Recrystallization to constant specific activity of estradiol-$^{14}$C synthesized in vitro from testosterone-$^{14}$C by the rat pineal gland

| Crystallization | Solvent system | Specific activity (cpm/mg) Estradiol | Estrone [a] |
|---|---|---|---|
| Initial | | 218 | 197 |
| 1 | Chloroform-ethyl acetate (1:1) | 204 | 201 |
| 2 | Chloroform | 215 | 200 |
| 3 | Chloroform-ethyl acetate (2:1) | 209 | 204 |
| Final mother liquor | — | 207 | 196 |

[a] Estradiol-like material was oxidized to estrone before crystallization.

*Material and methods.* Adult Wistar male rats were killed by decapitation and groups of 20 pineals were homogenized in 1 ml of Krebs-Ringer bicarbonate buffer pH 7.4 containing a NADPH-generating system[9]. The homogenate was incubated for 2 h with 0.5 μCi of testosterone-4-$^{14}$C (sp. act. 56 Ci/mole) at 37 °C under a 95% oxygen- 5% carbon dioxide atmosphere. Incubations were stopped by freezing on dry ice and the steroids were extracted into diethylether after adding 100 μg each of estradiol and estrone as carriers. The extracts were evaporated and the residue was partitioned between 1 *M* NaOH and toluene to yield a crude phenolic fraction. Phenolic extracts were chromatographed in the following thin layer chromatography systems: a) chloroform-ethyl acetate, 75:25; b) chloroform-ethanol, 90:10; c) benzene-ethanol, 80:20. The radioactive material behaving like estradiol was recrystallized to constant specific activity after adding 10 mg of the authentic steroid. In one experiment this material was subjected to a mild oxidation with 0.5% chromium trioxide in 95% acetic acid[9] and was recrystallized to constant specific activity after adding 10 mg of estrone.

*Results and discussion.* Phenolic extracts of rat pineal homogenates previously incubated with testosterone-$^{14}$C and subjected to thin-layer chromatography exhibited two peaks of radioactivity with the chromatographic behavior of estradiol and estrone (Rf = 0.25 and 0.46 in system *a*, 0.52 and 0.67 in system *b*, and 0.41 and 0.54 in system *c*). In 3 different experiments, the conversion of testosterone into phenolic steroids ranged from 0.19 to 0.27% for estradiol and from 0.02 to 0.04% for estrone; no corrections for losses were made. Estradiol-like material was recrystallized to constant specific activity as unmodified estradiol or after oxidation to estrone (Table); the radioactivity recovered from chromatograms as endogenously formed estrone (less than 350 cpm) did not allow further identification.

There is scanty knowledge concerning the metabolism of testosterone in pineal cells. $^{3}$H-Testosterone administered in vivo[2] or added in vitro to the incubation medium[7] becomes concentrated within the pinealocytes, in which it is metabolized by 5α-reductase into 5α-dihydrotestosterone and 5α-androstanediol; in addition 17β-reductase is present in pineal cells since androstenedione and 5α-androstanedione were detected in the incubates[7]. Data presented herein indicate that aromatization of androgens also occurs in the rat pineal gland. The conversion of testosterone into 5α-reduced metabolites and into estrogens by brain structures involved in gonadotropic regulation has been reported[10]. Therefore the pineal gland resembles these areas as far as the metabolism of testosterone is concerned. Moreover, the present results offer additional support to the hypothesis[2,7] that the pineal is an androgen target tissue in brain.

*Résumé.* Des homogénats de glande pineale de rat mâle métabolisèrent de la testostérone-C$^{14}$ in vitro en œstradiol-C$^{14}$ et en œstrone-C$^{14}$. Les métabolites furent identifiés chromatographiquement et dans le cas de l'œstradiol par recristallisation à activité spécifique constante. La conversion de la testostérone en œstradiol fut de 0.19–0.27% et en œstrone de 0.02–0.04%. Ces résultats-ci indiquent que la pinéale ressemble à d'autres régions du cerveau soumises au contrôle de la sécrétion de gonadotropines.

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## Changes in Serum Levels of Gonadotropins and Testosterone in the Male Rat in Response to Fasting, Surgery and Ether

It has recently become apparent that some of the procedures commonly used in endocrine studies on animals, such as ether anesthesia, fasting and surgery, may affect the hormone levels being studied. Surgery or frequent blood sampling has been reported to depress serum luteinizing hormone (LH) levels in rats[1,2] and stress factors associated with restraint appear to depress serum testosterone levels[3]. Stress of a more acute nature resulting from exposure to ether appears to elevate serum gonadotropin levels in rats[4,5] and may lead to reduced testosterone secretion[6]. Short periods of fasting have been found to depress serum levels of gonadotropins

Effects of fasting, surgery and ether anesthesia on serum levels of LH, FSH and testosterone in male rats

| Surgery | Fasted | LH (ng/ml) [a, b, c] | | FSH (ng/ml) [a, b] | | Testosterone (ng/ml) [d] | |
|---|---|---|---|---|---|---|---|
| | | No ether | Ether | No ether | Ether | No ether | Ether |
| None | No | 15.3 ± 2.6 [e] | 23.0 ± 3.9 | 272 ± 14 | 266 ± 11 | 4.35 ± 0.86 | 4.95 ± 1.40 |
| | Yes | 12.3 ± 2.3 | 16.5 ± 2.7 | 192 ± 14 | 204 ± 12 | 2.65 ± 0.50 | 2.26 ± 0.23 |
| Sham castration | No | 13.6 ± 2.3 | 19.1 ± 4.3 | 243 ± 10 | 273 ± 18 | 4.62 ± 1.44 | 4.18 ± 0.75 |
| | Yes | 6.9 ± 2.1 | 5.5 ± 1.1 | 184 ± 15 | 213 ± 11 | 2.46 ± 0.71 | 3.66 ± 1.08 |

[a] Ether $P < 0.05$. [b] Fasting $P < 0.01$. [c] Surgery $P < 0.01$. [d] Fasting $P < 0.05$. [e] Mean ± S.E., 12 rats/group.

without affecting testosterone levels[7,8] while prolonged starvation reduces serum levels of FSH, LH and testosterone[7,9].

The present experiment was designed to determine the effects of surgery, exposure to ether and fasting, either alone or in combination with each other, on serum levels of LH, FSH and testosterone in male rats.

*Materials and methods.* Male Long-Evans rats from our own colony were used in this study. Groups of 12 animals, 2–2½ months old, were assigned in replicates of 2 to a 2×2×2 factorial experiment. The factors studied were sham castration, fasting and ether anesthesia. The rats were assigned to treatment groups and placed in cages (2 rats/cage) in the morning. In the early afternoon, food was removed from fasted animals and sham castration was performed under ether anesthesia. 2 days later at 10.00 h, the rats were decapitated. Animals receiving the ether treatment were placed in an ether jar until unconscious and then removed, allowed to partially regain consciousness (attempting to crawl) and then were decapitated. An attempt was made to excite the animals as little as possible and to decapitate them quickly after removal from cages. Trunk blood was collected and allowed to clot. Serum was frozen and later assayed for LH, FSH and testosterone.

Serum LH levels were measured by the ovine-ovine radioimmunoassay described by NISWENDER et al.[10]. FSH concentrations were determined using the double antibody radioimmunoassay distributed by the National Institute of Arthritis and Metabolic Diseases (NIAMD), NIH. The standards used in the LH and FSH assays were NIAMD-rat-LH-RP-1 and NIAMD-rat-FSH-RP-1, respectively. Serum levels of testosterone were determined by radioimmunoassay[10] using an antiserum produced in rabbits immunized with testosterone-6-carboxy-methyloxime conjugated to bovine serum albumin. The data were statistically analyzed by factorial analysis of variance[11].

*Results and discussion.* The results are summarized in the Table. Rats that had been anesthetized with ether had higher ($P < 0.05$) levels of both LH and FSH than rats that had not been exposed to ether. Fasted rats had lower levels ($P < 0.01$) of both LH and FSH than non-fasted rats. Fasting also resulted in reduced ($P < 0.05$) testosterone levels. Surgery lowered serum levels of LH ($P < 0.01$) but did not significantly alter the levels of FSH or testosterone. There were no significant interactions noted which suggests that each factor acted independently to produce its effect on hormone secretion and/or release.

The reduced serum LH and FSH levels as a result of fasting confirm an earlier report from this laboratory[7]. Although in a previous study[12] an effect of surgery on serum LH levels in adult male rats could not be detected, the present study clearly demonstrates that surgery in adult males, as in females[1,13] and immature males[1] does depress LH levels.

The acute stress induced by ether resulted in elevated levels of both FSH and LH. DUNN et al.[4] previously reported a similar LH-response to ether and elevated levels of both LH and FSH were reported by AJIKA et al.[5] for ovariectomized females exposed to ether. Thus it would appear that serum gonadotropin levels that have been measured in experiments where ether anesthesia was used are probably artificially high.

Fasting in the present study was effective in reducing serum testosterone levels. Since both surgery and fasting depressed LH levels but only the latter affected testosterone, the effect of fasting on testosterone was presumably not a result of the reduced LH levels. Although with time, the reduced LH levels of fasted rats would undoubtedly have contributed to a reduction in testosterone secretion, during the fasting period imposed in this study, the drop in testosterone levels probably represented a direct response of the testes to fasting.

It was concluded that each of the factors studied does influence the levels of one or more of the reproductive hormones. Although no interactions were found among the factors studied, some of these factors could interact


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with strain, sex, season or age, characteristics which often vary from one experiment to another[14].

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*Résumé.* Chez des rats mâles anésthésiés à l'éther avant la décapitation, on a observé une augmentation du niveau sérique de LH et de FSH, sans effet sur le niveau de testostérone. L'intervention chirurgicale subie 2 jours avant le sacrifice a fait diminuer seulement le niveau de LH. Les niveaux de LH, de FSH et de testostérone ont également diminué après 2 jours d'absence de nourriture.

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## Absence of Sulphation Factor (Somatomedin) Activity in Preparations of Colony Stimulating Factor and Nerve Growth Factor

The term somatomedin (SM) has been proposed for a factor or group of factors in serum which are growth hormone dependent and which stimulate the in vitro incorporation of $^{35}S$ into cartilage proteoglycan[1]. The in vitro effects of SM, which are not species specific[2], do not appear to be limited to cartilage as partially purified preparations have also been shown to have insulin-like activity[3]. In addition, recent reports indicate that SM stimulates the growth of several cell-lines in culture[4–6].

As it was of interest to learn whether other growth factors present in serum shared some of the biological effects of SM, we have studied the effects of mouse nerve growth factor (NGF) (Wellcome Laboratories; K5740) and colony stimulating factor (CSF) on the in vitro incorporation of $^{35}S$ into embryonic chick catilage.

Colony stimulating factor was prepared from pregnant mouse uterus by the method of Bradley[7]. Colony stimulating activity was assayed by a modification of the method of Bradley and Metcalf[8] using mouse bone marrow culture in semi-solid agar. Equal volumes of 0.6% agar and double-strength modified Eagle's medium were mixed with mouse bone marrow cells to give a final cell-count of $2 \times 10^5$/ml. 1 ml aliquots of this mixture were added to plastic petri dishes containing test material. The number of colonies were counted after 7 days incubation at 37 °C in an atmosphere of 5% $CO_2$-95% air. Observations were made in duplicate. Approximately 200 colonies were formed when 0.05 ml of the CSF extract was used.

Proteoglycan synthesis was studied by incubating pelvic cartilages from 11-day chick embryos with $^{35}S$ for 18 h at 37 °C. Six cartilages, incubated in groups of three in 1 ml medium, were used for each observation. The medium contained dilute human serum in some experiments. Further details of the method are published elsewhere[9].

We found that addition of 0.1 ml of the preparation of mouse CSF had no significant effect on the incorporation of $^{35}S$ into chick pelvis, either in the absence of serum or in the presence of 2.5% or 40% serum (Table I). Similarly, NGF at concentrations of 10 U/ml–1000 U/ml had no effect on the incorporation of $^{35}S$ into cartilage (Table II). In addition, NGF at concentrations of up to 1000 U/ml did not stimulate colony formation by mouse bone-marrow cells in agar culture or inhibit the activity of CSF (Table III).

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Table I. Effect of colony stimulating factor prepared from mouse uterus on $^{35}S$ incorporation into chick pelvic cartilage

| Experiment | Test sample | $^{35}S$ incorporation ± SEM (cpm × $10^{-3}$/mg cartilage) | | |
|---|---|---|---|---|
| | | 0 [a] | 10% [a] | 40% [a] |
| 1 | 0.1 ml CSF | 5.7 ± 0.5 | — | — |
| | Control | 9.0 ± 1.4 | — | — |
| 2 | 0.1 ml CSF | — | 23.7 ± 0.9 | 37.1 ± 1.8 |
| | Control | — | 23.9 ± 2.1 | 35.9 ± 3.8 |

Six cartilages were used for each observation. Incubation volume = 1 ml. [a] Serum concentration in medium.