Shift with Age in Steady-State Concentrations of Androstenedione and Testosterone in Incubations of Guinea-Pig Testis

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(Received 25 August 1967)

Minces of testes from mature and immature guinea pigs were incubated with [^{14}C]androstenedione. Other portions of the same testes were simultaneously incubated with [^{14}C]testosterone. Under steady-state conditions, the predominant steroid was [^{14}C]androstenedione in the presence of tissue from young animals and [^{14}C]testosterone in that from mature animals, irrespective of the compound added initially. It is concluded that the steady-state equilibrium favours the weak androgen androstenedione in the presence of tissue from the young animals, but favours the potent testosterone in that from the mature animals. Portions of tissue were homogenized in phosphate buffer. The net reduction of [^{14}C]androstenedione to [^{14}C]testosterone was less by homogenates of testes from young animals than by those from mature animals. Doubling the concentration of the NADPH-generating system did not affect this difference between the homogenates.

The androgens androstenedione (androst-4-ene-3,17-dione) and testosterone (17 β -hydroxyandrost-4-en-3-one) differ structurally only in the functional group at the 17-position on the D ring, androstenedione having a carbonyl oxygen and testosterone having a hydroxyl group. This difference is important physiologically, however, in that in mammals androstenedione has 8–20 times less androgenic potency than testosterone (Dorfman & Shipley, 1956).

Lindner (1959) and Lindner & Mann (1960) have shown that the testosterone/androstenedione ratio in bovine testes changed from less than 1:1 at up to 3 months of age to greater than 10:1 in animals aged more than 9 months. The change was rather abrupt over the period from the fourth to the sixth month. Lindner (1961) also found that the androgen concentrations in bovine testes correlated well with their concentrations in the spermatic veins.

We previously studied the metabolism of [^{14}C]progesterone by minces of guinea-pig testes, as it varied with age of the animal from the age of the first appearance of a few sperm in the seminiferous tubules to complete maturity (Snipes, Becker & Migeon, 1965*a,b*). We found that, though the disappearance of progesterone and the appearance of 17 α -hydroxyprogesterone did not change consistently with age, there was an increase in the

ratio of label appearing in testosterone to label appearing in androstenedione with age of the animals. Further, when [^{14}C]androstenedione itself was incubated with testis minces from mature animals, three to four times as much was converted into [^{14}C]testosterone, compared with minces from immature animals. This finding was of considerable theoretical importance, since it afforded a simpler explanation for the changes with age in relative androgen concentration in bovine testes, found by Lindner (1959) and Lindner & Mann (1960), than that the changes were due either to an activation of a pathway of testosterone synthesis by-passing androstenedione in mature testes or to a further metabolism of formed testosterone in immature testes.

The present work is an investigation into the reasons for this change in net reduction of androstenedione to testosterone by minces of guinea-pig testes. It was particularly decided to test whether our previous findings could be due simply to a greater facility at reduction of the carbonyl group or whether the reoxidation of the hydroxyl group might also be changed at sexual maturity. An increase in reduction alone might have implied a simple increase in enzymic activity. However, it was found that the ratio of steady-state concentrations of the two androgens is shifted to a great preponderance of testosterone in incubations of testes of mature animals, no matter which androgen

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is added initially. A preliminary account of this work has been published (Snipes, Becker & Migeon, 1966b).

MATERIALS AND METHODS

Animals. Albino guinea pigs of the Hartley strain were used. Mature animals were 7 months old and had a mean body weight (seven animals) of 967.1 ± 59.4 (S.E.M.) g. The seven groups of immature animals were 10 weeks old and had a mean body weight of 357.7 ± 6.4 g. The animals were killed by cervical fracture and exsanguination.

Incubations. After removal of fat and tunic, the testes of each animal were minced together with scissors. In some cases testes from several immature animals were minced together. In these cases mean body and tissue weights were obtained, and the group was treated as one animal in the various calculations.

Portions (150 mg.) of testis minces from mature or immature animals were placed in 10 ml. of Krebs & Henseleit (1932) bicarbonate buffer, pH 7.4, containing glucose (10 mM) with either [^{14}C]androstenedione (0.6 μg . and 0.1 μC in 0.1 ml. of ethanol) or [^{14}C]testosterone (1.6 μg . and 0.1 μC in 0.1 ml. of ethanol). Incubation was carried out in a shaking water bath at 34° under $\text{O}_2 + \text{CO}_2$ (95:5). Reactions were stopped by the addition of 30 ml. of ethanol.

Portions of the minces were homogenized in Kontes Dual homogenization tubes, one part of mince to nine parts of an ice-cold buffer (80 mM-potassium phosphate, pH 7.4, and 4.8 mM- MgCl_2). The homogenates were then centrifuged at 1000g for 10 min. at 4° . Each incubation flask received 0.7 ml. of supernatant, 0.1 ml. of ethanol containing 80 μg . and 0.1 μC of [^{14}C]androstenedione and 1.2 ml. of the above buffer containing the following NADPH-generating system: NADP $^+$, 0.4 μmole ; glucose 6-phosphate, 0.49 μmole ; glucose 6-phosphate dehydrogenase, 1.75 Kornberg units (Kornberg & Horecker, 1955). Where indicated, the flasks contained double the concentration of this generating system. Flasks were incubated with shaking at 34° under air for 1 hr. Then 6 ml. of ethanol was added to stop the reaction. All incubation mixtures were stored at -20° until the extractions were to be performed.

Extraction. Flasks from incubations with minces and homogenates were extracted in the same manner. To each flask was added 40 μg . each of unlabelled androstenedione and of unlabelled testosterone. Also added were tritiated androstenedione and testosterone, which were counted at the end of the analysis to calculate recoveries. Samples of all the radioactive steroids used in this study had shown only the expected peak on chromatography and strip-scanning.

The contents of the flasks were then homogenized and centrifuged. Precipitates were washed twice with 10 ml. of 75% (v/v) ethanol, and combined supernatants were evaporated to dryness at 45° under a stream of air. Residues were dissolved in 8.0 ml. of water and extracted twice with 16 ml. of ethyl acetate and twice with 16 ml. of chloroform. More than 90% of the radioactivity was present in the combined organic solvent extract.

Chromatography and counting of radioactivity. Extracts were evaporated and streaked across 7.5 cm. of Whatman no. 2 chromatography paper. Chromatography (Bush, 1952) with a modified Bush B_3 system, heptane-benzene-methanol-water (10:5:12:3, by vol.), was carried out. The

two u.v.-absorbing spots containing androstenedione and testosterone were cut out and eluted with methanol. Portions (0.5 ml.) of samples dissolved in methanol were mixed with a toluene-base scintillation fluid and counted on a three-channel liquid-scintillation counter. The ^{14}C and ^3H content was calculated as described by Kowarski, Finkelstein, Loras & Migeon (1964). In preliminary experiments, counting of singly labelled samples indicated negligible quenching. All ^{14}C data given are expressed as ^{14}C isolated as a percentage of the ^{14}C incubated, corrected by the ^3H recovery.

Measurements of NADP $^+$ and NADPH. These compounds were determined by the modification by Lowry, Roberts, Kappahn & Lewis (1951) of the fluorimetric method of Kaplan, Colowick & Barnes (1951).

RESULTS

Tissue weights. The testes of mature animals (4.68 ± 0.25 g.) were markedly larger than those of the immature animals (1.09 ± 0.10 g.). Empty seminal vesicles of mature animals (2.19 ± 0.14 g.) also were larger than those of immature (0.60 ± 0.14 g.).

Kinetics of interconversion of androstenedione and testosterone by minces of testes. The testes of two mature guinea pigs and testes of two groups of three immature animals were minced together. There were thus two lots of mince from mature animals and two lots from immature animals. To study the kinetics of the interconversion of the two androgens, portions of testis mince from the same lot were incubated separately with both [^{14}C]androstenedione and [^{14}C]testosterone for 30, 60, 90, 135 or 180 min.

The percentage of label present in androstenedione after the several time-intervals is shown in Fig. 1. The lines at the top at longer incubation times are incubations with minces from immature animals. With [^{14}C]androstenedione most of the label disappeared from the compound in a manner resembling first-order kinetics in incubations with mature testes, but most remained in incubations with immature testes. With [^{14}C]testosterone, most of the label appeared in androstenedione in a manner resembling first-order kinetics in incubations with immature testes, but only a small amount did so in incubations with mature testes. It can be seen that a steady state had been reached and that the steady-state concentrations lay at different positions for the two ages of animals.

Fig. 2 is the same as Fig. 1 except that the ordinate now shows the percentage of the label present in testosterone. The lines at the top at longer incubation periods represent the incubations with mature testes. With [^{14}C]androstenedione, most of the label appeared in testosterone in incubations with mature testes, but only a small amount did so in incubations of immature tissue. With [^{14}C]testosterone, most of the label dis-

Fig. 1. Percentage of label in androstenedione after incubation of minces of testes with [^{14}C]androstenedione ($\circ$) or [^{14}C]testosterone ($\bullet$) for several time-intervals. All points are means of incubations of two lots of minces of testes. —, Immature testes; ----, mature testes.

Fig. 2. Percentage of label in testosterone after incubation of minces of testes with [^{14}C]androstenedione ($\circ$) or [^{14}C]testosterone ($\bullet$) for several time-intervals. All points are means of incubations of two lots of minces of testes. —, Immature testes; ----, mature testes.

appeared in incubations with immature testes, but most of the label remained in incubations with mature testes. The shift in steady-state concentration in the presence of tissue from the animals of different stages of sexual development is again apparent.

Replicate incubations of minces of testes. The above kinetic studies were done with two lots of minces of mature testes and two lots of minces of immature testes. To accumulate enough data for statistical analysis, the same types of incubations were done repeatedly at one incubation time: 90 min. was selected as being the first time-interval at which a steady state was reached.

Fig. 3 represents the combined results of the incubations for 90 min. of the testes minces of the animals of Figs. 1 and 2 plus another five lots of mature and immature guinea pigs. These additional lots were composed of one mature or two immature animals. All differences described below are statistically significant at the 1% level (Student's t test).

In incubations with [^{14}C]androstenedione (Fig. 3a), there was a large percentage of label remaining as that compound in incubations with immature testes, but a small percentage in the incubations with mature testes. This difference is accounted for by a greater conversion of [^{14}C]androstenedione into [^{14}C]testosterone in the mature than in the incubations with young testes.

Conversely, in incubations with [^{14}C]testosterone (Fig. 3b) there was a larger percentage of label remaining as that compound in incubations with mature testes than in incubations of tissue from young animals. This difference is accounted for by the smaller conversion of [^{14}C]testosterone into

Fig. 3. Replicate incubations of minces of testes for 90 min.: (a) with [^{14}C]androstenedione; (b) with [^{14}C]testosterone. A, Androstenedione; T, testosterone. The bars are the means of incubations of seven lots of minces of testes. The small vertical lines are $\pm$ s.e.m. The difference between each mean for incubation of immature testes and the mean for the corresponding incubation of mature testes is statistically significant ($P < 0.01$).

[^{14}C]androstenedione in the incubations with mature testes.

Identification of androgens. In several cases, chromatograms on which the androgens had been separated were radioautographed before elution. Radioautography for 2 days revealed no spots other than those corresponding to androstenedione and testosterone carrier. On a 3-day exposure to X-ray film, another faint spot was seen about 1 cm. behind testosterone in incubations with both [^{14}C]androstenedione and [^{14}C]testosterone for 90 min. However, it also contained considerable ^3H , suggesting incomplete separation from testosterone. When the eluates were rechromatographed with additional

testosterone carrier and radioautographed, the ^{14}C again appeared about 1 cm. behind the carrier. This area now contained about 3% of the initial ^{14}C and about 0.5% of the ^3H testosterone. There was no difference between incubations with tissue from the two age groups.

Areas corresponding to androstenedione and testosterone were eluted. Then 50mg. of appropriate non-radioactive steroid was added to each, and samples corresponding to all bars of Fig. 3 were crystallized three times. The $^{14}\text{C}/^3\text{H}$ ratio was not changed by crystallization. This procedure indicated that the radioactive compounds in the initial eluates from the chromatograms were essentially pure ^{14}C androstenedione and ^{14}C -testosterone respectively.

Incubations of ^{14}C androstenedione with homogenates. Portions of the testes of the five additional lots of guinea pigs were homogenized. Volumes of homogenates of mature and immature testes corresponding to 70mg. of tissue were incubated with ^{14}C androstenedione and an amount of non-radioactive androstenedione (280 μmoles) that, according to preliminary experiments, would result in zero-order kinetics. An influence of endogenous androstenedione on the reaction rate is thus improbable. Preliminary experiments also established that the concentration of NADPH-generating system used would reduce all the NAD^+ in less than 30sec.

On a 60min. incubation, homogenates of immature testes converted less ^{14}C androstenedione into ^{14}C testosterone than did homogenates of mature testes (Table 1). Doubling the concentration of the NADPH-generating system did not affect this difference (Table 1). In both cases, the difference was statistically significant at the 5% level for paired data. Radioautography of some of the chromatograms from this experiment for 3 days revealed no radioactivity other than that corresponding to androstenedione and testosterone carrier.

An attempt to measure activity in the reverse direction with testosterone and NAD^+ alone showed only a small conversion with no significant difference between the incubations with mature and immature testes. Further investigation showed that the NAD^+ was very unstable under the conditions used, being half gone at 10min. and undetectable at 60min. NADPH was stable under these conditions.

Control incubations. Since Scott, Baggett & White (1963) have reported an oxidation of ^{14}C -testosterone to ^{14}C androstenedione by semen of several species, the difference in androgen metabolism by minces of mature testes in our hands could conceivably have been due to the presence of sperm in the mature testes. To investigate this possibility, 100mg. portions of minces of sperm-containing

Table 1. *Incubation of homogenates of testes with androstenedione for 60min.*

Homogenate (0.7ml.) was incubated with 280 μmoles (80 $\mu\text{g.}$) and 0.1 μC of ^{14}C androstenedione, as described in the Materials and Methods section. The incubations denoted 'cofactor $\times 1$ ' contained the NADPH-generating system described in that section, and the incubations denoted 'cofactor $\times 2$ ' contained twice that concentration of NADPH-generating system. The data are expressed as μmoles of androstenedione appearing as testosterone, on the assumption that endogenous androstenedione was negligible relative to the 80 $\mu\text{g.}$ of exogenous steroid. Values are means ($\pm$ s.e.m.) of incubations of homogenates of five pairs of lots of testes. Both mean differences between incubations of homogenates of immature and mature testes are statistically significant for paired data ($P < 0.05$).

Cofactor	Immature testes	Mature testes	Difference
$\times 1$	43.2 $\pm$ 4.2	59.7 $\pm$ 7.8	16.4 $\pm$ 5.0
$\times 2$	48.0 $\pm$ 2.8	66.1 $\pm$ 8.1	18.0 $\pm$ 5.9

epididymis from mature animals were incubated simultaneously with ^{14}C androstenedione and ^3H testosterone for 3hr. No more than 2% of either isotope appeared in the area of the chromatogram corresponding to the other androgen; some or all of this could have been due to incomplete separation, since the extracts were not studied further. It was concluded that metabolism of the androgens by sperm could not be a significant factor in our studies.

That the androgens were not interconverted during our analytical procedure was shown by the immediate addition of ethanol to some flasks without incubation. The steroids were not changed by subsequent extraction and chromatography. Since testosterone can be sulphated but androstenedione cannot, there could have been a greater conjugation of sulphate to testosterone in incubations with mature testes followed by a loss of the conjugate during analysis. This possibility was investigated by the addition of ^3H testosterone sulphate to flasks identical with the above controls except for lack of radioactive steroid. Only 10% of the tritium was extracted from the aqueous phase and none of this migrated with androstenedione or testosterone on chromatography.

That the minces could still interconvert the androgens during the plateau time of Figs. 1 and 2 was shown by the incubation of minces of mature and immature testes for 90min. with 100 $\mu\text{l.}$ of ethanol without steroids. At that time, 50 $\mu\text{l.}$ of ethanol containing either ^{14}C androstenedione or ^{14}C testosterone was added; the minces were incubated for another 90min., with good steroid interconversion.

DISCUSSION

It is apparent that the equilibrium between androstenedione and testosterone in the presence of minces of testes is shifted from favouring the oxidized compound when minces of immature testes are used to favouring the reduced form in the presence of minces of mature testes. The findings reported in this paper, along with our previous work (Snipes *et al.* 1965*a,b*), lend further support to the hypothesis, derived from the work of Lindner & Mann (1960), that androgenization at puberty is in part due to changes in the proportions of androgens synthesized by the testes. In addition to this, the sum total of androgens would increase. Our use of radioactive steroids under steady-state conditions in the present work furnishes strong evidence against a number of mechanisms that could be postulated for this shift in proportions of androgens.

Lindner & Mann (1960) quantitatively determined materials moving as androstenedione and testosterone on paper chromatography by measuring the absorption of u.v. light. In addition to its lack of sensitivity, this approach cannot give information about the reasons for a change in proportion of androgens. It has, however, the advantage of being an actual measure of the androgens present, assuming that the tests for identity described by Lindner & Mann (1960) were performed on samples from cattle of all ages.

Incubations with radioactive precursors allow the study of the conversion of one molecule into another. However, usually precursors are introduced at some point and the rate of conversion of one direction is measured. Results gained in this way have the disadvantage of being only a measure of enzymic potentiality.

The approach we have adopted, that of allowing the system to reach a steady state, has many of the advantages of both the above methods. This approach simplifies the question of the number of Leydig cells. It may be that in rats (Niemi & Ikonen, 1963) and humans (Sniffen, 1950) there are two different generations of Leydig cells. That the present work was done under steady-state conditions removes any necessity to consider the number of Leydig cells, if one assumes only that the enzymes catalysing oxidation and reduction of the androgens are located in the same cells. The steady-state conditions also eliminate the possibility of a simple saturation effect due to a difference in endogenous content of the two androgens. Moreover, in a steady-state equilibrium such as this, membrane effects on the steroid molecules themselves would be a factor only if there were somewhere a membrane more permeable in one direction and its direction of greatest permeability changed with age. Compartmental changes in the extent of cofactor

reduction or pH could, however, be envisaged. Both cofactors and H^+ are stoichiometric reactants in this system.

The equilibrium shift would seem to rule out also a simple increase in activity of an easily reversible 17-dehydrogenase as the sole factor. It is noteworthy that a similar alteration in steady-state concentration of androgens has been found in testes from isoimmune aspermatogenic guinea pigs (Becker, Snipes & Migeon, 1966; Snipes, Becker & Migeon, 1966*a*).

The present work also confirms our previous experiments (Snipes *et al.* 1965*a,b*), indicating that in the guinea pig the change in proportion of androgens is not due to a further metabolism of either androstenedione or testosterone to some other steroid and suggesting that it need not be due in part to changes in a pathway by-passing androstenedione. The latter possibility is not, however, precluded.

Nayfeh, Barefoot & Baggett (1966) and Inano & Tamaoki (1966) have found a greater reduction of the double bond of the A ring of androgens by homogenates of immature rat testes than by homogenates of mature testes. The end products obtained by the two groups of workers differed, but the 5α -androstan- $3\alpha,17\beta$ -diol obtained by the first group differs from some of the products obtained by the second group only by the state of reduction of the two oxygen-containing functions. The latter workers also reported a more rapid disappearance of pregnenolone after incubation with fractions of mature testes than with those of immature testes. Whether the difference between testicular androgen metabolism found with rat and guinea-pig preparations is due to a species difference awaits further work. There is, however, indeed a change in androgen ratio at puberty in the rat. Hashimoto & Suzuki (1966) have found an increase at this time of the testosterone/androstenedione ratio in spermatic-vein blood. Their immature rats had been pretreated with gonadotrophin. A similar change in ratio after puberty has been found in blood of the general circulation of humans (Frasier & Horton, 1966) and rats (Rivarola, Snipes & Migeon, 1968), although changes in extragonadal production or catabolism of these androgens may influence their concentration in systemic plasma. In fact, it was not rigorously proved that the changes in concentration of these androgens noted by Hashimoto & Suzuki (1966) in plasma of the spermatic vein are to be attributed to the testis; even though they quantitatively determined the androgens in testicular tissue and venous efflux, they did not determine their concentrations in arterial plasma supplying the gland.

Foetal testes of rabbits (Lipsett & Tullner, 1965) and rats (Noumura, Weisz & Lloyd, 1966) have also

been incubated with labelled progesterone. Both groups of workers found an increase in the testosterone/androstenedione ratio with age of the foetus. The percentage of progesterone disappearing per milligram of testis increased with age in the incubations with foetal rat testes; it was not measured in the other species. Rabbit testis mince was also incubated with labelled androstenedione. The percentage converted into testosterone per testis, but not per milligram of testis, increased with foetal age. The conversion of C₂₁ precursors into testosterone by foetal human testes *in vitro* increases with foetal age during the first part of foetal life, as summarized by Solomon (1966).

The relevance of studies of androstenedione and testosterone in guinea-pig testis to the physiology of that animal might be questioned. Although they are formed from radioactive precursors by homogenates (Lynn & Brown, 1958) and whole-cell preparations (Snipes *et al.* 1965*b*) of guinea-pig testis, these compounds had never been identified in the blood of this species. However, we have found (Rivarola *et al.* 1968) that their concentrations in systemic plasma of adult male guinea pigs are similar to that in adult male humans. The identification of testosterone is based on one column, three paper and two thin-layer chromatography systems, plus two derivatives. The same procedures plus one more derivative were used for androstenedione.

This investigation has been supported by Grants AM-00180 and TI-AM-5219 from the National Institutes of Health, U.S. Public Health Service. W.G.B. was a Henry Strong Denison Scholar for 1965-66.

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