior as indicated by significant increases in number of puffs per cigarette and duration of each puff. Increases in the number of cigarettes smoked per session were also observed, but were small and not

statistically significant.

Only one study failed to find a significant effect of stress on smoking. Glad and Adesso (1976) monitored the smoking behavior (number of cigarettes, number of puffs and number of minutes of smoking) of 144 light (less than 10 cigarettes per day) and heavy (more than 15 cigarettes per day) smoking college students (72 male, 72 female) placed in one of four conditions: high arousal with nonsmoking confederates; high arousal with smoking confederates; low arousal with nonsmoking confederates; low arousal with smoking confederates. Subjects in the high arousal conditions were asked to give a speech and were told that their performance would be rated by evaluators trained in a procedure capable of predicting academic success. Subjects in the low arousal condition were not asked to give a speech. Although the anxiety manipulation was effective, only the presence of others smoking significantly increased the number of subjects who smoked; this result applied to "light" smokers only. However, the presence of confederates who were smoking significantly increased the number of minutes subjects smoked in all conditions for both light and heavy smokers, suggesting a ceiling effect for heavy smokers.

Taken together these data suggest that there is a relationship between stress and smoking. Smokers appear to smoke more in situations characterized by high stress than situations characterized by low stress, particularly among moderate and heavy smokers.

Effects of Stress on the Effects of Nicotine

Schachter and his coworkers (Schachter, 1978; Schachter, Silverstein, Kozlowski, Perlick, Herman & Liebling, 1977) suggested that stress might alter the effects of nicotine by altering levels of nicotine in the body. Specifically, they proposed that stress decreases urinary pH which, in turn, increases excretion of unmetabolized nicotine. Based on earlier studies which examined the effects of urinary pH changes on the excretion of nicotine (Beckett, Rowland & Triggs, 1965; Beckett & Triggs, 1967), Schachter estimated that acidification of the urine increases the excretion of unmetabolized nicotine by 28 percent. Given the magnitude of this change he reasoned that acidification of the urine could potentially result in decreases in circulating levels of nicotine which would then lead to increased smoking rates as the smoker seeks to maintain steady-state levels of nicotine (Schachter, Kozlowski & Silverstein, 1977). In addition, he reasoned that because the smoking behavior of heavy smokers is much more sensitive to changes in nicotine levels than light smokers (Schachter, 1977, 1978), the effect of stress on smoking behavior would be greater among heavy compared to light smokers.

In a series of human laboratory studies designed to test this hypothesis, Schachter and his coworkers demonstrated that: experimentally induced increases in urinary acidity were accompanied by increases in cigarette

smoking (Schachter, Kozlowski & Silverstein, 1977); stress induced by threat of electric shock and academic evaluation was accompanied by increases in urinary acidity and smoking, particularly among heavy smokers (Schachter, Silverstein, Kozlowski, Herman & Liebling, 1977); and when urinary pH was kept alkaline, the effects of stress on smoking behavior were eliminated (Schachter, Silverstein & Perlick, 1977). Based on these findings Schachter concluded that stress affects circulating levels of nicotine through its effects on urinary pH (Schachter, 1978; Schachter, Silverstein, Kozlowski, Perlick, Herman & Liebling, 1977). Unfortunately, Schachter never directly measured nicotine levels in the blood. Based on the effects of pH on nicotine excretion demonstrated by Beckett and his colleagues (Beckett, Rowland & Triggs, 1965; Beckett & Triggs, 1967), Schachter assumed that changes in pH would also change circulating levels of nicotine, but he never tested this hypothesis.

Since 1977, many investigators have replicated or extended portions of Schachter's work, but none have tested the hypothesis that stress alters nicotine levels in the body. For example, two investigators examined the effects of stress on urinary pH and or smoking behavior, but did not measure nicotine levels in the body. These studies found that stress was accompanied by decreased pH in the urine (Dobbs, Strickler & Maxwell, 1981; Sandin & Chorot, 1985), skin and saliva (Sandin & Chorot, 1985), as well as by increases in smoking behavior (Dobbs, Strickler & Maxwell,

1981). Although these studies suggest that stress affects both urinary pH and smoking behavior, they do not address the more central question of whether stress affects nicotine levels in the body.

Other investigators measured circulating nicotine levels under conditions of controlled pH, but have not measured or manipulated stress. These studies confirmed the finding that acidification of the urine results in significant increases in urinary excretion of nicotine (Benowitz & Jacob, 1985; Feyerabend & Russell, 1978; Fix, Daughton & Issenberg, 1986; Matsukura, Sakamoto, Takahashi, Matsuyama & Muranaka, 1979; Rosenberg, Benowitz, Jacob & Wilson, 1980), but have failed to confirm the finding that changes in urinary pH are related to either changes in smoking behavior (Cherek, Lowe & Friedman, 1981; Fix, Daughton & Issenberg, 1986; Marshall, Green, Epstein, Rogers & McCoy, 1980) or changes in circulating levels of nicotine (Feyerabend & Russell, 1978; Rosenberg, Benowitz, Jacob & Wilson, 1980). These studies suggest that acidification of the urine may not significantly affect blood levels of nicotine or smoking behavior, but they do not directly address the effects of stress on smoking or on blood levels of nicotine.

Other studies have examined the relationship between urinary pH and nicotine using a very different approach. One human study examined the effect of pH manipulations on performance and inferred changes in nicotine levels from

changes in performance. Taylor and Blezard (1979) measured performance on a detection task of smokers and nonsmokers under conditions of acidic or basic pH. The pH manipulation had no effect on the performance of the non-smokers, who improved steadily throughout the task. Performance of smokers whose urine was acidified showed significantly less improvement on the task over time. The authors suggested that the decrement in performance of the smokers whose urine had been acidified was indicative of nicotine deprivation. Thus, they inferred changes in nicotine levels, but they did not measure nicotine.

In addition to the human study presented above, two animal studies have examined the effects of nicotine on behavior under conditions of controlled urinary pH. Latiff, Smith, and Lang (1979) examined the effects of urinary pH on the self-administration of nicotine. Urinary pH was altered in one of two ways: prior to the acquisition of responding, or 10 days after the responding to nicotine had been established. Results indicated that animals whose urine had been acidified prior to the acquisition of responding self-administered nicotine at a significantly greater rate than rats whose urine had been alkalinized and than rats in the control group. However, acidification of the urine had no effect on rats whose urinary pH was manipulated subsequent to the establishment of an initial rate of responding suggesting that it is difficult to change rates of nicotine self-administration once they have been established.

In another study, Grunberg, Morse, and Barrett (1983) examined the effects of nicotine on responding of squirrel monkeys under a multiple fixed interval (FI), fixed ratio (FR) schedule of stimulus shock termination under conditions that manipulated urinary pH. Nicotine administration increased responding under FI and FR schedules. Acidification of the urine resulted in attenuation of the effects of nicotine on responding under these schedules, while alkalinization potentiated the effects.

Based on these results Grunberg, Morse, and Barrett (1983) postulated that changes in smoking behavior that occur in response to situations and stimuli that alter urinary pH may affect smoking behavior not by altering levels of nicotine in the blood, but by altering the acute effects of nicotine on behavior. Grunberg and Kozlowski (1986) further developed this idea and suggested that alterations in pH, while not noticeably affecting blood levels of nicotine, might result in large changes in the percentage of nicotine in the free base form which would change the distribution of nicotine across membranes and might alter body fluid nicotine distribution and brain levels of nicotine. Despite this theoretical development these ideas have never been tested empirically.

In summary, the available literature supports the generalization that stress is associated with decreased urinary pH and increased smoking, and that decreased urinary pH is associated with increased urinary excretion of nicotine

and changes in the acute effects of nicotine. Based on these data researchers have speculated that stress alters nicotine levels in the body. Alternate formulations suggest changes in nicotine levels in the blood or brain. However, there is no direct empirical evidence to support any of these specific notions. Although these possibilities are consistent with the available research literature, they have not been examined.

THE PRESENT STUDY

Overview

The current study measured and compared levels of nicotine in the blood of stressed and nonstressed rats in order to directly test Schachter's (1977) notion that stress affects circulating levels of nicotine. In addition, levels of nicotine in the other tissues to which nicotine is primarily distributed (brain, muscle, and fat) were compared in these animals in order to test Grunberg and Kozlowski's (1986) theory that stress alters the distribution of nicotine to other tissues in the body. Finally, *in vivo* and *in vitro* nicotine metabolism were also measured in order to determine if stress might alter body nicotine levels directly by altering nicotine metabolism. Three dosages of nicotine were used in order to determine if there is an interaction between the dosage of nicotine and the effect of stress on nicotine levels in the body.

Subjects were 90 male Sprague-Dawley rats receiving

one of three nicotine dosages (0, 6, and 12 mg nicotine/kg/day) administered chronically via osmotic minipumps. After 14 days of drug administration, 10 animals from each drug condition were subjected to one of three stressors: physical stress (hind-leg rubber ligature), environmental stress (loud bursts of white noise), or no stress (left undisturbed) for 2.5 hours. In order to validate the stress manipulation both behavioral and biological measures of stress were taken. The behavioral measure consisted of three ratings (taken once every 45 minutes beginning on minute 15 during the 2.5 hour stress period) of five behaviors previously shown to indicate fear: activity, trembling, urination, defecation, and grooming (Singer, 1961, 1963). The biological measure consisted of the level of corticosterone found in plasma taken immediately following the stress period.

Immediately following administration of the stressor, blood, brain, muscle, fat, and liver samples were collected from each animal. In order to determine the effects of stress on levels of nicotine in the rat's body, blood, brain, muscle, and fat, tissues were analyzed for nicotine by gas chromatography as described in Jacob, Wilson, and Benowitz (1981). In order to determine the effects of stress on *in vivo* nicotine metabolism, blood samples were also analyzed for cotinine, nicotine's primary metaboite, by gas chromatography. In order to determine the effects of stress on *in vitro* nicotine metabolism, liver samples were incubated

with nicotine for 15 minutes and the incubate was analyzed for cotinine by gas chromatography as described in Jacob, Wilson, and Benowitz (1981).

Specific Hypotheses

Hypothesized drug effect

Tissue nicotine levels

Hypothesis. It was hypothesized that animals having received nicotine would have higher levels of nicotine in their blood and tissues than animals having received saline. Further, animals that were in the 12 mg nicotine/kg body weight/day condition would have higher levels of nicotine in their blood and tissues than animals that had been in the 6 mg nicotine/kg body weight/day condition.

Rationale. A pilot study using similar animals and method of nicotine administration found that animals administered 12 mg nicotine/kg body weight/day had significantly higher mean circulating levels of nicotine and cotinine (319.29 $\pm$ 87.19, 1347.70 $\pm$ 243.07, ng/ml blood, nicotine and cotinine, respectively) compared to animals administered 6 mg nicotine/kg body weight/day (150.95 $\pm$ 40.57, 728.88 $\pm$ 155.60 ng/ml blood, nicotine and cotinine, respectively). Given that tissue levels of a given drug generally reach equilibrium with plasma levels in less than a day (Gilman, Goodman, Rall, & Murad, 1985), it would follow that after 14 days of chronic nicotine administration, animals in the 12 mg nicotine/kg body weight/day condition would have higher levels of nicotine in all of the tissues

being sampled compared to animals in the 6 mg nicotine/kg body weight/day condition.

Blood and liver cotinine levels

Hypothesis. It was hypothesized that higher levels of cotinine would be found in the blood and liver incubated with nicotine of animals that had received nicotine compared to animals that had received saline. It was further hypothesized that cotinine levels in these tissues would be higher comparing animals in the 12 mg/kg/day group to animals in the 6 mg/kg/day group.

Rationale. Previous studies have demonstrated that nicotine administration is associated with an induction of drug-metabolizing enzymes in man (Beckett & Triggs, 1967) and animals (Wenzel & Broadie, 1966; Yamamoto, Nagai, Kimura, & Iwatsubo, 1966).

Hypothesized stress effect

Tissue nicotine levels

Hypothesis. It was hypothesized that tissue levels of nicotine would be lower in animals exposed to stress compared to animals not exposed to stress.

Rationale. If stress increases nicotine excretion as Schachter (1977, 1978) has postulated, then levels of nicotine in animals receiving a steady infusion of nicotine should decrease as nicotine excretion increases due to stress.

Levels of cotinine in blood and liver incubated with nicotine

Hypothesis. It was hypothesized that levels of

cotinine found in blood and liver incubated with nicotine would be higher comparing animals that had been stressed to animals that had not been stressed.

Rationale. Ninety-one percent of injested nicotine is metabolized (Jacob, Benowitz & Shulgin, 1988). The majority of this metabolism occurs in the liver (Gilman, Goodman, Rall & Murad, 1985) where nicotine is broken down by a class of enzymes known as microsomal monooxygenases (Beckett & Triggs, 1967; Katzung, 1987 cf table 3-2). Stress has been found to stimulate the activity of these enzymes (Stitzel & Furner, 1967), and thus might conceivably facilitate metabolism of nicotine. In addition, metabolism of other drugs broken down in the liver has been found to be enhanced by stress (Bousquet, Rupe & Miya, 1964; Chung & Brown, 1976; Driever & Bousquet, 1965; Rupe, Bousquet & Miya, 1964) lending further empirical support to the notion that stress might induce liver metabolism of nicotine.

The primary known metabolites of nicotine are nicotine N-oxide and cotinine. Conversion to nicotine N-oxide accounts for about four percent of nicotine metabolism while conversion to cotinine accounts for approximately 70 percent of nicotine metabolism (the remaining 17 percent is broken down through yet unknown pathways). If stress increases the functioning of the liver enzymes and that conversion of nicotine to cotinine is the primary liver metabolism pathway, then levels of cotinine in blood and liver incubated with nicotine of animals exposed to stress should be higher than

those of nonstressed animals.

Hypothesized stress by drug interaction

Tissue nicotine levels

Hypothesis. It was hypothesized that the effect of stress on tissue levels of nicotine would be similar comparing animals in the 12 and 6 mg nicotine/kg/day groups (e.g., there would be no stress by drug interaction).

Rationale. Because stress appears to have a lesser effect on the smoking behavior of light smokers compared to heavy smokers (Billings & Moos, 1983; Schachter Silverstein, Kozlowski, Herman, & Liebling, 1977) it is possible that there might be an interaction between stress and nicotine dosage. However, if, as Schachter (1977, 1978) proposed, the difference between heavy and light smokers is due to the fact that light smokers are poor regulators of nicotine, then the effect of stress on tissue levels of nicotine would not be expected to differ across dosage levels.

Blood and liver cotinine levels

Hypothesis. It was hypothesized that the effect of stress on levels of cotinine found in blood and liver incubated with nicotine would be similar comparing animals in the 12 and 6 mg nicotine/kg/day groups (e.g., there would be no stress by drug interaction).

Rationale. Previous research examining the effect of stress on metabolism of other drugs broken down by the liver has not found any stress by drug dosage interactions (Bousquet, Rubpe & Miya, 1964; Chung & Brown, 1976; Driever &

Bousquet, 1965; Rupe, Bousquet & Miya, 1964).

Methods

Subjects and Housing

Subjects were 90 male Sprague-Dawley rats obtained from Charles River weighing between 373 and 435 grams (approximately 76-84 days old). Animals were individually housed in standard polypropylene cages (35.56 cm x 15.24 cm x 20.32 cm) with absorbent wood pine-dri shavings and metal grill lids. All cages were placed on six, four-shelved, single-sided racks in a 20' x 10' room with overhead fluorescent illumination. The room was maintained on a 12 hour light/dark cycle at approximately 72° F and 50 percent humidity.

Rat chow (Charles River RMH 3500 pellets) and tap water were continuously available. Food pellets were placed on the wire lids of the cages within easy reach of the animals. Water was presented in plastic bottles fitted with stainless steel stopper tubes. Bottles were suspended on the wire lids of the cages with the spout protruding into the cage. Cages, wire lids, and water bottles were changed twice a week.

An animal model was chosen because, with the exception of blood samples, the samples to be obtained in the proposed study cannot easily be obtained from humans due to the prohibitive costs and excessive risks associated with the required sampling procedures. Rats were chosen as an appropriate animal model for the current study because of the

approximation of their physiology to that of humans and their widespread usage in drug distribution and metabolism studies of this type (Mitruka, Rawnsley & Vadehra, 1976). In addition, rats have proven to be an excellent model of several effects of nicotine, providing comparable data to human studies of the effects of cigarette smoking (Winders & Grunberg, 1989).

Drug administration

Nicotine was administered using Alzet mini osmotic pumps (model 2002, Alza Corp., CA). A saline control solution was also administered via this method. The minipump released its contents at a rate of approximately 0.5 µl/h to the subject for 14 days. Physiological saline was used to make nicotine solutions (made from nicotine dihydrochloride synthesized by Dr. Edward Cone at the Addiction Research Center, NIDA) and served as the control solution. Minipumps were used because animals may receive drug each day without the trauma of daily injections, and because the slow infusion rate establishes and maintains fairly constant concentrations of drug for many days. Both the drug dosages and method of administration were chosen because previous research using these procedures has produced animal results comparable to studies of human smokers (Winders & Grunberg, 1989; Grunberg, 1982; Grunberg & Morse, 1984; Grunberg, Bowen & Winders, 1986; Grunberg, Winders & Popp, 1987).

Determination of tissue nicotine and blood cotinine

Nicotine was measured in the body tissues to which

nicotine is primarily distributed: blood, brain, fat, and muscle. This measurement was used to determine whether changes in nicotine levels caused by stress were a result of decreased blood levels of nicotine as Schachter and his colleagues argued (Schachter, 1978; Schachter, Silverstein, Kozlowski, Perlick, Herman & Liebling, 1977) or were the result of stress-induced changes in relative body-wide distribution of nicotine as Grunberg and his colleagues argued (Grunberg & Kozlowski, 1986; Grunberg, Morse & Barrett, 1983). In addition, levels of cotinine, nicotine's primary and inactive metabolite (Benowitz, 1986), were measured in blood. Blood cotinine data was used as an estimate of the total amount of drug metabolized in the body.

Tissue preparation

Blood. Immediately following decapitation, trunk blood was collected. Four ml of the blood was then transferred to labeled, 12 x 75 mm, five ml polypropylene tubes containing 0.5 g of potassium oxalate anticoagulant. The tubes were then capped, inverted gently and placed in ice. The tubes were then placed in a -70°C freezer for storage until later nicotine and cotinine assay.

Muscle, fat and brain tissue. Following decapitation, the brain, a representative abdominal fat sample (weighing approximately 1g) and a quadriceps muscle were removed from each animal. Whole brains were taken in order to determine overall brain levels of nicotine rather than levels at particular brain loci. Because nicotine distributes equally

to similar tissues, fat, and muscle tissues could be taken from a variety of locations. However, abdominal fat and quadriceps muscles were specified in order to assure that a standardized sample of sufficient size (as specified by the assay procedure) were taken from each subject. Following removal, tissues were transferred to pre-weighed, labeled, 12 x 75 mm, five ml polypropylene tubes containing a strong alkali solution (2 ml 5N NaOH) and weighed. The tissue samples were placed in a strong alkali solution in order to dissolve the tissue because the assay requires that substances to be assayed are in solution. In addition, the alkali solution served to convert the nicotine and cotinine contained in the tissues into their more stable freebase forms for the purposes of transport and storage. Weights were taken so that nicotine levels could be determined per gram of tissue. The tubes containing the sample and alkali solution were then placed in a water bath set at 37°C until the tissue was completely dissolved (approximately five days). Once tissue dissolution was complete, the tubes were placed in a -70°C freezer for storage for later nicotine and cotinine assay.

Determination of nicotine and cotinine in blood and tissues

The steps involved in the assay procedure are presented in the following text and Appendix 1. The methods described for determination of nicotine and cotinine in blood are a modified version of the method described in Jacob, Wilson, and Benowitz (1981) of the Division of Clinical

Pharmacology, Department of Medicine, University of California, San Francisco (UCSF). Because the modifications on the original assay are extensive, the procedure is described, step-by-step, below.

Because it was especially difficult to extract nicotine from brain, fat, and muscle tissues, a preliminary extraction step was necessary for these tissues before they could be extracted according to the methods described for blood samples. The preliminary extraction is based on the unpublished research of Dr. Chin Savidiprani, a member of the research team at UCSF. Because data on the percentage of nicotine recovered from tissues analyzed via this procedure were not established at the time the study was conducted, tissues were collected from 6 animals who were not part of the study but who were similar to the animals used in the study in all respects. Just after the tissues were collected and placed in test tubes containing the alkali solution, but before they were placed in the water bath, nicotine (approximately 900 ng/g of tissue) was added to each tissue sample. In order to determine the percentage of nicotine recovered using this procedure, the concentration of nicotine measured in the sample (corrected for assay interference) was calculated as a percentage of the amount of nicotine which had been added to the sample. Based on the data from these six animals, the mean recovery rates of the three tissue assays were 100, 92, and 82 for the brain, fat, and muscle assays, respectively. All assays were performed by the

author at the UCSF clinical pharmacology laboratory under the supervision of Doctors Benowitz, Jacob, Savidiprani, and Wilson.

Addition of internal standards. After thawing, blood and tissue samples were aliquotted into 0.3 ml (brain) or 0.5 ml (blood, fat, and muscle) samples. To each sample, 30 µl (brain) or 50 µl (blood, fat, and muscle) of an internal standard containing 20 ng Ortho-nicotine Perchlorate and 200 ng Ortho-cotinine Perchlorate (internal standards for nicotine and cotinine respectively) was added.

Preliminary extraction for brain, fat, and muscle tissues. Following addition of the internal standards, 0.5 ml 4N H_2SO_4 and 3.0 ml toluene:butanol (70:30) were added to brain, fat, and muscle samples. The samples were then vortexed, centrifuged, and frozen in an acetone dry ice bath. The organic layer was then discarded and the remaining aqueous layer containing nicotine and cotinine was then extracted according to the procedures described below for blood samples.

Blood. After addition of internal standards and the preliminary extraction step (brain, fat, and muscle only), 0.5 ml of either 2N NaOH (blood) or 5N NaOH (brain, fat, and muscle) and 0.2 N NH_4OH and 3 ml toluene:butanol (70:30) was added to each sample. The samples were vortexed, centrifuged, and frozen in an acetone dry ice bath. The organic layer containing nicotine and cotinine was transferred to tubes containing 0.5 ml 1 N H_2SO_4 and the

aqueous layer was discarded. The tubes were then vortexed, centrifuged, and frozen. After freezing, the organic layer was discarded and the aqueous layer containing nicotine and cotinine was poured into new tubes and 0.5 ml 50% K_2CO_3/0.2 N NH_4OH and 0.4 ml toluene:butanol (90:10) were added. The resulting mixture was again vortexed, centrifuged and frozen. Aliquots (1-5 µl) of the organic layer were transfered into autosampler vials and analyzed by gas chromatography on 1.8 m x 2 mm I.D. Carbowax-KOH or SP-2250 DB columns at 145°C as described in Jacob, Wilson, and Benowitz (1981). Quantitation was achieved by calculating peak height ratios of nicotine to the internal standard and referring to the standard curve.

Determination of cotinine in liver incubated with nicotine

To provide evidence of a direct nature that nicotine metabolism is altered in stressed rats, livers from control and stressed rats were incubated with nicotine and 15 minutes later the incubate was analyzed for nicotine's primary metabolite cotinine.

Liver Preparation. Immediately following decapitation, livers were quickly removed from the body, rinsed in ice-cold isotonic KCl solution and weighed. Livers were homogenized in 2 volumes of ice-cold 0.25 M tris-KCl (pH 7.4), using a polytron. The homogenate was centrifuged at 10,000 x g for 30 minutes in a Sorval refrigerated centrifuge in cold (0°C) and the resulting supernate was stored frozen in a -70°C freezer.

Incubation and Assay Procedure. Drug-metabolizing enzyme activity was determined by incubating the 10,000 x g supernatant fractions with nicotine based on the procedure described in Jenner, Gorrod, and Beckett (1973). Incubations were carried out in open 20 ml scintillation vials, each containing 1.1 ml of 1.15% KCl, 1.5 ml of buffer solution, pH 7.4 (10 µmol $MgCl_2 \cdot H_2O$; 2,000 µmol Na_2HPO_4; 2,000 µmol KH_2PO_4); 0.2 ml cofactor solution (2 µmol NADP, 20 µmol Glucose-6-Phosphate); 0.2 ml glucose-6-phosphate dehydrogenase (2 units), nicotine dihydrochloride (0.05 µmol) in water (1.0 ml) and liver 10,000 x g supernatant (1.0 ml equivalent to 0.25 g liver) giving a total incubation volume of 5.0 ml.

Samples were then incubated at 37°C (+/- 0.5°C) in air using a metabolic shaker. After 15 minutes, the microsomal reaction was stopped by chilling the flasks in an ice bath and adding 5 M HCL (1.0 ml). The suspensions were then diluted to 200 ml and aliquots (5.0 ml) were analyzed in duplicate for cotinine by gas chromatography on 1.8 m x 2 mm I.D. Carbowax-KOH or SP-2250 DB columns at 145°C as described in Jacob, Wilson, and Benowitz (1981).

Stress Induction

Two types of stressors (physical and environmental) were used in this study. A physical stressor was chosen because the majority of studies which have examined the effect of stress on drug metabolism have used physical stressors. An environmental stressor was used in order to

determine if this type of stressor, which is more typical of stressors reported in humans and which has previously been used to provide a model of psychological stress in humans (Glass & Singer, 1972), might also affect nicotine levels in the body. Both stressors were administered acutely, rather than chronically, because the majority of research covered in the preceding section on stress and human smoking has employed acute stressors. Therefore, it seemed logical to begin an investigation of this nature with an acute stressor.

Physical stressor

Animals in the physical stress condition were stressed by the application of a rubber band (7 to 8 cm in circumference) wrapped three times around the distal end of the left hind-leg femur for 2.5 hours. This form of stress was employed because previous studies have demonstrated that it is sufficient to induce a stress response as indicated by depletion of adrenal ascorbic acid (Driever & Bousquet, 1965; Rupe, Bousquet & Miya, 1963) and peak elevation of blood corticosterone in rats (Chung & Brown, 1976; Smith, Maickel & Brodie, 1963). In addition, it has been found to reduce circulating levels and or induce inhibition of other drugs metabolized in rat hepatic microsomes (Bousquet, Rupe & Miya, 1965; Chung & Brown, 1976; Chung & Brown, 1974; Driever & Bousquet, 1965; Rupe, Bousquet & Miya, 1963).

Environmental stressor

Rats in the psychological stress condition were exposed to periods of silence followed by bursts of white

noise played at 100 dBA for 2.5 hours. The source of the white noise was the output of a Gebrands masking noise generator (model G4651) powered by a Gebrands power supply (Model G4660) recorded onto a cassette tape. The length of each silence/noise burst cycle was randomly determined with the stipulation that each period of silence last between 5 and 90 seconds and each burst of noise last between 5 and 15 seconds. The tape was played, fed via an amplifier into a loudspeaker placed approximately 3 feet away from each animal. The tape was played continuously for 2.5 hours. The noise level was monitored throughout the procedure to insure that it remained at 100 dBA. White noise was chosen because it has been demonstrated to induce stress in rats (Bindra & Thompson, 1953; Broadhurst, 1957; Patrick, 1931). In addition, this volume was below the intensity that Morgan and Galambos (1942) found would cause audiogenic seizures with low frequencies in rats.

Stress validation

In order to determine the effectiveness of the stressors, behavioral and endocrine measures of stress were taken. Behavioral measures allowed for repeated assessment of stress throughout the 2.5 hour stress period. This was particularly important in light of the possibility that animals might habituate to the stressor at some point during the 2.5 hour period. However, due to the nature of the stressors employed in this study, the experimenter could not be kept blind to the stress condition of the subject and thus

experimenter bias could not be ruled out as an explanation for the behavioral observation results. Although endocrine measures could not be taken periodically throughout the stress period because blood collection by its very nature is stressful, blood collected at the end of the stress period was taken and analyzed for corticosterone, thereby offering an objective index of stress.

Behavioral stress measures

Beginning on minute 15 of the 120 minute stress period, animals were observed once every 45 minutes for 90 seconds. During each of the three 90-second observation periods, five behaviors (activity, trembling, urination, defecation, and grooming) previously shown to indicate fear (Hall, 1934; Singer, 1961, 1963) were coded on a standard observation form (See Appendix 2 for copy of observation form). The observation form consisted of a series of rating scales, on which responses were coded during each of the three 90-second rating periods.

Activity and trembling were rated on separate five-point scales ranging from one to five. "One" represented little activity or trembling and a "five" represented a lot of activity or trembling. Ratings were made once every 30 seconds during the 90 second observation period and the mean of the three ratings represented activity or trembling during the entire 90 second observation period.

Defecation was scored as the total number of fecal bolae excreted during the 90 second observation period.

Urination was scored as the total number of eliminations during the 90 second period.

Face washing was counted as the number of times a subject engaged in this behavior, e.g., each time a subject began face washing it was counted as one incidence regardless of how long the behavior lasted.

Endocrine stress measure

The endocrine measure used in this study was level of corticosterone, an indicator of adrenal cortical activity. Several studies have reported increased adrenal cortical activity among experimental animals following exposure to a variety of stressors including noise (Elmadjian & Pincus, 1945; Herringtonn & Nelbach; 1942) and application of a rubber ligature (Chung & Brown, 1976; Szot & Murphy, 1970). Measurement of corticosterone was chosen over measurement of catecholamines (an index of adrenal medullary activity) because corticosterone has a longer half-life than catecholamines and is less sensitive to rapid changes in the environment (Baum, Grunberg, & Singer, 1982) and, therefore, would be more likely to reflect the preceding 2.5 hour stress period and less likely to be influenced by the effects of the decapitation procedure immediately following the stressor.

Corticosterone concentration was measured in plasma taken immediately following the 2.5 hour stress period. Blood was collected in heparinized 4 ml polypropylene tubes and centrifuged. The resulting plasma was frozen in a -70° C freezer for later corticosterone assay. Assays were

performed on the thawed plasma using ^{125}I-labeled corticosterone and a specific anti-corticosterone antiserum to determine the corticosterone in specimens using the double antibody technique. Reagents and instructions for this radioimmunoassay were purchased from Cambridge Medical Technology (Billerica, MA).

Procedure

Rats were gentled daily for a week before the study. On day one of the study, rats were assigned to one of three drug conditions (0, 6, 12 mg nicotine/kg) each containing 30 subjects. Subjects from each drug group were then assigned in equal numbers to either a physical stress, environmental stress, or a no stress control group. In sum, there were nine experimental groups (3 levels of nicotine x 3 levels of stress) each containing 10 subjects. Group assignments were made randomly with the stipulation that each group have the same initial mean body weight.

On the first day of the study, animals were transported to a procedure room, anesthetized with methoxyfluorane, and Alzet miniosmotic pumps containing the appropriate concentrations of nicotine solution or saline were implanted. Pumps were implanted subcutaneously in each rat between the shoulder blades by making a small incision (roughly 1 cm), inserting the pump, and closing the incision with 9 mm wound clips. Following surgery, animals were returned to their living quarters where they received their usual care for 14 days.

On day 14 (the last day of the study), animals in the stress conditions were taken to separate procedure rooms where the appropriate stressor was administered for 2.5 hours. Animals in the no stress condition remained undisturbed in their living quarters during this same period. During the 2.5 hour period, the three previously described behavioral observations were made. At the end of the 2.5 hour period animals were taken to a nearby procedure room where they were decapitated and the previously described tissue samples were collected.

RESULTS

Overview

The rubber ligature of one subject in the 6 mg/kg/day rubber ligature condition came off during the stress period. Because it was unclear when the ligature had been removed, the data from this subject were not included in any of the following analyses. Nonparametric tests were used to analyze the behavioral data because the data violated several assumptions necessary for parametric analysis. A separate two-way (stress x drug) analyses of variance was performed on blood corticosterone data. Multivariate analyses were used to analyze tissue (blood, brain, fat, and muscle) nicotine and blood cotinine data. Data obtained from the tissue nicotine and blood cotinine assays were found to violate the assumption of homogeneity of variance necessary for analysis of variance. Therefore, these data were transformed using

square root transformations (Kirk, 1982). All analyses were performed on both raw and transformed data. Because analyses performed on the raw and transformed data yielded similar results in all but one comparison, the analyses based on raw data are presented in the text and the analyses based on the transformed data are presented in the appendix. In the one case that analysis of the raw and transformed data yielded different results, this difference is indicated in a footnote in the text. A separate two-way (stress x drug) analyses of variance was performed on the liver cotinine data. All results were based on a two-tailed test of significance, and the alpha level for all analyses was set at 0.05.

Stress validation

Behavioral stress measures

Activity and trembling

To determine the effects of drug and stress condition on activity and trembling, the data from each of the three 90-second observation periods were analyzed using separate Kruskal-Wallis one-way Analysis of Variance Tests. A non-parametric test was used because examination of the means and error variances revealed that the means within each treatment condition were approximately equal, but the variances of the error were heterogeneous. Transformations to make data of this description suitable for parametric analysis are not available and therefore nonparametric tests are recommended (Kirk, 1982). Kruskal-Wallis one-way ANOVAs

were selected because they are considered the most efficient non-parametric test available to compare three or more independent samples (Siegel, 1956).

Table 1 presents the mean activity scores for each drug condition during each time period. Kruskal-Wallis one-way ANOVAs performed on the data broken down by stress indicated that there was a significant effect of stress on activity, $H = 217.9948$, $df = 2$, $p < 0.00001$, at Time 1 (the observation period which began 15 minutes after the stressor began). Follow-up analyses revealed that animals in both stress conditions were significantly more active compared to controls. In addition, animals in the rubber ligature condition were significantly more active compared to animals in the noise condition. At Time 2 (the observation period which began 60 minutes after the onset of the stressor), animals in the two stress conditions were more active compared to control animals; however, these differences were only marginally significant, $H = 5.2166$, $df = 2$, $p < .07$. There were no significant effects of stress condition on activity levels at Time 3 (the observation period beginning 105 minutes after the onset of the stressor). The results of these analyses are summarized in Table 2.

Although the amount of activity recorded among animals in the two stress conditions was similar, the type of behavior observed during these periods was quite different comparing the two stress conditions. Specifically, among animals in the noise stress condition, the increased activity

took the form of increased levels of general activity (e.g., running, rearing, sniffing, etc.). Among animals in the rubber ligature condition, the increased activity was mainly directed towards removing the rubber ligature (e.g., scratching, clawing, and biting at the rubber ligature). In contrast, the most common behavior exhibited by the majority of the animals in the no stress condition was sleep.

Analyses of these data broken down by drug condition revealed no effect of drug condition on activity during any time period. Similar analyses performed on the trembling data (presented in Table 3) revealed no significant effects of stress or drug condition at any time period.

Defecation, urination and face washing

Tables 4, 5, and 6, respectively present the mean defecation, urination and face washing scores for each drug condition during each time period. To determine the effect of stress and drug on defecation, urination, and face washing, the frequency of the occurrence of each of these behaviors was compared at each of the three 90-second observation periods using separate chi-square tests. Nonparametric tests were used because nominal data are not suitable for parametric analysis. Chi-square tests were chosen because they are recommended when comparing three or more independent samples (Siegel, 1956). There were no significant effects of either stress or drug condition on any of these measures during any of the observation periods.

Endocrine stress measure

The mean corticosterone levels for each drug condition during each time period are presented in Table 7 and Figure 1. To determine the effect of stress on circulating corticosterone levels, the results were submitted to a three (no stress control, noise, and rubber ligature) by three (0, 6, and 12 mg nicotine/kg/day) ANOVA. The results of this analysis indicated that there was a significant main effect of stress on corticosterone levels, $F(2,80) = 4.75$, $p < 0.01$. Follow-up tests (Tukey's HSD) revealed that this effect was due to animals in the rubber ligature condition. Specifically, mean corticosterone levels were significantly higher among animals in the rubber ligature condition (76.095, s.d. = 31.387) compared to animals in either the no stress (54.023, s.d. = 34.735) or noise stress (53.82, s.d. = 27.647) condition. No significant main effect of drug or stress by drug interactions were observed.

Tissue nicotine and blood cotinine levels

Table 8 presents the mean levels of nicotine (blood, brain, muscle and fat tissues) and cotinine (blood) for each drug and stress condition. To determine the effects of stress and drug condition on tissue levels of nicotine and blood cotinine, a three (0, 6, and 12 mg nicotine/kg/day) by three (no stress control, noise, and rubber ligature) MANOVA with five dependent variables (blood, brain, muscle, and fat nicotine and blood cotinine) was performed. Because preliminary analyses revealed that the means of the treatment

levels and the variances of the error effects were proportional, square root transformations were performed on these data to achieve homogeneity of error variance (Winer, 1962). All analyses were performed on both the raw and transformed data. Analyses performed on the raw data are presented in the text and Table 9 and depicted in Figures 2 through 6. Analyses performed on the raw data are presented in the text because although analyses performed on transformed data may be more statistically correct, the transformed values are conceptually difficult to interpret. In addition, Analysis of Variance procedures are robust to heterogeneity of variance when there are an equal number of observations for each treatment group (Edwards, 1972). Analyses performed on the transformed data are presented in Appendix 2 and Table 10. Analyses performed on the transformed data are presented in the appendix because, for the reasons listed above, the trend among statisticians in recent years has been to present analyses performed on both transformed and untransformed data and to allow the reader to interpret any differences in results obtained from the two procedures (George Relyea, personal communication, April 28, 1989). Analyses on the original and transformed data yielded similar results in all but one comparison. This difference is described in a footnote in the text.

Main effect for drug

The results of the multivariate and univariate analyses of the main effect for drug are presented in Table 9. As

expected, the multivariate analyses revealed a significant main effect for drug condition. Examination of the univariate analyses performed on each tissue found that these differences were significant across all tissues. Follow-up tests (Tukey HSD Procedure) indicated that animals in the 12 mg nicotine/kg/day group had significantly higher levels of blood, fat, brain, and muscle nicotine and blood cotinine compared to animals in the saline and 6 mg nicotine/kg/day conditions. Similarly, animals in the 6 mg nicotine/kg/day group had significantly higher levels of blood, fat, brain, and muscle nicotine and blood cotinine compared to animals in the saline condition.

Main effect for stress

The multivariate and univariate analyses of the main effect of stress are also presented in Table 9. In contrast to what was predicted, examination of the multivariate analyses indicated that there was no significant main effect for stress considering all tissues together. However, examination of the univariate analysis suggest that there was a main effect for stress on levels of nicotine and cotinine in the blood (e.g., blood levels were lower among stressed animals compared to nonstressed animals).

Stress by drug interactions

The multivariate and univariate analyses of the stress by drug interaction are presented in Table 9. Examination of the multivariate analyses revealed a significant stress by drug interaction. Although the trends were similar across

all tissues, examination of the univariate analyses performed on each tissue revealed that the stress by drug interaction was significant comparing blood levels of nicotine and cotinine only. To determine the precise nature of this interaction, simple main effects ANOVAs were performed on the blood nicotine and cotinine data comparing the three stressors at each level of drug. There were no significant differences between stress groups in the saline or 6 mg nicotine/kg/day condition. The analysis indicated a significant effect for animals in the 12 mg nicotine/kg/day group, $F(2,80) = 10.804$, $p < 0.0001$. Specifically, comparing animals in the 12 mg/kg/day condition using a Tukey HSD follow-up test, blood nicotine levels of animals in the rubber ligature and noise conditions were significantly lower than animals in the no stress condition.[1] Animals in the noise and rubber ligature groups did not significantly differ from one another.

Comparing blood levels of cotinine of animals in the 12 mg/kg/day condition, animals in the noise condition had significantly higher levels of cotinine compared to animals in either the no stress or rubber ligature conditions, F (2, 80) = 7.26, $p < 0.001$.

Hepatic microsomal nicotine metabolism rates

The mean levels of cotinine obtained in liver incubated with nicotine for each drug and stress condition are presented in Table 11 and Figure 7. Liver samples from two subjects in the 6 mg/kg/day rubber ligature condition

were destroyed during the assay procedure. As mentioned previously, the rubber ligature of one subject in this condition came off during the stress period. Therefore, the following analyses include data from only seven subjects in the 6 mg/kg/day rubber ligature condition. To determine the effects of stress and drug administration on *in vitro* nicotine metabolism, cotinine levels obtained from liver tissue incubated with nicotine were compared via a three (no stress control, noise, and rubber ligature) by three (0, 6, and 12 mg nicotine/kg/day) ANOVA. The liver cotinine data were not included in the tissue MANOVA described in the previous section for three reasons. First, conceptually the *in vitro* liver metabolism measure differed from the tissue measures in that it was an indirect measure of what was happening in the body and, therefore, was not necessarily related to those measures. Second, nicotine exposure per gram of tissue *in vivo* and *in vitro* may be different. And finally, when statistical correlations were computed between liver cotinine and tissue levels of nicotine (blood, brain, fat, and muscle) and cotinine (blood), the correlations were low (ranging from -.153 to -.057) suggesting that liver cotinine levels were not correlated with tissue levels and, therefore, were not appropriate for inclusion in the tissue MANOVA.

The results of the ANOVA performed on cotinine levels obtained from liver tissue incubated with nicotine indicated a significant main effect of drug dosage on liver cotinine

levels, $F(2,78) = 17.30$, $p < 0.0001$. Follow-up tests (Tukey HSD Procedure) revealed that liver cotinine levels were significantly higher comparing animals in the 6 and 12 mg/kg groups to control animals. Although cotinine levels obtained from liver tissue incubated with nicotine were generally higher among stressed animals compared to nonstressed animals, there was no significant main effect of stress on liver cotinine levels. In addition, no significant interaction was observed.

DISCUSSION

For animals receiving 12 mg nicotine/kg/day, physical and environmental stressors significantly decreased blood levels of nicotine. This finding is consistent with the idea first postulated by Schachter and his coworkers (Schachter, 1978; Schachter, Silverstein, Kozlowski, Perlick, Herman & Liebling, 1977) that smokers smoke more under stress in order to replace lost nicotine. In addition, although not statistically significant, these stressors consistently decreased levels of nicotine in the brain and muscle tissues in these same animals. This finding is consistent with the notion suggested by Grunberg and his associates (Grunberg, Morse & Barrett, 1983; Grunberg & Kozlowski, 1986) that stress increases smoking as a result of changes in body-wide distribution of nicotine. The fact that decreased levels of nicotine were observed while nicotine was being infused continuously suggests that the effects of stress on nicotine

levels must be fairly robust.

Stress did not noticeably affect levels of nicotine in animals receiving 6 mg nicotine/kg/day. This finding suggests that a critical level of nicotine in the system is necessary in order for stress to appreciably alter levels of nicotine in the blood. This finding is consistent with the finding that light smokers do not tend to alter their smoking patterns in response to stress to the extent that heavy smokers do, but does not support Schachter's notion that this difference in behavior is due to differences between light and heavy smokers in their ability to regulate nicotine.

Stress did not significantly increase *in vitro* nicotine metabolism as measured by levels of cotinine obtained from liver tissue incubated with nicotine. In addition, stress had no consistent effect on *in vivo* nicotine metabolism as measured by blood cotinine levels. Taken together, these results suggest that reduced levels of nicotine in the body *are not* the result of increased conversion of nicotine to cotinine in the liver or elsewhere, suggesting that either stress has no effect on nicotine metabolism, or that stress affects nicotine metabolism through some other pathway.

Consistent with the previous studies (Singer 1961, 1963) activity levels were found to reliably discriminate between stressed and nonstressed animals. Specifically, activity levels were higher among animals in the stress conditions compared to their no stress controls. Among

animals in the two stress conditions, activity decreased over time, suggesting that some degree of habituation occurred over the course of the 2.5 hour stress period.

In contrast to previous studies using the same behavioral stress measures (Singer, 1961, 1963), neither trembling, defecation, urination, or face washing reliably discriminated between stressed and nonstressed animals in the present study. There are two possible explanations for the failure of these behaviors to discriminate stress from nonstress. The first explanation is that the stressors used in this study were not sufficient to induce these behaviors. However, given the ability of these stressors to increase general activity levels, this explanation seems unlikely. The second and most likely explanation is that these behaviors occurred, but that they had already habituated by the time the first observation period took place. In the present study, the first observation took place between minutes 15 and 20 of the stress period. Previous studies using these measures (Singer, 1961, 1963) observed these behaviors during the first 90 seconds of stress. Therefore, it is not known how enduring these resonses to stress are.

There was no significant effect of noise on circulating corticosterone levels measured at the end of the stress period suggesting that either this stressor was somewhat less stressful than the rubber ligature or that habituation to this stressor occurred earlier or to a much greater degree. Further examination of the corticosterone

data reveal some interesting patterns that suggest that perhaps the corticosterone levels obtained from animals in the noise condition are the result of some complex interactions between the noise stressor and nicotine and *not* solely because the noise stressor was, in and of itself, less stressful or easier to habituate to. Specifically, among animals which received no nicotine, corticosterone levels of animals in the noise condition were 50 percent *higher* than the those of their non-stressed counterparts. In contrast, among animals in the 6 and 12 mg nicotine/kg/day conditions, corticosterone levels of animals in the noise conditions were 0 and 29 percent *lower*, respectively, compared to those of their non-stressed counterparts. Taken together these results suggest that nicotine may actually reduce the stressfulness of or increase the rate of habituation to noise among animals receiving high dosages of nicotine. The latter possibility has received some support in the literature. Friedman, Horrath, and Meares (1974) found that the smokers habituated to intermittent bursts of loud noise (90 dB) significantly more quickly after smoking two cigarettes. Similar results were reported in Friedman and Meares (1974).

The implications of these data are many. Among animals receiving 12 mg/kg/nicotine/day, stress decreased blood levels of nicotine. If these results generalize to human smokers, then it seems that they might indeed smoke more during stress to compensate for nicotine lost as a result of stress. This supports Schachter's claim that

smokers smoke during stress to ward off symptoms of withdrawal which occur as a result of decreased blood levels of nicotine. In addition, among animals receiving 12 mg nicotine/kg/day in the noise condition, nicotine appeared to decrease corticosterone levels. In as much as this result generalizes to humans, it suggests that smoking may act to reduce the sympathetic activation and, by association, the symptoms which normally accompany stress. This supports the claim frequently made by smokers that they smoke to reduce the symptoms of their stress.

Among animals receiving 6 mg nicotine/kg/day, there was no effect of stress on levels of nicotine. If these results generalize to human smokers, it might explain why stress has little or no affect on smoking behavior of light smokers (Billings & Moos, 1983; Schachter, Silverstein, Kozlowski, Herman & Liebling, 1977). This suggests that a critical level of nicotine in the system is necessary in order for stress to alter levels of nicotine in the blood. Light smokers have considerably lower blood levels of nicotine compared to heavy smokers (USDHHS, 1988). These results suggest that the blood levels achieved by light smokers are below the critical level necessary to be affected by stress. If this is true, it suggests that the smoking behavior of light smokers would be unaffected by stress because blood nicotine levels are unaffected by stress among these smokers. In addition, among animals in the 6 mg nicotine/kg/day condition, nicotine did not appear to reduce

corticosterone levels. In as much as animals in this condition are analogous to light smokers, this suggests that light smokers would not receive the tension-reducing benefit from smoking that heavy smokers receive.

The results of this study highlight the need for future research. The present study suggests that stress does indeed reduce circulating levels of nicotine as Schachter postulated. However, the mechanisms by which nicotine levels are decreased remain unclear. The data presented herein suggest that lower nicotine levels are not the result of increased conversion of nicotine to cotinine in the liver or elsewhere in the body. However, nicotine has two primary metabolites: cotinine and nicotine N-oxide as well as a number of lesser metabolites. Therefore, it is still possible that stress might exert its effects on nicotine levels through its effects on nicotine metabolism, but through a different pathway than the one investigated in the current study. In addition, the present study did not directly test Schachter's notion that decreases in circulating nicotine levels during stress were the result of increased urinary excretion rates. In order to distinguish between these competing hypotheses, future studies should assess urinary nicotine levels and blood levels of nicotine's other metabolites in addition to blood and nicotine cotinine levels.

The results of this study suggest that the relationship between stress and smoking is dose-specific.

However, the present study used only two dosages of nicotine; therefore, the critical level of nicotine necessary for stress to have an effect on levels of nicotine in the blood cannot be determined from these data. In addition, in the present study the highest dosage used was 12 mg nicotine/kg. It is possible that the relationship between stress and nicotine levels is linear (e.g., animals receiving higher dosages would have even lower levels of nicotine when exposed to stress) or curvilinear (e.g., animals receiving dosages would have nicotine reductions similar to those observed among animals receiving doses lower that 12 mg nicotine/kg) or that the effect levels off after a certain dosage. Future studies are needed which employ a greater range of dosages in order to determine the exact nature of the dose-response relationship.

In addition to being dose-specific, the results of this study suggest that the relationship between stress and nicotine may also be stress-specific. It cannot be assumed that one stressor will have the same effect on blood levels of nicotine as another. Therefore, future studies need to distinguish between the effects of different types of stressors on nicotine levels.

The present study examined the effects of stress on nicotine levels in male animals only. Several studies have found that women are more likely to report that they smoke more in situations of high arousal than men (Frith 1969; Barnes & Fishlinski, 1976) and that this self-reported sex

difference is particularly strong comparing male and female heavy smokers (Elgerot, 1977). These data suggest that women smoke more under stress compared to men. Several studies report sex differences in nicotine metabolism in both humans (Beckett, Gorrod, & Jenner, 1971; Benowitz & Jacob, 1984; Kozlowski, Frecker, & Lee, 1982) and animals (Kyerematen, Owens, Chattopadhyay, deBethizy, & Vessel, 1988; Winders & Benowitz, unpublished data) and increased sensitivity to the behavioral effects of nicotine has also been found in both humans (Battig, Buzzi, & Nil, 1982; Kozlowski, Director, & Harford, 1981; Silverstein, Feld, & Kozlowski, 1980) and animals (Battig, 1981; Rosecrans, 1971, 1972). Therefore, it is possible that stress might potentiate existing sex differences in nicotine metabolism and sensitivity. Future studies should directly compare the effects of stress on smoking behavior and blood levels of nicotine in males and females.

Although the nicotine administration paradigm used with rats in this study has been found to provide data comparable to human studies on the effects of cigarette smoking (Winders & Grunberg, 1989), there is no substitute for studies using human smokers; such studies are urgently needed. First, in order to document in a straightforward manner that stress reduces circulating levels of nicotine in human smokers and to examine several possible mechanisms, studies comparing levels of nicotine and its metabolites in the blood and urine of stressed and nonstressed human smokers

administered identical dosages of nicotine should be conducted. Second, in order to determine if the increases in smoking rates that accompany stress actually compensate for nicotine lost during stress, studies tracking nicotine levels before, during, and after stress in smokers allowed to smoke could be conducted. Third, the results of the present study highlight the need to compare the relationship between stress and blood levels of nicotine in smokers administered different dosages of nicotine in order to determine the precise nature of the dose-response relationship in humans. Fourth, studies examining the effects of different stressors in humans are needed. And finally, in order to determine if there are sex differences in the relationship between stress and smoking in humans, all studies should use both male and female smokers.

Although these data provide some support for the notion that nicotine reduces the symptoms of stress, the most consistent finding of this study was that stress acts to reduce circulating nicotine levels. This finding has clinical significance. Many smokers, particularly heavy smokers, report that they smoke because smoking reduces their stress (Barnes & Fishlinski, 1976; Frith, 1971; Ikard & Thomkins, 1973; Kleinke, Staneski, & Meeker, 1983; Linn & Stein, 1985; McKennell, 1970; Russell, Peto & Patel, 1974). In addition, many smokers report stress as the primary reason for relapsing after smoking cessation (Klesges & Klesges, 1988). It is likely that smokers who report this reason for

continuing smoking and for beginning again after they have quit assume that smoking reduces the physiological and psychological manifestations of stress. Yet, the fact that stress reduces nicotine levels suggests that rather than reducing stress, smoking may actually increase smokers' stress by placing them in a state of partial nicotine withdrawal which can only be relieved by smoking. To the extent that smoking and smoking relapse are based upon the smokers' belief that smoking reduces stress, it is possible that provision of more accurate information to these smokers regarding the relationship between stress and smoking might convince these smokers to quit once and for all. Given the health consequences associated with smoking, anything that can be done to assist smokers in their efforts to quit is worthy of continued research attention.

TABLES

Table 1

Mean Frequency of Activity at Each Time Period by Drug and Stress Condition (Mean ± Standard Deviation)

Drug Condition	Time 1	Time 2	Time 3
Saline			
No Stress	1.42 ± 0.59	1.32 ± 0.57	1.20 ± 0.42
Rubber Ligature	2.14 ± 0.79	1.45 ± 0.63	1.22 ± 0.37
Noise	2.46 ± 0.56	1.54 ± 0.69	1.62 ± 0.86
6 MG/KG/DAY			
No Stress	1.39 ± 0.53	1.00 ± 0.00	1.09 ± 0.20
Rubber Ligature	2.52 ± 0.83	1.29 ± 0.45	1.39 ± 0.56
Noise	2.15 ± 0.77	1.28 ± 0.35	1.15 ± 0.25
12 MG/KG/DAY			
No Stress	1.20 ± 0.63	1.13 ± 0.32	1.35 ± 0.66
Rubber Ligature	2.31 ± 0.70	1.28 ± 0.35	1.25 ± 0.36
Noise	2.32 ± 0.80	1.23 ± 0.42	1.06 ± 0.19

Table 2

Mean Rank of Activity for Each Stress Condition During Each Time Period (Means ± Standard Deviation)

Stress Condition	Time 1	Time 2	Time 3
No Stress	25.22^{ab*}	37.63^{de}	41.57
Rubber Ligature	54.62^{ac}	49.00^{d}	48.47
Noise	55.52^{bc}	48.48^{e}	43.57

*Like letters indicate significant differences.

Comparisons d-e, $p < 0.07$.

Table 3

Mean Frequency of Trembling at Each Time Period by Drug and Stress Condition (Mean ± Standard Deviation)

Drug Condition	Time 1	Time 2	Time 3
Saline			
No Stress	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Rubber Ligature	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Noise	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
6 MG/KG/DAY			
No Stress	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Rubber Ligature	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Noise	1.10 ± 0.32	1.00 ± 0.00	1.00 ± 0.00
12 MG/KG/DAY			
No Stress	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Rubber Ligature	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Noise	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00

Table 4

Mean Number of Fecal Bolae at Each Time Period by Drug and Stress Condition (Mean ± Standard Deviation)

Drug Condition	Time 1	Time 2	Time 3
Saline			
No Stress	0.00 ± 0.00	0.00 ± 0.00	2.00 ± 0.63
Rubber Ligature	0.30 ± 0.67	1.00 ± 0.32	1.00 ± 0.32
Noise	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
6 MG/KG/DAY			
No Stress	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Rubber Ligature	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Noise	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
12 MG/KG/DAY			
No Stress	0.00 ± 0.00	0.00 ± 0.00	1.35 ± 0.66
Rubber Ligature	0.00 ± 0.00	0.00 ± 0.00	1.25 ± 0.36
Noise	0.00 ± 0.00	0.00 ± 0.00	1.06 ± 0.19

Table 5

Mean Number of Urinations at Each Time Period by Drug and Stress Condition (Mean ± Standard Deviation)

Drug Condition	Time 1	Time 2	Time 3
Saline			
No Stress	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Rubber Ligature	0.10 ± 0.32	0.10 ± 0.32	0.10 ± 0.32
Noise	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
6 MG/KG/DAY			
No Stress	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Rubber Ligature	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Noise	0.10 ± 0.32	0.00 ± 0.00	0.00 ± 0.00
12 MG/KG/DAY			
No Stress	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Rubber Ligature	0.10 ± 0.32	0.00 ± 0.00	0.00 ± 0.00
Noise	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 6

Mean Number of Face Washings at Each Time Period by Drug and Stress Condition (Mean ± Standard Deviation)

Drug Condition	Time 1	Time 2	Time 3
Saline			
No Stress	1.20 ± 2.82	0.90 ± 1.59	1.10 ± 2.42
Rubber Ligature	0.70 ± 1.25	0.10 ± 0.32	0.30 ± 0.67
Noise	1.00 ± 1.41	0.90 ± 1.66	1.00 ± 2.65
6 MG/KG/DAY			
No Stress	0.70 ± 1.89	0.00 ± 0.00	0.40 ± 0.96
Rubber Ligature	0.22 ± 0.67	0.00 ± 0.00	0.00 ± 0.00
Noise	0.80 ± 2.20	0.80 ± 1.62	0.30 ± 0.95
12 MG/KG/DAY			
No Stress	0.20 ± 0.63	0.10 ± 0.32	1.00 ± 2.49
Rubber Ligature	0.30 ± 0.67	0.30 ± 0.95	0.30 ± 0.68
Noise	1.20 ± 2.90	0.00 ± 0.00	0.00 ± 0.00

Table 7

Mean Corticosterone Levels (ng/ml) Measured in Blood Taken at the End of the 2.5 Hour Stress Period by Drug and Stress Condition (Mean ± Standard Deviation)

	Stress Condition		
Drug Condition	No Stress	Rubber Ligature	Noise
Saline	38.97 ± 23.24	76.65 ± 38.98	56.57 ± 26.91
6 MG/KG/DAY	59.60 ± 32.92	76.30 ± 23.93	59.15 ± 34.06
12 MG/KG/DAY	63.49 ± 43.34	75.36 ± 33.08	45.83 ± 21.63
Total	54.02 ± 34.73[a*]	76.09 ± 31.39[ab]	58.82 ± 27.65[b]

*Like letters indicate significant differences.

Table 8 (Continued on next page)

Mean Tissue Nicotine and Blood Cotinine Levels (ng/g)

(Mean ± Standard Deviation)

Drug Condition	Blood Nicotine	Blood Cotinine
Saline		
No Stress	4.83 ± 4.78	0.00 ± 00.00
Rubber Ligature	7.09 ± 6.03	5.12 ± 10.88
Noise	3.57 ± 5.62	0.60 ± 1.89
6 MG/KG/DAY		
No Stress	149.61 ± 26.71	814.24 ± 91.63
Rubber Ligature	149.72 ± 29.19	856.04 ± 104.42
Noise	145.58 ± 41.57	784.17 ± 94.53
12 MG/KG/DAY		
No Stress	296.81 ± 49.19^{ab*}	1374.85 ± 244.39^{c}
Rubber Ligature	219.30 ± 68.82^{a}	1269.00 ± 164.99^{d}
Noise	255.77 ± 43.87^{b}	1527.14 ± 303.42^{cd}

*Like letters indicate significant differences.

Table 8 (Continued on next page)

Tissue Nicotine and Blood Cotinine Levels (ng/g)

(Mean ± Standard Deviation)

Drug Condition	Brain Nicotine	Fat Nicotine
Saline		
No Stress	22.56 ± 20.64	9.33 ± 13.01
Rubber Ligature	31.46 ± 30.49	6.01 ± 8.82
Noise	17.48 ± 28.67	15.60 ± 18.91
6 MG/KG/DAY		
No Stress	294.16 ± 65.39	52.63 ± 43.81
Rubber Ligature	284.32 ± 46.41	55.99 ± 20.51
Noise	290.80 ± 62.25	65.40 ± 37.08
12 MG/KG/DAY		
No Stress	651.55 ± 121.17	109.59 ± 43.17
Rubber Ligature	578.75 ± 132.77	109.07 ± 60.49
Noise	649.76 ± 143.58	93.34 ± 50.71

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Table 8 and all other text complete.

Table 8 (Last page of Table 8)

Tissue Nicotine and Blood Cotinine Levels (ng/g)

(Mean ± Standard Deviation)

Drug Condition	Muscle Nicotine
Saline	
No Stress	44.69 ± 25.16
Rubber Ligature	43.86 ± 25.99
Noise	51.91 ± 20.17
6 MG/KG/DAY	
No Stress	193.84 ± 47.55
Rubber Ligature	173.79 ± 37.52
Noise	174.38 ± 35.53
12 MG/KG/DAY	
No Stress	342.36 ± 92.53
Rubber Ligature	305.32 ± 66.04
Noise	300.54 ± 61.67

Table 9

MANOVA Tissue Levels of Nicotine and Cotinine

(Raw Data)

Multivariate Analyses

Drug Condition: Wilks Approximate F (10,152) = 67.17293, p = 0.0001

Stress Condition: Wilks Approximate F (10,152) = 1.3345, p = 0.217

Interaction: Wilks Approximate F (20,253) = 1.7878, p = 0.022

Univariate Analyses

Main Effect for Drug Condition

Blood Nicotine: Wilks Approximate F(2,80) = 344.59674, p = 0.0001

Blood Cotinine: Wilks Approximate F(2,80) = 629.67475, p = 0.0001

Brain Nicotine: Wilks Approximate F(2,80) = 373.46801, p = 0.0001

Fat Nicotine: Wilks Approximate F(2,80) = 47.32147, p = 0.0001

Muscle Nicotine: Wilks Approximate F(2,80) = 208.15554, p = 0.0001

Main Effect for Stress Condition

Blood Nicotine: Wilks Approximate F(2,80) = 3.38980, p = 0.039

Blood Cotinine: Wilks Approximate F(2,80) = 1.21891, p = 0.301

Brain Nicotine: Wilks Approximate F(2,80) = 0.70841, p = 0.495

Fat Nicotine: Wilks Approximate F(2,80) = 0.00740, p = 0.993

Muscle Nicotine: Wilks Approximate F(2,80) = 1.32885, p = 0.271

Interaction

Blood Nicotine: Wilks Approximate F(2,80) = 3.68062, p = 0.008

Blood Cotinine: Wilks Approximate F(2,80) = 3.24733, p = 0.016

Brain Nicotine: Wilks Approximate F(2,80) = 0.86099, p = 0.491

Fat Nicotine: Wilks Approximate F(2,80) = 0.54469, p = 0.700

Muscle Nicotine: Wilks Approximate F(2,80) = 0.62028, p = 0.649

Table 10

MANOVA Tissue Levels of Nicotine and Cotinine

(Transformed Data)

Multivariate Analyses

Drug Condition: Wilks Approximate F (10,152) = 125.25698, p = 0.0001
Stress Condition: Wilks Approximate F (10,152) = 0.91546, p = 0.521
Interaction: Wilks Approximate F (20,253) = 1.6791, p = 0.037

Univariate Analyses

Main Effect for Drug Condition

Blood Nicotine: Wilks Approximate F(2,80) = 661.86726, p = 0.0001
Blood Cotinine: Wilks Approximate F(2,80) = 2295.63402, p = 0.0001
Brain Nicotine: Wilks Approximate F(2,80) = 468.22164, p = 0.0001
Fat Nicotine: Wilks Approximate F(2,80) = 81.77636, p = 0.0001
Muscle Nicotine: Wilks Approximate F(2,80) = 297.48817, p = 0.0001

Main Effect for Stress Condition

Blood Nicotine: Wilks Approximate F(2,80) = 1.72258, p = 0.185
Blood Cotinine: Wilks Approximate F(2,80) = 0.52219, p = 0.589
Brain Nicotine: Wilks Approximate F(2,80) = 0.28639, p = 0.752
Fat Nicotine: Wilks Approximate F(2,80) = 0.18819, p = 0.829
Muscle Nicotine: Wilks Approximate F(2,80) = 91522, p = 0.405

Interaction

Blood Nicotine: Wilks Approximate F(2,80) = 3.12475, p = 0.019
Blood Cotinine: Wilks Approximate F(2,80) = 3.47575, p = 0.011
Brain Nicotine: Wilks Approximate F(2,80) = 1.04209, p = 0.391
Fat Nicotine: Wilks Approximate F(2,80) = 0.81417, p = 0.520
Muscle Nicotine: Wilks Approximate F(2,80) = 0.63300, p = 0.640

Table 11

Cotinine Levels Obtained from Liver Tissue Incubated with Nicotine (ng/0.25 g liver sample) (Mean ± Standard Deviation)

Drug Condition	Liver Cotinine
Saline	
No Stress	43.76 ± 20.51
Rubber Ligature	53.66 ± 24.33
Noise	55.55 ± 17.71
6 MG/KG/DAY	
No Stress	70.34 ± 28.19
Rubber Ligature	83.83 ± 43.43
Noise	70.77 ± 12.17
12 MG/KG/DAY	
No Stress	83.38 ± 35.56
Rubber Ligature	92.85 ± 22.69
Noise	94.12 ± 23.17

FIGURES

Figure 1

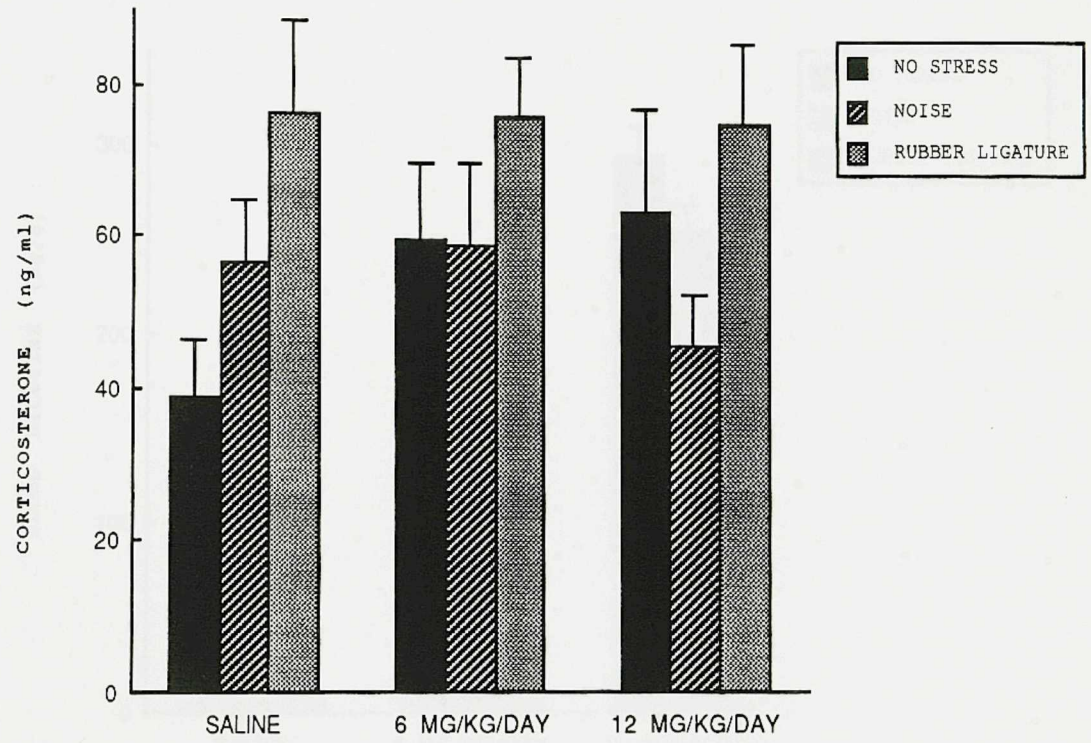


FIGURE 1: Corticosterone levels (ng/ml) by stress and drug condition (means and standard errors).

Figure 2

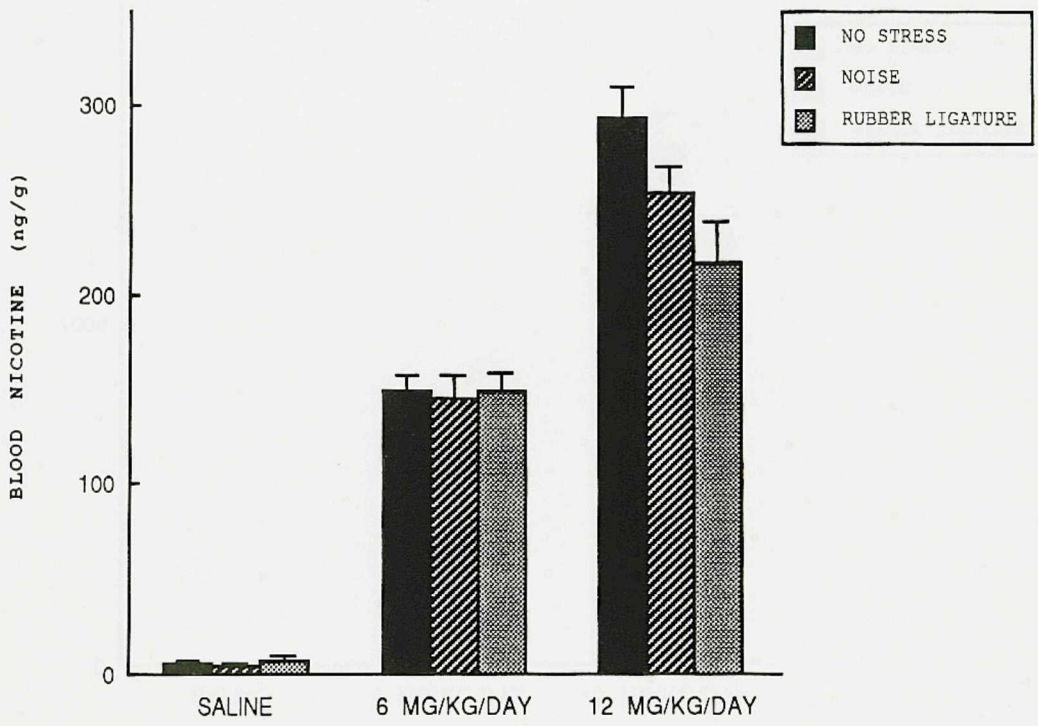


FIGURE 2: Blood nicotine levels (ng/g) by stress and drug condition (means and standard errors).

Figure 3

FIGURE 3: Blood cotinine levels (ng/g) by stress and drug condition (means and standard errors).

Figure 4

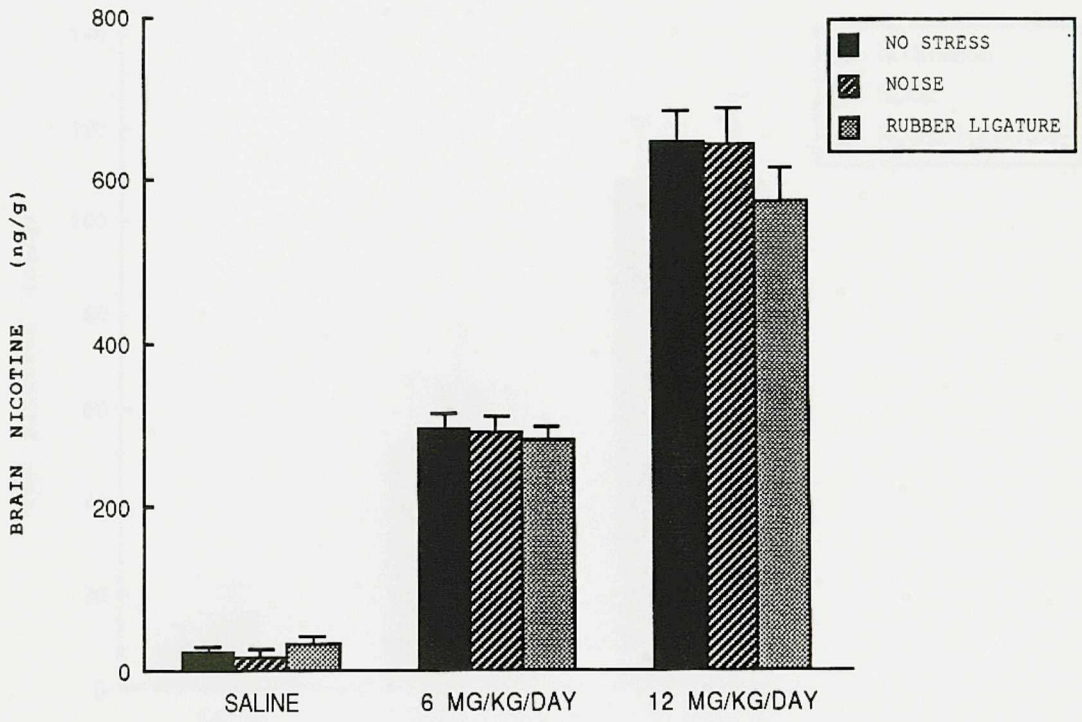


FIGURE 4: Brain nicotine levels (ng/g) by stress and drug condition (means and standard errors).

Figure 5

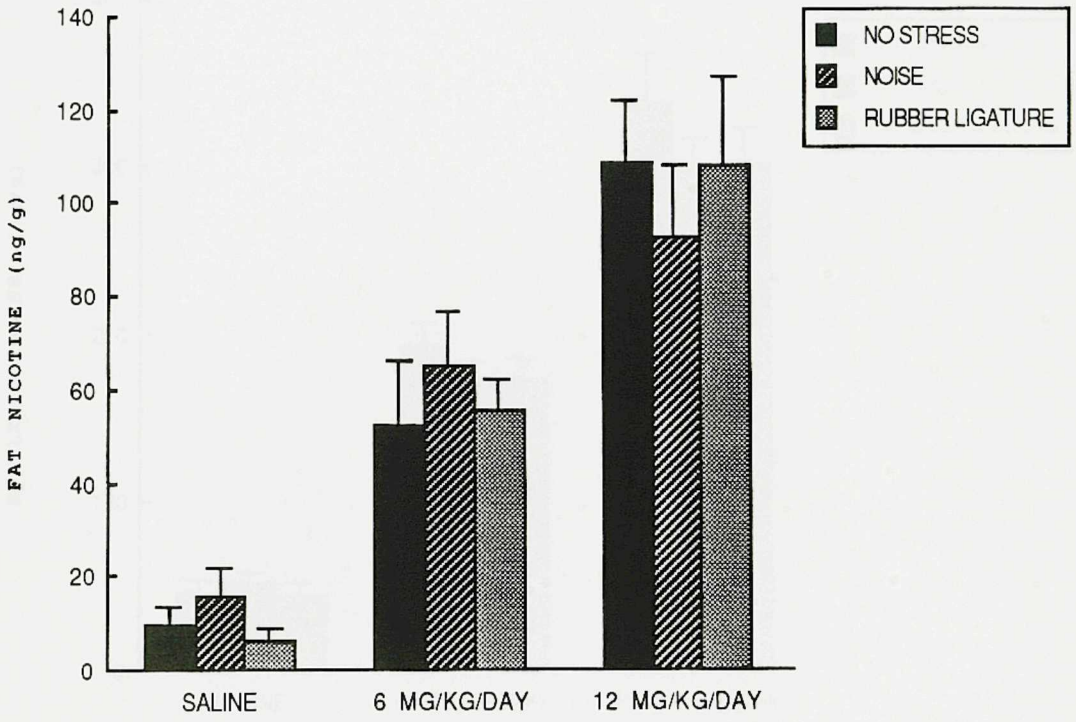


FIGURE 5: Fat nicotine (ng/g) by stress and drug condition (means and standard errors).

Figure 6

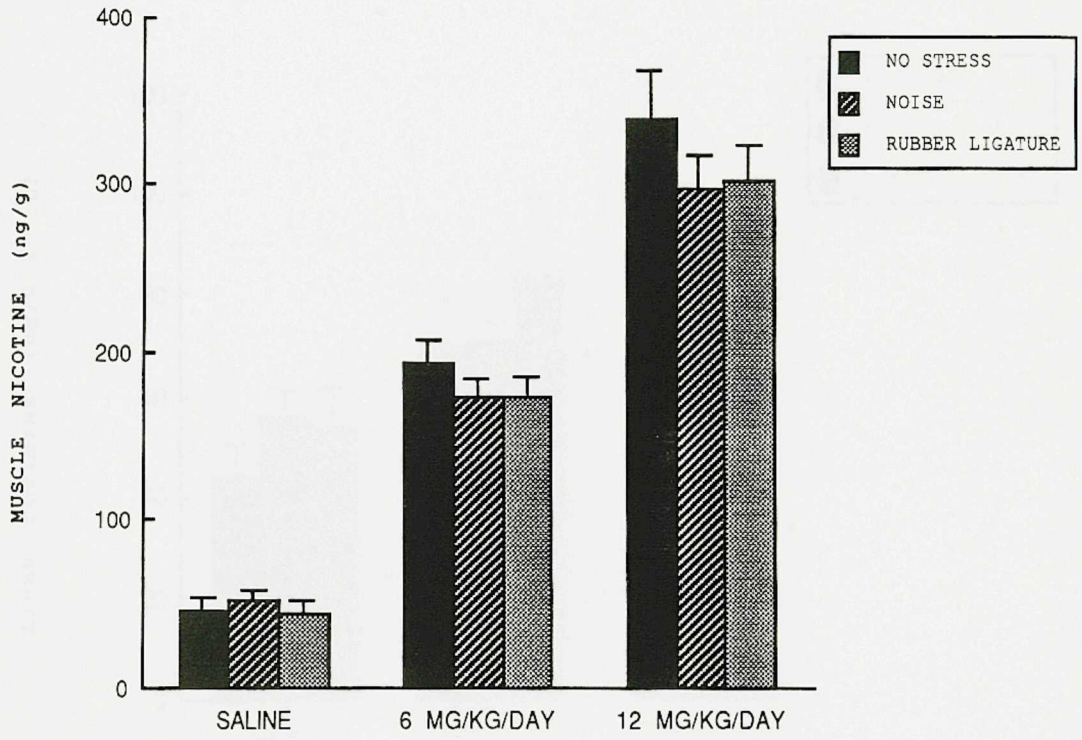


FIGURE 6: Muscle nicotine levels (ng/g) by stress and drug condition (means and standard errors).

Figure 7

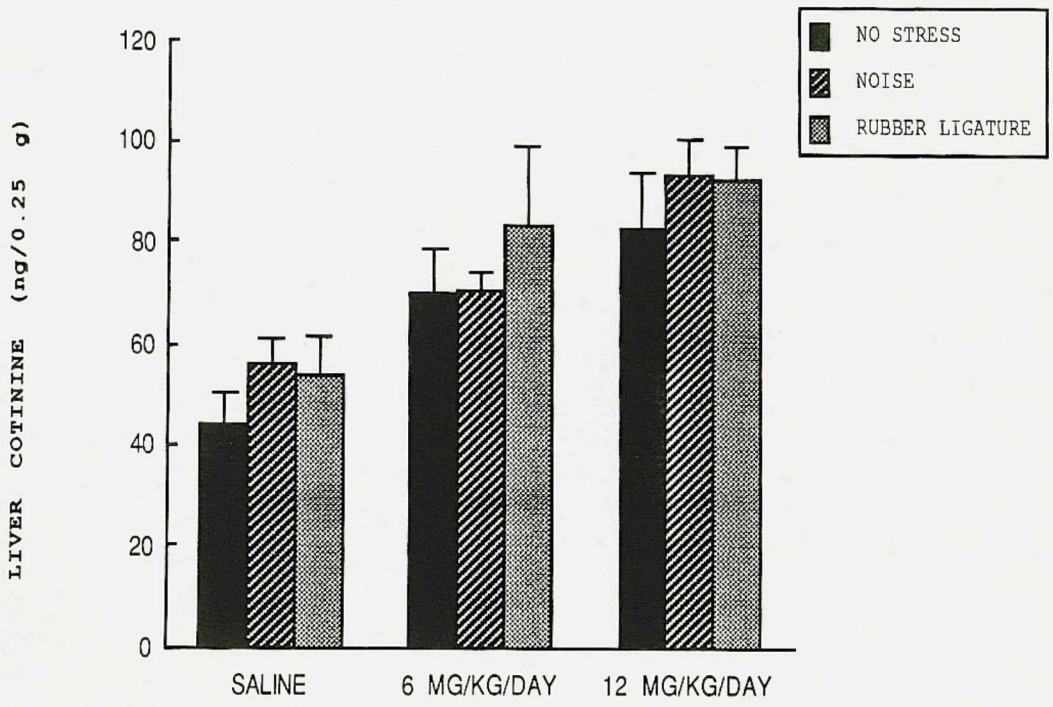
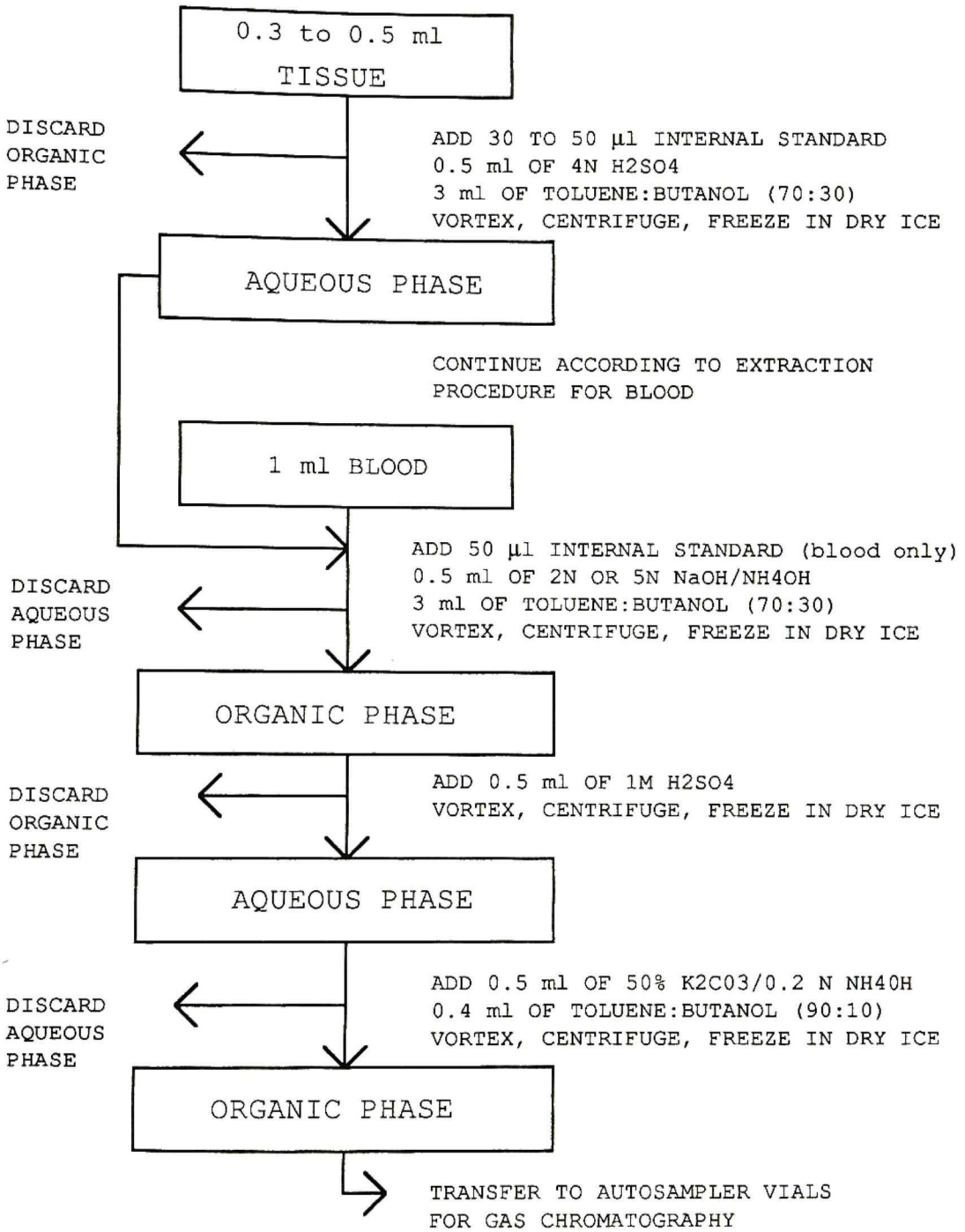


FIGURE 7: Cotinine levels (ng/0.25 g) in liver tissue incubated with nicotine (means and standard errors).

APPENDICES

Appendix 1

Simultaneous Extraction of Nicotine and Cotinine from Tissue and Blood



Appendix 2

BEHAVIORAL OBSERVATION SCALE

Date _____ Subject # _____

Time One_____

ACTIVITY

1 2 3 4 5

none large amount

TREMBLING

1 2 3 4 5

not at all a great deal

DEFECATION (Total number of boli during 90 s observation period)

URINATION (Number of eliminations during 90 s observation period)

FACE WASHING AND SCRATCHING (Record number of times behaviors occur during 90 s observation period)

Time Two_____

ACTIVITY

1 2 3 4 5

none large amount

TREMBLING

1 2 3 4 5

not at all a great deal

DEFECATION (Total number of boli excreted during 90 s observation period)

URINATION (Number of eliminations during 90 s observation period)

FACE WASHING AND SCRATCHING (Record number of times behaviors occur during 90 s observation period)

Time Three_____

ACTIVITY

1 2 3 4 5

none large amount

TREMBLING

1 2 3 4 5

not at all a great deal

DEFECATION (Total number of boli excreted during 90 s observation period)

URINATION (Number of eliminations during 90 s observation period)

FACE WASHING AND SCRATCHING (Record number of times behaviors occur during 90 s observation period)

Appendix 3

Results (transformed data)

Main effect for drug

The multivariate and univariate analyses of the main effects of drug for the transformed data are presented in Table 10. As in the untransformed analyses, a significant main effect for drug condition was observed. Examination of the univariate analyses performed on each tissue revealed these differences were significant across all tissues. Follow-up tests (Tukey HSD Procedure) revealed that animals in the 12 mg/kg nicotine group had significantly higher levels of blood, fat, brain, and muscle nicotine and blood cotinine compared to animals in the saline and 6 mg/kg nicotine conditions. Similarly, animals in the 6 mg/kg nicotine group had significantly higher levels of blood, fat, brain, and muscle nicotine and blood cotinine compared to animals in the saline condition. All comparisons were significant at the $p < 0.05$ level.

Main effect for stress

The multivariate and univariate analyses of the main effect of stress are also presented in table 10. As in the untransformed analyses, examination of the multivariate analyses revealed no significant main effects for stress. However, examination of the univariate analyses suggest that there was a main effect for stress on levels of nicotine and cotinine in the blood.

Stress by drug interactions

The multivariate and univariate analyses of the stress by drug interaction performed on the transformed data are presented in Table 10. As in the untransformed analyses, examination of the multivariate analyses revealed a significant stress by drug interaction. Examination of the univariate analyses performed on each tissue revealed that there was a significant stress by drug interaction on blood levels of nicotine and cotinine. To determine the precise nature of the interaction, separate one-way ANOVAs were performed comparing the three stressors at each level of drug. Results of these analyses indicated that this effect was limited to animals in the 12 mg/kg nicotine per day group only. Specifically, comparing animals in the 12 mg/kg/day condition, blood levels of nicotine of animals in the rubber ligature condition were significantly lower than animals in either the no stress or noise stress conditions ($F(2,80) = 6.59$, $p < 0.01$). Comparing blood levels of cotinine of animals in the 12 mg/kg/day condition, animals in the noise condition had significantly higher levels of cotinine compared to animals in either the no stress or rubber ligature conditions ($F(2, 80) = 5.97$, $p < 0.01$).

ENDNOTES

1. This comparison performed on the transformed data yielded somewhat different results. Specifically, comparing animals in the 12 mg/kg/day condition, blood levels of nicotine of animals in the rubber ligature condition were significantly lower than animals in either the no stress or noise stress conditions ($F(2,80) = 6.59$, $p < .01$) who did not differ significantly from each other.

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