Plasma concentrations of testosterone in adult male rats following acquisition of copulation-illness associations

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Plasma concentrations of testosterone in adult male rats following acquisition of copulation-illness associations

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Plasma concentrations of testosterone in adult male rats following acquisition of copulation-illness associations

by

Paul Charles Koch

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Prior research has demonstrated that testosterone is necessary for the expression of copulatory behaviors in adult male rats. Further, stimuli paired with copulatory experiences elevate circulating levels of testosterone. The purpose of the present experiment was to assess testosterone levels in adult male rats whose copulatory behaviors had been associatively inhibited. Each male rat was exposed to an inaccessible estrous female for seven min and was then given an opportunity to copulate. Two groups received an injection of either lithium chloride (LiCl: 0.3 M, 20 ml/kg, ip; n = 9) or saline (0.3 M, 20 ml/kg, ip; n = 8) immediately after each of 12 such pairings spaced at 3-4 day intervals. A third group (n = 8) received a noncontingent injection of LiCl 24 hr after each pairing. On the thirteenth trial male rats were returned to their home cages after seven min of exposure to an inaccessible female. Blood was collected by decapitation 38 min later. Testosterone levels were measured using radioimmunoassay techniques. Male copulatory behaviors of rats that received contingent administration of LiCl gradually declined during successive test sessions and rats that received noncontingent LiCl or saline remained vigorous copulators. Mean levels of plasma testosterone were
comparable for rats that received either contingent or noncontingent LiCl, and were significantly lower than that of rats receiving saline. Thus, the gradual decline in copulatory behaviors in rats that received LiCl immediately after each pairing with an estrous female cannot be attributed to inadequate levels of circulating testosterone. Male rats that received LiCl 24 hr after each pairing with an estrous female had comparable levels of testosterone, yet copulated during all test sessions.
INTRODUCTION

The copulatory behaviors of adult rodents provide prototypical examples of the activational effects of hormones on behavior. Thus, the reflexive yet integrated sequence of activities that comprise the copulatory behaviors of the male rat are dependent upon appropriate levels of the circulating hormone testosterone (Bermant & Davidson, 1974). Testosterone is a steroid hormone whose primary source in the male is the testes, and whose release is regulated primarily by luteinizing hormone (LH), a pituitary gonadotropin (Gray, 1978). Male rats cease to copulate, usually within a few weeks, following castration (Davidson, 1966; Stone, 1927). Ando, Giacchetto, Canonaco, Aquila, Valenti, Beraldi, Piro, and Dessi-Fulgheri (1986) demonstrated that plasma levels of testosterone decrease dramatically (by a factor of 17) five days after castration. The decline in copulatory activities following castration can be prevented or reversed by treatment with testosterone (Beach & Holz-Tucker, 1949; Stone, 1939). Further, testosterone facilitates ejaculation-related reflexes in castrated male rats with spinal transections (Hart, 1967) and in intact male rats (Malmnas, 1977).

Successful copulation by the male rat acutely
increases plasma concentrations of both LH and testosterone (Kamel, Mock, Wright, & Frankel, 1975; Purvis & Haynes, 1974). Although these increments in plasma hormone levels are associated with mating, they do not necessarily depend upon successful copulation (Kamel, Wright, Mock, & Frankel, 1977). Whereas attempts to mate with a nonreceptive female increase both plasma hormone levels, nontactile contact with and/or the odor of an estrous female increases LH, but not testosterone (also see Purvis & Haynes, 1972).

Static cues present in an environment in which mating behaviors occur gain stimulus control over endocrine responses associated with copulatory activity. Placement of sexually experienced male rats in a familiar mating arena increases plasma levels of both LH and testosterone, whether the arena contains an estrous, anestrous, or no female (Kamel et al., 1975). These increments in hormone levels are not observed when male rats are placed in a novel mating arena (Kamel et al., 1977).

Graham and Desjardins (1980) recently provided evidence that the secretion of LH and testosterone can be altered systematically by conditioned stimuli. Sexually naive adult male rats were exposed to an odor conditioned stimulus followed by a sexually receptive
female rat once daily for 14 days. Exposure to the conditioned stimulus alone on the 15th day increased both plasma LH and testosterone concentrations. These increments in LH and testosterone levels are comparable to those observed in male rats exposed to a sexually receptive female. These results are consistent with the hypothesis that increments in plasma hormone levels (LH and testosterone) are in anticipation of, or associated with copulatory activity and may serve to energize copulatory behavior in the adult male rat (Graham & Desjardins, 1980; Kamel et al., 1975).

Recent research has demonstrated that excitatory Pavlovian conditioned stimuli can increase the rate of copulation in the male rat (Zamble, Hadad, & Mitchell, 1985a; Zamble, Hadad, Mitchell, & Cutmore, 1985b; Zamble, Mitchell, & Findlay, 1986). In contrast, copulatory behaviors of adult male rats can be associatively inhibited by aversive contingencies (Peters, 1983; Peters, Blythe, Koch, & Kueker, 1987; Peters, Koch, & Blythe, in press; Lawrence & Kiefer, 1987; also see Johnston, Zahorik, Immler, & Zakon, 1978; Koch & Peters, in press). Following each encounter with a sexually receptive female, male rats received an injection of lithium chloride (LiCl, 3.0 mEq/kg). This procedure inhibited copulatory responding entirely after
five to ten such pairings. Further, during acquisition of copulation-illness associations (C-IAs), male rats frequently display "agitated" behaviors (paw-treading and chin-rubbing; Peters, 1983; Peters et al., 1987) which may represent a characteristic component of all aversion responses in rats (Grill & Norgren, 1978; Hall & Bryan, 1981).

The general purpose of the present research was to partially characterize the endocrine state of male rats whose copulatory behaviors have been associatively inhibited. The hormone of present interest was testosterone.

Although male rats fail to copulate following C-IA acquisition, the presence of an estrous female may elicit increments in testosterone in a manner similar to that seen in copulating males. Thus, learned behaviors may override a hormonal influence and the behavioral state of the animal may not correspond to the hormonal state. Conversely, testosterone levels may be depressed in animals whose copulatory behaviors have been associatively inhibited.

Plasma levels of testosterone were measured in four groups of rats. One group received LiCl immediately after each copulatory opportunity and a second group received saline. A third group received LiCl 24 hr
later to provide a control for possible pharmacologic effects of LiCl on testosterone. Because noncontingent LiCl administration does not affect copulatory behaviors (Peters et al., 1987), it was anticipated that noncontingent administration of LiCl would not affect plasma testosterone levels. Finally, a fourth group of rats was used to validate the hormone assay procedure. These rats received only one copulatory opportunity to insure that they were good copulators and subsequently had no contact with the cues of estrous females. Consequently, rats in this group should have lower levels of testosterone than rats that copulate during repeated exposures to estrous females.
METHOD

Subjects. Male Long-Evans hooded rats were purchased from Blue-Spruce Farms, Altamont, New York and were approximately 70 days old upon arrival at the laboratory. Male rats were housed in suspended wire-mesh triple cages (four or five animals per cage) in a temperature-controlled (22-24 °C) room on a reversed 12:12 hr light/dark cycle with lights off at 0800 hr. Food (Simonsen 1526 rat/mice diet) and water were continuously available except during testing.

Stimulus Females. Female Long-Evans hooded rats were obtained from the same supplier and were approximately 70 days old upon arrival at the laboratory. They were individually housed in suspended wire-mesh cages with food and water continuously available except during testing.

Stimulus females were ovariectomized under pentobarbital anesthesia (Chloropent, 3 ml/kg ip). Estrus was induced with sc injections of estradiol benzoate (0.5 µg 24 and 48 hr before testing) and progesterone (0.5 mg 5 hr before testing) in a 0.1 ml sesame oil carrier. All stimulus females were screened for sexual receptivity and proceptivity (Beach, 1976) with sexually vigorous males prior to pairing with males in the various treatment groups. Each male was paired
Behavioral procedures. Male rats without prior heterosexual copulatory experience received their first pairing with a sexually experienced estrous female when both sexes were approximately 90 days old. Immediately after their first ejaculation, the males received their randomly assigned treatment and were housed individually thereafter. Male rats that did not ejaculate during their first pairing with a female were discarded from the study.

Copulation tests were conducted during approximately the middle third of the dark cycle at 3-4 day intervals. Data were recorded by trained observers viewing identically constructed test chambers (74 x 56 x 38 cm, painted black with a glass front wall). The length of each chamber was divided into equal halves by 2 x 2 hardware cloth. Each of nine chambers was illuminated from above with a 10-w red bulb, insulated on all sides except the front with 2.54-cm styrofoam, and ventilated with an exhaust fan. Chamber floors were covered with wood shavings.

At the beginning of each copulation test males were placed in one half of a randomly assigned testing chamber with an inaccessible estrous female in the other half. After 7 min, the male was moved to the side
containing the female. Thus, the female in the opposite half of the testing chamber served as a reliable predictor of an ensuing copulatory opportunity for the male. Each copulation test was terminated following an ejaculation, a failure to intromit within 900 sec, or a failure to ejaculate within 1800 sec.

Latencies used to determine the intervals between introduction of the stimulus female and the first mount (mount latency, ML), intromission (intromission latency, IL), and ejaculation (ejaculation latency, EL) were recorded from electronic timers. Frequency counts were made of the number of mounts and intromissions. If a male failed to intromit and/or mount the female during the testing session, its IL and ML were recorded as 900 sec. Presence or absence of paw treading and chin rubbing was recorded for each male.

Within 1 min after their first ejaculation and after each of 11 subsequent pairings with an estrous female, male rats received either a 0.3 M (20 ml/kg) ip injection of LiCl (n = 9) or a 0.3 M (20 ml/kg) ip injection of saline (n = 8). Males in a third group (n = 8) were returned to their home cage following each copulation test and received a noncontingent injection of LiCl 24 hr later to control for nonassociative effects of LiCl on hormone secretion. All injections
were made using a 23 g needle and males were returned to their home cage following each injection. Finally, a fourth group of males (n = 8) was paired with an estrous female only during the first copulatory test to ensure that they were good copulators. These males never received an injection of either saline or LiCl and were subsequently housed in a room that did not contain female rats. These animals were handled briefly each day to reduce the possible stress due to handling prior to blood collection. Each male rat in all groups was briefly handled on days in which copulation tests were not run to diminish the likelihood that the experimenter and/or the handling procedure would become discriminative cues for copulatory opportunities.

Rats that received saline should display elevated levels of testosterone compared to the fourth group that was not exposed to cues associated with copulatory experiences. If differences in plasma testosterone concentrations were not detected in rats that did (saline and noncontingent LiCl groups) and did not (contingent LiCl group) copulate, it was necessary to demonstrate that the assay procedure detected differences when they were reasonably expected to exist.

Blood collection and hormone assay. On trial 13 males in the contingent LiCl, noncontingent LiCl, and
saline groups were exposed to an inaccessible female for 7 min and then returned to their home cages. Males in the handled only group were briefly handled, returned to their home cage for 7 min, briefly handled again and returned to their home cage. Blood samples for determination of testosterone concentrations were collected from all animals 38 min later by decapitation. These delay parameters in blood sampling were used to maximize the likelihood of obtaining a female-elicited surge in testosterone concentration (Graham & Desjardins, 1980). Blood collection was alternated among and randomized within groups. Blood samples were collected in refrigerated EDTA tubes and kept in an ice bath until they were centrifuged (within 1 hr of collection). Plasma samples were stored at -20 °C until assay one week later.

The concentration of testosterone in plasma was measured by radioimmunoassay procedure using a commercial kit (Diagnostic Products Corporation, Los Angeles, CA; see the Appendix).
RESULTS

There were no significant differences among the four groups for any measure of copulatory behavior during the initial screening trial. Male rats that received contingent LiCl gradually decreased copulatory behaviors during subsequent trials until they failed to initiate the copulatory sequence with either a mount or intromission. Males in the saline and noncontingent LiCl groups remained vigorous copulators throughout (see Figure 1). Analysis of IL data yielded a significant effect for drug treatment ($F(2,22) = 111.59, p < .001$), trials ($F(10,220) = 19.34, p < .001$), and drug x trials interaction ($F(20,220) = 17.96, p < .001$; see Figure 2). Post hoc analyses indicated that males in the contingent LiCl group had significantly longer intromission latencies on trials 2-11 ($p < .05$ on trial 2, $p < .001$ on trials 3-11) than males in either the saline or noncontingent LiCl groups. There were no significant IL differences between the latter two groups. Analysis of ML data yielded a similar pattern of results.

Male rats in the contingent LiCl group engaged in paw-treading and chin-rubbing behaviors during 87% and 38% of the trials, respectively. During the final trial, all males in the contingent LiCl group engaged in paw-treading behavior. In no instance did a male in
Figure 1. Percentage of contingent LiCl-, noncontingent LiCl-, and saline-treated rats that ejaculated on each trial.
Trials

Ejaculations (%)

- ○ Saline
- • Noncontingent LiCl
- △ Contingent LiCl
Figure 2. Mean intromission latency for contingent LiCl−, noncontingent LiCl−, and saline-treated rats
either the saline or noncontingent LiCl groups display these behaviors during copulatory tests.

Analysis of variance of plasma testosterone concentrations revealed significant differences among the four groups ($F(3,29) = 4.27, p < .02$; see Figure 3). Post hoc analyses indicated that males in the saline group had significantly higher concentrations of plasma testosterone than males in both the contingent and noncontingent LiCl groups ($p < .01$ and $.02$ respectively). A median test indicated that males in the saline group had significantly higher plasma concentrations of testosterone than males in the handled group ($p < .05$). This nonparametric test was used because an extreme value (6.6 standard errors above the mean; see Figure 3) inflated the mean of the handled group (with this score removed, the difference between the saline and handled groups was significant using a parametric test, $p < .01$). There were no significant differences in plasma testosterone concentrations for the contingent LiCl, noncontingent LiCl, and handled groups using either parametric or nonparametric tests.
Figure 3. Testosterone concentration (mean and standard error) in the blood plasm of male rats (H: handled; S: saline; C-L: contingent LiCl; NC-L: noncontingent LiCl). Individual points represent plasma testosterone concentrations of group members.
Testosterone (ng/ml)
DISCUSSION

The copulatory behaviors of male rats that received LiCl immediately after each pairing with an estrous female were associatively inhibited. That is, the number of mounts, intromissions, and ejaculations displayed by these rats gradually declined until they failed to initiate the copulatory sequence. In contrast, noncontingent administration was ineffective; these rats continued to copulate throughout all copulatory test sessions. The copulatory behaviors of rats in the saline and noncontingent LiCl groups were virtually indistinguishable.

Plasma testosterone concentrations were not significantly different for rats that received contingent or noncontingent LiCl and in both instances were significantly less than testosterone concentrations for saline control rats. LiCl may have some unknown pharmacologic interaction that directly suppresses circulating levels of testosterone in adult male rats. This supposition, however, has no empirical support. Further, such a direct pharmacologic effect of LiCl on testosterone, if documented, cannot account for the differential copulatory behaviors displayed by rats in the contingent and noncontingent LiCl groups.

Male rats that received saline immediately after
pairings with estrous females had a mean plasma testosterone concentration of 2.73 ± 0.47 ng/ml (mean ± standard error). This value is comparable to those (2-3 ng/ml) reported for males that were either a) allowed to copulate with an estrous female, b) permitted only nontactile exposure to an estrous female (female on the far side of a wire mesh barrier), or c) exposed only to the odors of estrous females (Kamel et al., 1977). It is also comparable to the value (approximately 2 ng/ml) reported for males exposed to an odor conditioned stimulus that had been previously paired with copulatory experiences (Graham & Desjardins, 1980). Males that received contingent or noncontingent LiCl, or that were only handled had mean plasma testosterone concentrations of 0.89 ± 0.21 ng/ml, 1.16 ± 0.26 ng/ml, and 1.37 ± 0.24 ng/ml (with extreme score removed), respectively. These values are comparable to those reported for males exposed to an empty mating arena lined with clean shavings (1-1.5 ng/ml; Kamel et al., 1977) or males that received only a handling procedure (approximately 1 ng/ml; Graham & Desjardins, 1980). These comparisons suggest that males that received saline in the present experiment exhibited a female-elicited surge in testosterone, whereas males in the contingent and noncontingent groups did not.
Clark, Smith, and Davidson (1985) reported that four of nine castrated male rats with implanted silastic capsules containing testosterone copulated to ejaculation with a mean level of plasma testosterone of only $0.17 \pm 0.02$ ng/ml. The mean plasma testosterone concentration for rats in the contingent LiCl group was more than five times larger than this value, yet all rats in this group failed to copulate on the final copulatory test. Thus, CI-As cannot be simply attributed to inadequate levels of circulating testosterone.

All rats in the contingent LiCl group engaged in paw-treading and chin-rubbing behaviors during CI-A acquisition. It is likely that these behaviors are part of a constellation of behavioral and physiological responses elicited by stressful events (e.g., LiCl-induced malaise). Consequently, adrenocorticotropic (ACTH) levels may be elevated in these animals as a result of stress. De Souza and Van Loon (1982, 1985) demonstrated that restraint stress applied for two min in both intact and adrenalectomized rats increases plasma ACTH levels that peak within 2.5-5 min after the onset of stress. Plasma ACTH levels are similarly elevated after both escapable and inescapable shock (Maier, Ryan, Barksdale, & Kalin, 1986). Recent
research has also demonstrated a clear relationship between ACTH or stress and testosterone levels. Both chronic stress (induced by immobilization or surgical procedures) or ACTH administration reduce levels of circulating testosterone (Gray, Smith, Damassa, Ehrenkranz, & Davidson, 1978; Repcekova & Mikulaj, 1977; Stahl, Gotz, & Dorner, 1984; Tache, Ducharme, Charpenet, Haour, Saez, & Collu, 1980).

Periodic administration of LiCl (both contingent and noncontingent) in the present paradigm may have caused a stress-induced elevation in ACTH that was conditioned to environmental cues (e.g., experimenters, handling procedures, cues of an estrous females). Alternatively, these procedures may have caused a chronic stress-induced ACTH-mediated suppression of testosterone. Regardless of the specific source of possible ACTH-mediated effects of stress on testosterone concentrations, the groups that received contingent and noncontingent LiCl had similar levels of circulating testosterone. It is possible, however, that contingent and noncontingent administration of LiCl may differentially affect other hormonal systems (e.g., LH and gonadotropin releasing hormone) which may in turn account for the decline in copulatory behaviors in rats that received contingent LiCl. It is also possible that
the associative decrements in copulatory behaviors in rats receiving contingent administration of LiCl are not mediated by changes in the endocrine status of the rats.

The present experiment demonstrates the necessity of combining behavioral and nonbehavioral measures of copulatory behaviors. Elevations in testosterone may energize copulatory behavior in the male rat (Graham & Desjardins, 1980; Kamel et al., 1975) but failures to induce female-elicited elevations in circulating testosterone do not necessarily decrease these behaviors. Plasma testosterone concentrations were similar in rats that received either contingent or noncontingent LiCl, yet these procedures induced differential behavioral outcomes. Although copulatory measures were not recorded on the day of blood collection, it is reasonable to assume that the rats in the various groups would have performed in a manner similar to the previous test session when copulatory measures were recorded.

In summary, male rats that received both contingent and noncontingent administration of LiCl following pairings with estrous females had lower levels of testosterone compared to saline controls. Copulatory behaviors were associatively inhibited only in males that received contingent LiCl. Lower levels of
circulating testosterone cannot account for the associative inhibition of copulatory behaviors observed in this aversive conditioning paradigm.
REFERENCES


APPENDIX:

RADIOIMMUNOASSAY PROCEDURES

Testosterone. Plasma testosterone was determined by radioimmunoassay using a commercially available kit (Coat-A-Count Total Testosterone, Diagnostic Products Corporation, Los Angeles, CA). Prior to assay, all plasma samples were allowed to come to room temperature and were mixed by gentle swirling. Four uncoated 12 x 75 mm polypropylene tubes were labelled total counts (T) and nonspecific binding (NSB) in duplicate. Testosterone Antibody-Coated tubes were labelled, in duplicate, A (maximum binding, 0 ng/ml), B (0.30 ng/ml), C (1.00 ng/ml), D (3.00 ng/ml), E (10.00 ng/ml), and F (30.00 ng/ml). Additional antibody-coated tubes were labelled in duplicate for plasma samples. Fifty μl of the zero calibrator A was dispensed into the NSB and A tubes and 50 μl of each remaining calibrator (B through F) was dispensed into the prepared tubes. Fifty μl of each plasma sample was dispensed into appropriately labelled antibody-coated tubes. Within a period of 10 min, 1.0 ml of [125I] Testosterone was added to each tube and mixed with a vortex mixer. The T tubes were set aside for counting and the remaining tubes were incubated for 3 hr at 37 °C.
Following incubation, all tubes (except the T tubes) were thoroughly decanted, allowed to drain for 2-3 min, and then struck sharply on absorbent paper to remove as much residual moisture as possible. All tubes were subsequently counted for 1 min in a gamma counter (Gamma Trac 1191) and the data were recorded as counts per min (CPM).

Total testosterone concentrations were calculated from a logit-log representation of the calibration curve (see Figure 3). The 30.00 ng/ml calibrator was not included in the calibration curve to improve the accuracy of the curve-fitting procedure because this value was well outside the range of the obtained values of testosterone concentration in the unknown samples (0.23 - 5.8 ng/ml). The average NSB-corrected counts per min was calculated for each duplicate pair of tubes using the following formula:

\[
\text{Net Counts} = \text{Average CPM} - \text{Average NSB CPM.}
\]

The binding of each pair of tubes was determined as a percent of maximum binding (MB), with the NSB-corrected counts of tube A taken as 100% using the following formula:
Percent Bound = Net Counts/Net MB Counts x 100.

Percent Bound was plotted on the vertical axis of logit-log paper against concentration on the horizontal axis for each of the calibrators B through E and a straight line approximating the path of these four points was drawn (see Figure 4). Total testosterone concentrations for the plasma samples were estimated from the line by interpolation. The mean intra-assay coefficient of variation was 2.39%. Because all assays were conducted on the same day, there was no inter-assay variation.
Figure 4. Logit-log plot used to estimate plasma concentrations of testosterone. Percent bound on the vertical axis is plotted against concentration on the horizontal axis.
Calibrator Levels: 0.30:1.0:3.0:10 Units: ng/ml

Percent Bound

95%
90%
80%
70%
60%
50%
40%
30%
20%
10%
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