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# NEONATAL STERILIZATION OF RODENTS WITH STEROID HORMONES

## 3. INFLUENCE OF NEONATAL TREATMENT WITH TESTOSTERONE PROPIONATE OR ESTRADIOL BENZOATE ON STEROIDS IN PLASMA OF ADULT MALE RATS

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SUMMARY

The influence of neonatal estrogen or androgen treatment was compared in adult male rats by measuring disappearance of testosterone, 4-androstene-3,17-dione, 3β-hydroxy-5-androsten-17-one and 3β-hydroxy-5-pregnen-20-one from plasma. It was found that neonatal treatment with estradiol benzoate altered the rate of transformation of testosterone in plasma when measured 20 min after intravenous injection; the relative specific activity of 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one was apparently decreased, whereas that of testosterone and androstenedione was increased.

In sexually mature rodents, treatment with androgenic or estrogenic hormones results in a temporary suppression of the hypothalamo-pituitary-gonadal axis; after cessation of therapy there is a return to normal reproductive function. A different response, however, is seen in very young animals. A single subcutaneous injection of testosterone propionate to female mice [1] or rats [2] within ten days of birth produces sterility in the adult animals. Estradiol benzoate given to male animals under similar conditions will evoke irreversible changes manifested by azoospermia and decrease in the weights of the accessory sex organs [3, 4]. It has been suggested by several investigators that treatment with steroid hormones shortly after birth may produce alterations in those central nervous system centers which in later life direct gonadal functions [cf. 5] but other modalities may be involved.

We have noticed that the uptake of radioactivity from injected steroids was much higher in the gonads than in the hypothalamus [6]. In male pups radioactivity uptake (expressed in dpm/mg wet tissue) of injected 4-$^{14}$C estradiol was 20 times greater in the testis than in the hypothalamus; in females injected with $^{14}$C testosterone propionate the ratio hypothalamus: ovaries was 1 : 16. Additional studies revealed that neonatal treatment with steroid hormones may influence testosterone turnover time [7]. This was studied in adult male rats injected with labeled testosterone. The specific activity of testosterone extracted from peripheral plasma was 2·3 times higher in males injected with estradiol benzoate (EB) and 3·1 times higher in animals injected with testosterone propionate (TP). We have postulated that such increase in the specific activity could result either from a decrease in the endogenous production of testosterone or from increased

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biological half-life and decreased clearance rates. Previous measurements of testosterone plasma levels in adult male rats [8] showed that in EB treated males, peripheral testosterone plasma levels were low. Thus, a decrease in the endogenous production of testosterone by the testes could result in a higher specific activity of intravenously injected testosterone. In TP treated males peripheral testosterone plasma levels were comparable to controls. The observed increase in the specific activity of testosterone isolated from plasma of similarly treated animals indicates that the turnover of circulating testosterone is decreased. The observation [9] that androgenization of male rats (neonatal TP treatment of castrated rats) resulted in greater sensitivity of the accessory sex organs to subsequent treatment with TP is of importance since it may indicate an increased sensitivity due to a reduced capacity of the animals to inactivate testosterone.

The present studies were undertaken to elucidate further this problem.

## MATERIALS AND METHODS

Holtzman strain male pups bred in our laboratories were used. Testosterone propionate (1000 $\mu$g) or estradiol benzoate (250 $\mu$g) dissolved in 0·05 ml of sesame oil were injected at the age of 5 days. Control males received oil alone. The pups were weaned at the age of 24–26 days and maintained in light and temperature controlled rooms with water and food *ad libitum*. At the age of 200 days the animals were divided into two groups. In one group (group 1) each animal was injected intravenously with a mixture of [7$\alpha$-$^3$H]3$\beta$-hydroxy-5-pregnen-20-one (S.A., 14·7 Ci/mmol), and [4-$^{14}$C]3$\beta$-hydroxy-5-androsten-17-one (S.A. 57·1 mCi/mmol), dissolved in 0·2 ml of 20% ethanol in propylene glycol. The average dose per animal was about 90 $\mu$Ci of $^3$H labeled steroid and 4·5 $\mu$Ci of $^{14}$C labeled steroid. The $^{14}$C/$^3$H ratio determined by counting of an aliquot sample was 0·050. In the second group each animal was injected intravenously with approximately the same dose of a mixture of [1,2-$^3$H]4-androstene-3,17-dione (S.A. 15 Ci/mmol) and [4-$^{14}$C] testosterone (S.A. 58·2 mCi/mmol). The $^{14}$C/$^3$H ratio determined by counting an aliquot was 0·073. The solvents and volume used for injection were the same as used in the first group. Twenty min after injection blood was drawn from the vena cava into heparinized syringes (ether anesthesia) and centrifuged; plasma was separated and kept frozen until processed. The animals were autopsied and selected organs were removed, weighed and frozen for further study. Total plasma volume which was used for experimentation was as follows: group 1 controls, 36·2 ml; TP injected, 12·5 ml; and EB injected, 43·9 ml; group 2 controls, 47·7 ml; TP injected, 12·5 ml; and EB injected, 35·7 ml.

*Chemicals.* Solvents were of reagent grade. Radioactive steroids were obtained from Amersham/Searle, Chicago, Illinois and New England Nuclear Corp., Boston, Massachusetts. Prior to use the purity was checked by descending paper chromatography. Inert steroids obtained from Steroloids, Inc., Pawling, New York, were crystallized.

*Extraction and purification.* Plasma was diluted with absolute ethanol to a 70% aqueous solution. The solids were separated by centrifugation, the residue was washed with 70% aqueous ethanol and the process repeated. All supernatants were combined and ethanol was removed under reduced pressure in a rotatory evaporator. The volume was brought to 40–50 ml with water and was extracted with ether by refluxing 4 hrs in a continuous extractor. Total radioactivity (Bray type scintillation media) was determined in both the aqueous and ether portions.

The aqueous phase was not processed further. Ether extracts from individual animals in the six experimental groups were combined. The crude extracts were purified first by a descending paper chromatography (about 20 h) on Whatman paper No. 1 using the solvent system ligroin: methanol: water (5:4:1, by vol.). The zones corresponding to 3β-hydroxy-5-pregnen-20-one, 3β-hydroxy-5-androsten-17-one, 4-androstene-3,17-dione and testosterone standards run at the same time were cut out, eluted with methanol and the solvent was removed by distillation *in vacuo*. Further purification of individual fractions was achieved using thin layer chromatography (Eastman Kodak Silica Gel sheets No. 6060) in a solvent system containing ethyl acetate : n-hexane: acetic acid (75 : 20 : 5 by vol.). The zone corresponding to respective standards was eluted with methanol (5 ml) and an aliquot (0·5 ml) was used to determine the radioactivity present. An authentic specimen (50–100 mg) was then added and each sample was crystallized to constant specific activity using, in succession, the following solvents: ethyl acetate–methanol, aqueous acetone, ethyl acetate and aqueous methanol for testosterone and 4-androstene-3,17-dione; aqueous methanol, ethyl acetate: methanol (1 : 1, v/v), aqueous ethanol and ethyl acetate for the other two steroids. All samples were crystallized four times except testosterone which had to be crystallized 5 times. Mother liquors were taken to dryness under nitrogen. In each crystallization step 1–6 mg of crystals, and 3–6 mg of mother liquor solids were weighed on an electric microbalance (Cahn Instrument Co., Paramount, California, U.S.A.) and dried under high vacuum (Abderhalden drying apparatus) at 80°C for 4–8 h.

*Measurement of radioactivity.* For detection of radioactivity the samples dissolved in 10 ml of toluene containing 1% of 2-(4-tert-butylphenyl)-5-(4-biphenyl)-1,3,4-oxidiazole were counted in liquid scintillation spectrophotometer (Packard Model No. 3375). $^{14}$Carbon and tritium were counted simultaneously. Appropriate standards ($^{14}$C and $^{3}$H toluene) were used to establish counting efficiency. Paper chromatograms were scanned using Actigraph III (Nuclear Chicago Corp., Des Plaines, Illinois, U.S.A.). Aqueous solutions were counted as described previously [10].

## RESULTS

Table 1 listing mean body and organ weights of control and steroid treated animals shows that treatment of 5 day old pups with 250 μg of estradiol benzoate (EB) or 1000 μg of testosterone propionate (TP) results in a significant weight decrease of testes, liver, and kidneys in the adult animals.

Table 1. Body and organ weights of 200 day old rats injected at the age of 5 days with estradiol benzoate testosterone propionate

| Treatment μg | No. of rats | Body wt., g ± S.E. | Organ weights, mg ± S.E. Testes | Liver | Kidneys |
|---|---|---|---|---|---|
| 0 | 15 | 459 ± 20 | 3459 ± 116 | 15490 ± 468 | 3120 ± 162 |
| Estradiol benzoate. 250 | 9 | 489 ± 11 | 2631 ± 107* | 12600 ± 616* | 2561 ± 107* |
| Testosterone propionate. 1000 | 6 | 472 ± 14 | 2987 ± 244* | 10930 ± 514* | 2209 ± 146* |

*$P \leqq 0{\cdot}05$.

Table 2. Total radioactivity from intravenously injected steroids in plasma of 200 day old male rats treated neonatally with estradiol benzoate or testosterone propionate

| Steroid injected | Radioactivity detected dpm.[c] × $10^3$/ml plasma Control | EB treated | TP treated |
|---|---|---|---|
| [$^3$H]Pregnenolone/[$^{14}$C]DHA | 12997[a](791)[b] | 8026(705) | 6798(456) |
| % ether fraction | 44%(61%) | 30%(60%) | * |
| [$^3$H]Androstenedione/ [$^{14}$C]Testosterone | 8835(615) | 17157(1204) | 13405(919) |
| % ether fraction | 56%(61%) | 39%(47%) | * |

[a] $^3$H label; [b] $^{14}$C label.
* Not determined. See text.
[c] All animals combined.

Table 2 shows total radioactivity detected in pooled plasma expressed in disintegrations per minute (dpm) × $10^3$ per ml. In addition the table shows (in per cent) the amount of radioactivity obtained in distributing the total radioactivity between ether and water. Compared to controls, total radioactivity in EB treated groups associated with tritium (pregnenolone)* was decreased. It was about the same associated with $^{14}$C label (DHA). In the second group injected with androstenedione and testosterone total radioactivity was increased about twofold as compared to controls. The portion of total radioactivity associated with water soluble substances, presumably conjugated steroids, was decreased in EB treated groups with the exception of fractions associated with DHA. In animals treated neonatally with TP, total radioactivity distribution was similar to that seen in EB treated groups. Distribution between ether and water could not be established in the two groups since a portion of the aqueous phase was lost.

During the first purification step of ether soluble material (descending paper chromatography) 5–30% of radioactivity was found in zones corresponding to the four standards. Further purification by thin layer chromatography removed an additional 60–80% of radioactive material. The results of the final purification by crystallization to a constant specific radioactivity are given in Table 3. Values (expressed in dpm/mg) are given only for the first and the last two products of crystallization and the last mother liquors. In one sample (testosterone isolated from plasma of TP treated animals) a portion of the crystalline material was lost and only 2 crystallizations were possible. This datum is hence only preliminary.

Radioactivity of the pure steroids expressed as total dpm × $10^3$ per 100 ml of plasma is given in Table 4. The given values were calculated by taking into account plasma volume taken for extraction and the weight of authentic specimens added prior to crystallization. No $^{14}$C radioactive material was found associated with purified pregnenolone. For the other three steroids, which contained both $^3$H and $^{14}$C labels, the first figure represents $^3$H radioactivity; radioactivity associated with $^{14}$C is given in parentheses. As shown in Table 5, the conversion of pregnenolone into DHA at 20 min after injection was low in all three groups. Based on tritium determination this was 1·5% in the control group, 1% in EB treated

* Trivial names used in this paper: *Pregnenolone*: 3β-hydroxy-5-pregnen-20-one; *DHA*: 3β-hydroxy-5-androsten-17-one; *androstenedione*: 4-androstene-3,17-dione; *testosterone*: 17β-hydroxy-4-androsten-3-one.

Table 3. Crystallization of radioactive steroids isolated from plasma of 200 day old rats injected neonatally with estradiol benzoate or testosterone propionate

| Steroid | | dpm per mg of steroid Controls | EB treated | TP treated |
|---|---|---|---|---|
| 3β-Hydroxy-5-pregnen-20-one | C1 | 3128[a] (16)[b] | 9433 (18) | 180 (0) |
| | C3 | 2968 (0) | 9069 (0) | 165 (0) |
| | C4 | 2838 (0) | 8650 (0) | 159 (0) |
| | ML4 | 2644 (0) | 8465 (0) | 188 (0) |
| 3β-Hydroxy-5-androsten-17-one | C1 | 89 (387) | 134 (438) | 32 (58) |
| | C3 | 28 (604) | 29 (769) | 4 (54) |
| | C4 | 30 (640) | 30 (852) | 4 (54) |
| | ML4 | 25 (544) | 34 (827) | 6 (63) |
| 4-Androstene-3,17-dione | C1 | 4884 (308) | 5046 (307) | 2254 (65) |
| | C3 | 3930 (222) | 4178 (244) | 2198 (64) |
| | C4 | 3867 (226) | 4149 (239) | 2234 (61) |
| | ML4 | 3990 (224) | 4395 (268) | 2179 (64) |
| Testosterone | C1 | 3255 (269) | 3019 (331) | 1600 (300)* |
| | C4 | 1398 (181) | 2897 (316) | |
| | C5 | 1367 (174) | 2863 (323) | |
| | ML4 | 1419 (193) | 3006 (336) | |

*See text.
[a] $^{3}H$ label.
[b] $^{14}C$ label.

Table 4. Radioactivity of four steroids isolated from plasma

| Steroid | Total dpm × $10^3$/100 ml of plasma Control | EB treated | TP treated |
|---|---|---|---|
| [$^{3}H$]-3β-Hydroxy-5-pregnen-20-one | 814[a] | 859 | 218 |
| [$^{14}C$]-3β-Hydroxy-5-androsten-17-one | 12[a] (265)[b] | 8·7 (246) | 1·5 (214) |
| [$^{3}H$]-4-Androstene-3,17-dione | 794[a] (46)[b] | 1131 (65) | 1750 (48) |
| [$^{14}C$]*Testosterone | 281[a] (36)[b] | 779 (88) | 1258 (235)* |

*See text.
[a] $^{3}H$ label.
[b] $^{14}C$ label.

animals and 0·7% in the TP group. Conversion of androstenedione into testosterone was 35% in controls, 69% in the EB group and about 70% in the TP group. The reversal of the reaction, androstenedione to testosterone, proceeded about twice as fast in the EB groups (ratio 1·35) and even faster in TP group (ratio about 5·0) as compared to controls (ratio 0·77).

## DISCUSSION

The effect of neonatal injection of estradiol benzoate or testosterone propionate into 5 day old male rats was evaluated 195 days later by comparing plasma levels of testosterone and three precursors, 20 min after intravenous injection[21]. The specific activity of testosterone isolated from peripheral plasma of EB treated rats was 2·5 times higher than controls. This is in good agreement with the pre-

viously reported value of 2·1[7]. The specific activity of testosterone isolated from the TP group was 6·6 times higher than controls, whereas our previous value for this group was 3·1. The discrepancy between the two values can be explained on the basis of difference in the time allowed for the steroids to circulate (20 min vs. 120 min in the previous study) and possibly by incomplete purification of the testosterone fraction. It is known that ether anesthesia causes a rapid decrease, within 5 minutes, of the secretion of testosterone in the spermatic vein blood of rats[12]. However, since both controls and experimental animals were exposed to about the same amount of ether this possibility may most likely be neglected.

Interpretation of the data concerning the disappearance of the three precursors, or their interconversion, is more difficult. We have obtained our data after a single interval (20 min) following the intravenous injection and have no assurance

Table 5. Relative specific activity of four steroids isolated from plasma of adult rats injected neonatally with estradiol benzoate or testosterone propionate

| Steroid | Radioactivity associated with label: | Ratio of treatment groups to controls: EB treated/control $^{3}H$ | EB treated/control $^{14}C$ | TP treated/control $^{3}H$ | TP treated/control $^{14}C$ |
|---|---|---|---|---|---|
| [$^{3}$H]-3$\beta$-Hydroxy-5-pregnen-20-one | | 1·06 | | 0·27 | |
| [$^{14}$C]-3$\beta$-Hydroxy-5-androsten-17-one | | 0·70 | | 0·12 | |
| [$^{3}$H]-4-Androstene-3,17-dione | | | 1·41 | | 1·03 |
| [$^{14}$C]-Testosterone | | 2·77 | | 4·48* | |

*See text.

that the conversions of pregnenolone into dehydroepiandrosterone, and the equilibration of androstenedione and testosterone, are linear and parallel for the three groups. In addition our data are based on the specific activity of the steroids after adding a carrier and recrystallization, not in terms of endogenous steroid mass. Hence, the data must be viewed only as comparison between controls and treatment groups and no conclusions can be made concerning the dynamics of the biosynthetic processes. Despite this limitation a few general comments may be made.

Table 5 shows the relative rate of transformation of the four steroids studied, expressed as ratio in specific activity between treatment and control groups. This comparison shows that the SA of testosterone precursors was apparently influenced by neonatal steroid treatment. In TP treated animals the SA of pregnenolone was about 5 times higher and only about 12% of the label was found in DHA. In contrast the values for EB treated animals were about the same as in untreated animals. The reduction of androstenedione to testosterone may have proceeded at a higher rate as indicated by higher ratios which were 2·8 and 4·5 times for EB and TP groups, respectively.

The influence of neonatal steroid treatment on steroid biosynthesis *in vitro* has been studied by several groups[13–15]. It was reported that ovaries of adult female rats injected at the age of 5 days with testosterone propionate produced abnormal amounts of androgens and that the aromatizing enzyme activity was increased. The pattern of steroidogenesis was thought to reflect an abnormal pattern of gonadotropin secretion and/or release due to neonatal steroid treatment.

An abnormal pattern of production of a steroid hormone may not necessarily reflect an altered stimulatory function of the pituitary gland. An example was provided by Kitay and co-workers who found that corticosterone production by adrenal homogenates of castrated female[16] and male[17] rats was decreased independent of changes in endogenous ACTH secretion. De Moor and co-workers have reported[18–20] that cortisol metabolizing enzymes in the liver of male rats become 'sexually differentiated' under the influence of testosterone. The pattern of cortisol metabolism can be changed by injecting estradiol benzoate on the 5th day of life[21].

Our present results raise the possibility that neonatal treatment of male rats with steroid hormones induces changes in injected testosterone and precursors of testosterone. It is difficult to speculate that such results could be due to an abnormal pattern of gonadotropin secretion and/or release directed by higher central nervous centers. To our knowledge an interrelationship between the central nervous system and steroid metabolism has not yet been established. In the absence of such information it may be assumed that neonatal administration of androgens or estrogens induces permanent changes either in enzymes concerned with the synthesis and degradation of gonadal hormones or in the binding of steroids to plasma proteins.

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