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SUPERFUSION OF RAT TESTES WITH MIXTURES OF LABELED TESTOSTERONE AND ANDROSTENEDIONE

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SUMMARY

A tracer superfusion experimental design was applied to the study of testicular steroidogenesis. Decapsulated testes from mature and immature rats were superfused with mixtures of [^{3}H]-testosterone and [^{14}C]-androstenedione. The steady state concentrations of the labeled hormones in superfusate (c.p.m./ml) and in tissue (c.p.m./g) were measured. Isotope ratios in intracellular metabolities were also determined. The levels (ng/g) of testosterone (T) in tissue superfused in parallel, under identical conditions and at the same concentrations of T and androstenedione (A) in the medium, were measured by radioimmunoassay. These data were used to estimate rates of entry, interconversion, metabolism, and release of T and A, as well as rates at which endogenous T was synthesized *de novo* and secreted during the superfusions.

Endogenously formed T and A were found to be preferentially secreted as T by mature testes; a much lower T/A secretion ratio was seen with immature tissue. The conversion factor of T to A is larger in mature than in immature testes as a consequence of 5α reductase acting directly on T, as revealed by the similarity of the isotope ratios in intracellular T, 5α dihydrotestosterone and 3α, 17β androstanediol. The intracellular T/A ratio derived from either of these hormones was always greater than 1. The rate of production (ng/h × g) of T in superfused testes was not very much higher in mature than in immature rats; much greater differences were noted, however, in the rates of secretion of the hormone.

INTRODUCTION

Most of the available factual information and concepts on steroidogenesis in the rat testes derive from *in vitro* studies in which labeled steroidal precursors were used. Recent experiments have dealt with the metabolism of progesterone (P) and testosterone (T) by testicular tissue taken from prepubertal and adult rats[1–9]. Labeled metabolites formed during incubations of whole testis, as well as of seminiferous tubules or preparations rich in Leydig cells, have been described [10–15]. The studies have shown a high testosterone 5α reductase activity reaching a maximum at about day 20 and declining to very low levels after puberty. On the basis of these observations, the concept that testicular levels of testosterone and rates of secretion of the hormone can be regulated by the activity of its metabolizing enzymes has been put forward[4, 6, 7, 13, 16].

The *in vitro* studies with rat testis that we are now reporting were motivated by our belief that the tracer superfusion method, developed to study the dynamics of estrogen and progestagens in human endometrium [17–19], could yield useful quantitative information when applied to a steroidogenic system. In particular, we hoped to be able to contribute to the elucidation of changes in rates of synthesis and metabolism that lead to the increase in the ratio of secreted testosterone to androstenedione (A) which has been reported to occur after puberty[20, 21].

The reasons to consider that constant flow superfusion of testicular tissue with a mixture of labeled T and A could yield new information include the following.

Steady state isotopic data obtained during superfusion of tissue with two metabolically related tracers, one labeled with ^{3}H and other with ^{14}C, allow the calculation of a variety of parameters describing the dynamics of the hormone[17, 18] which are not measurable by any other current procedures.

Rates of release of hormones from the cells, e.g., secretory processes, can be calculated from the isotopic data. In contrast to batch incubations in which net uptake or utilization of the substrate is measured, the rate of entry of the hormone into cells and of its release to the medium can be estimated from tracer superfusion experiments.

The application of this method allows the study of the relative importance of branched pathways. For

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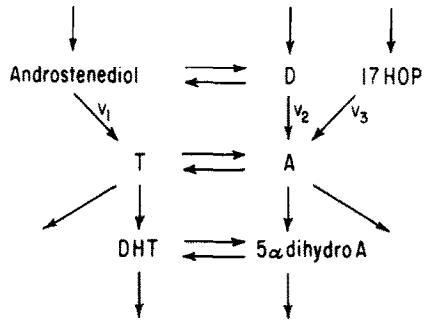

Fig. 1. Partial biosynthetic scheme illustrating the multiple immediate precursors of testosterone and 5α dihydrotestosterone. The rates v_1 and $(v_2 + v_3)$ shown in the figure can be estimated from measurements of the specific activities of intracellular T and A and the concentrations of labeled T and A in superfusion medium and superfusate, at the steady state during superfusion of tissue with a mixture of [^{3}H]- T and [^{14}C]-A.

instance, as shown in Fig. 1, T may be formed by oxidation of androstenediol or by reduction of A. Similarly, A may be directly derived from T, from dehydroisoandrosterone (D), or from 17 hydroxyprogesterone (17 HOP). If the steady state specific activities of T and A are measured in the superfused tissue, the rates v_1 and $(v_2 + v_3)$ shown in Fig. 1, can be estimated. Furthermore, the relative contribution of T and A to any of their metabolites can be calculated from the steady state specific activities of T and A and the isotope ratio in the metabolite, as indicated in Fig. 2[22].

The results shown in this report were obtained by superfusing a mixture of [^{3}H]-T and [^{14}C]-A over decapsulated rat testis and measuring the concentrations of labeled T and A in tissue and superfusate as well as the isotope ratios in 5α dihydrotestosterone (DHT) and 3α, 17β androstanediol isolated from the tissue. Some of the calculations include data on the levels of T measured in another portion of tissue, superfused in parallel with unlabeled steroids.

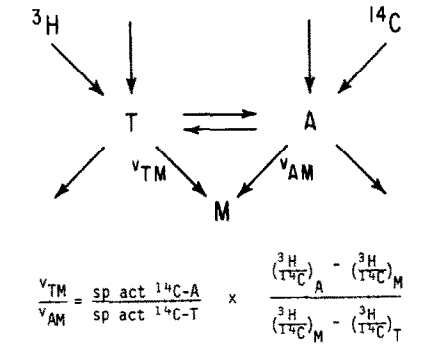

Fig. 2. Scheme indicating that T and A are the sole precursors of a metabolite M. The relative contribution of the rates v_{TM} and v_{AM} to the formation of M, can be estimated from measurements of steady state specific activities of intracellular T and A and isotope ratios in M isolated from the superfused tissue.

EXPERIMENTAL

[1,2-^{3}H]-testosterone (45 Ci/mmole) and [4-^{14}C]-androstenedione (57·5 mCi/mmole) were purchased from New England Nuclear Corporation (Boston, Mass.). Non-radioactive T, A, DHT and 3α,17β-androstanediol were supplied by Steraloids, Inc. (Pawling, N.Y.).

The tracers were purified by paper chromatography, using the system hexane–90% aq methanol, before use.

The superfusion chamber is shown in Fig. 3. It consists of a section of a chromatography column of the type used for gel filtration (Chromatronix Inc., Berkeley, Calif.) to which a sintered glass disk of coarse porosity has been fitted[18]. The capillary glass tube leads to a glass syringe containing a Krebs-Ringer bicarbonate buffer solution of the tracers to which glucose (1 mg/ml) and excess 95% O_2–5% CO_2 have been added. The Teflon outlet tubing leads to a collecting test tube

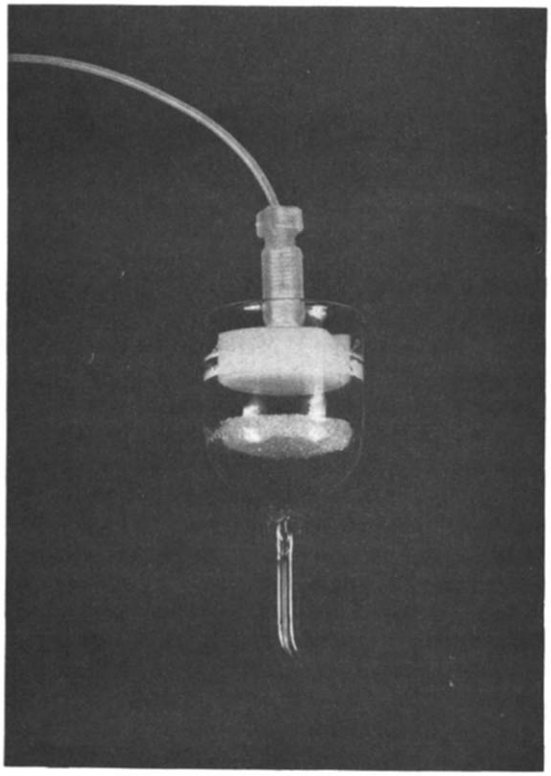

Fig. 3. Superfusion chamber. Support for the tissue is provided by a sintered glass disk (coarse) fitted to a short section of a chromatography column: the outlet consists of a plunger to which a small diameter Teflon tubing is attached (Chromatronix Inc., Berkeley, Calif.).

immersed in a chilling solution. The chamber is held inside a water bath maintained at 37°C.

The testes are removed from ether anesthesized Sprague–Dawley rat, the capsule is slit and a portion of the contents (approximately 150 mg) is weighed and transferred to the superfusion chamber.

The buffer solution is forced through the chamber at a rate of 20 ml/h using a pump (Sage Instruments, Cambridge, Mass., Model 352) which drives the piston of the glass syringe. The superfusion is continued for 2 h, collecting separately fractions of superfusate every 20 min. When the superfusion is stopped, the tissue is rapidly removed from the chamber, placed on a Miracloth filter (Chicopee Mills, N.Y. City) and rapidly washed with cold buffer under suction (all labeled medium is removed within the first minute). The washed tissue is immediately transferred to a glass homogenizer which contains a methanolic solution of unlabeled T and A (200 μg each). After homogenization, the precipitated protein is separated by centrifugation for eventual measurement by colorimetry using Folin's reagent[23]. The supernatant is taken to dryness and chromatographed on Silica Gel GF (Analtech, Inc., Newark, Del.) thin layer plates using the system chloroform : acetone : hexane (4 : 1 : 3). The bands corresponding to T and A are localized under ultraviolet light, eluted and chromatographed separately on paper using the hexane–90% aq. methanol system. The specific activities of T and A are measured using a Beckman DU-2 spectrophotometer and a Nuclear Chicago Isocap 300 liquid scintillation spectrometer. Some of the purified hormones are crystallized from ethyl acetate-hexane after adding 3 mg of authentic carrier. The specific activity of the twice-crystallized material is used to evaluate the radiochemical homogeneity of the eluates from the paper chromatogram. The metabolites DHT and 3α,17β androstanediol, when detected by scanning the TLC plates for radioactivity (Radiochromatogram Scanner, Packard Instruments), are eluted separately from the plates and mixed with the corresponding crystalline compound. The mixtures are then recrystallized to constant $^3H/^{14}C$ ratios.

The last two superfusate samples and a sample of the superfusion medium remaining in the syringe are similarly analyzed. A known volume of superfusate is extracted with ethyl acetate containing T and A carriers. After evaporation of the solvent, the residue is worked up as indicated above.

Testosterone levels were measured by radioimmunoassay in another aliquot of the same tissue superfused under identical conditions and same concentrations of unlabeled T and A. The antiserum prepared by immunizing rabbits against testosterone-3-carbomethyloxime conjugated to BSA was kindly made available to us by L. Speroff and B. Caldwell, Yale University.

RESULTS

Metabolic changes at puberty

Typical experimental data obtained by superfusion of testicular tissue from immature and mature rats are shown in Table 1.

These data are used to calculate the parameters shown in Fig. 4 according to formulae previously presented[17, 18]. The values obtained from these calculations, listed in Table 2, are representative of the results obtained in similar experiments. Some of the conclusions about the dynamics of metabolism and secretion of T and A that can be drawn from these values are the following.

The increased T/A secretion ratio observed after puberty is reflected in the pattern of release of labeled hormones to the medium. Thus, the ratio of the amounts of superfused [^{3}H]-released either as [^{3}H]-T (β_T) and as [^{3}H]-A (γ_{TA}) is higher in mature than in immature testicular tissue.

Table 1. Concentrations of labeled compounds (c.p.m./ml or c.p.m./g)

	Immature			Mature		
	^3H	^{14}C	$^3H/^{14}C$	^3H	^{14}C	$^3H/^{14}C$
Superfusion medium						
T	112000	–	4·0	114000	–	4·2
A	–	27800			27000	
Superfusate (100–120 min)						
T	89400	1330	67	103000	2640	39
A	5000	25000	0·20	4340	21700	0·20
Tissue						
T	190000	41300	4·6	428000	83500	5·1
A	32500	23900	1·4	61800	25700	2·4
DHT	–	–	4·3	–	–	–
3α, 17β Androstanediol	–	–	4·8	–	–	–

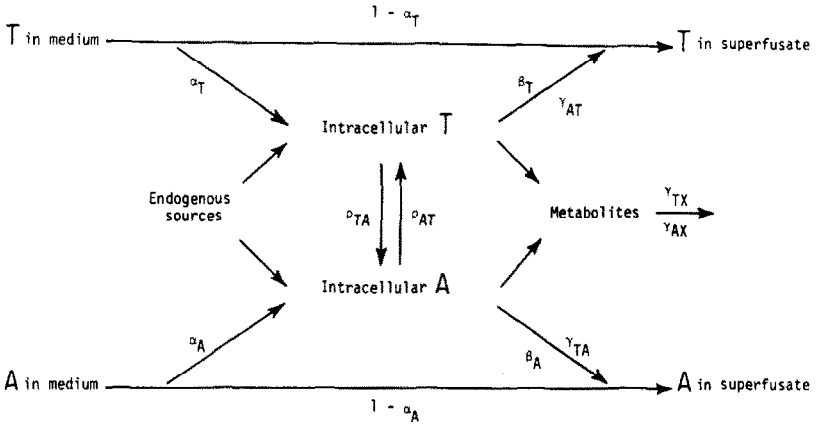

Fig. 4. Diagram representing the fate of the superfused tracers. A fraction of each tracer (α_T and α_A) enters the cells and the remainder (1-α_T, 1-α_A) bypasses the tissue. The intracellular labeled hormones are released back to the medium (fractions β_T and β_A) or leave the tissue after conversion to the other compounds (fractions γ_{TA} and γ_{AT}) or to metabolites (fractions γ_{TX} and γ_{AX}). T and A isolated from the superfusate are doubly labeled. The values of α's, β's, ρ's and γ's can be estimated from the steady state concentrations of the labeled hormones in the superfusion medium, in the superfusate and in the tissue[17, 18].

$$\left(\frac{\beta_T}{\gamma_{TA}}\right)_{mat} = 3{\cdot}2 > \left(\frac{\beta_T}{\gamma_{TA}}\right)_{immat} = 1{\cdot}4 \qquad \left(\frac{\gamma_{AT}}{\beta_A}\right)_{mat} = 1{\cdot}3 > \left(\frac{\gamma_{AT}}{\beta_A}\right)_{immat} = 0{\cdot}4.$$

Furthermore, the ratio of the amounts of superfused [^{14}C]-A released to the medium as [^{14}C]-T (γ_{AT}) and as [^{14}C]-A (β_A) is higher in mature testes:

As pointed out before, the change in the pattern of secretion which occurs after puberty can result from a decline in the 5α reductase activity. We were able to

Table 2. Calculated parameters

Fraction	Symbol	Values Immature	Mature
Medium to tissue (entry)			
T	α_T	0·26	0·22
A	α_A	0·23	0·26
Tissue to medium (secretion)			
T → T	β_T/α_T	0·21	0·55
T → A	γ_{TA}/α_T	0·15	0·17
T → X (all other metabolites)	γ_{TX}/α_T	0·64	0·27
A → A	β_A/α_A	0·57	0·27
A → T	γ_{AT}/α_A	0·22	0·38
A → X (all other metabolites)	γ_{AX}/α_A	0·23	0·35
Intracellular			
T → A	ρ_{TA}	0·3	0·7
A → T	ρ_{AT}	0·9	0·7
Isotope distribution			
From superfused T:			
$\frac{\text{T in tissue (c.p.m./g)}}{\text{T in medium (c.p.m./ml)}}$	$\left(\frac{\text{Tiss}}{\text{Med}}\right)_T$	1·7	3·7
$\frac{\text{T in tissue}}{\text{A in tissue}}$	$(T/A)_T$	5·8	6·9
From superfused A:			
$\frac{\text{A in tissue (c.p.m./g)}}{\text{A in medium (c.p.m./ml)}}$	$\left(\frac{\text{Tiss}}{\text{Med}}\right)_A$	1·2	0·9
$\frac{\text{T in tissue}}{\text{A in tissue}}$	$(T/A)_A$	1·7	3·2

confirm this decline by direct measurement of enzymatic activity in testicular homogenates using [^{14}C]-T as substrate. The high 5α reductase activity in immature testes is also clearly evident from the superfusion isotopic data. That T, and not A, is the direct substrate for the reduction can be concluded from the similarity of the $^{3}H/^{14}C$ ratios of T, DHT and 3α,17β androstanediol isolated from the superfused tissue (Table 1). The ratios found in these compounds and in A were definitely different.

The high rate of metabolism of T to compounds other than A prior to puberty is also evident from several other parameters calculated from isotopic data. For instance, the fraction of T converted to A (ρ_{TA}) is smaller in the immature than in the mature rat:

$$(\rho_{TA})_{immat} = 0{\cdot}3 < (\rho_{TA})_{mat} = 0{\cdot}7.$$

Note from the data in Table 1 that the intracellular concentration of T derived from the superfused hormone is smaller in the immature rat. Furthermore, the T/A ratio of concentrations of the labeled hormones in tissue, either derived from superfused T or A, is smaller in immature than in mature testes. These ratios describe the contribution of newly synthesized T and A to each of the two intracellular pools.

Another parameter reflecting the extent of metabolism of T is the fraction of superfused T converted to metabolites other than A (γ_{TX}). This fraction declines after puberty (Table 2). As already mentioned, the conversion of T to the metabolites X (γ_{TX}) can result from enzymatic actions on T (γ_{TTX}) or on A (γ_{TAX}). The relative importance of these pathways of metabolism of T can be estimated by solving the following intuitively evident system of equations:

$$\gamma_{TX} = \gamma_{TTX} + \gamma_{TAX}$$

$$\gamma_{TTX}/\alpha_T = \gamma_{ATX}/(\rho_{AT}\alpha_A)$$

$$\gamma_{AX} = \gamma_{ATX} + \gamma_{AAX}$$

$$\gamma_{AAX}/\alpha_A = \gamma_{TAX}/(\rho_{TA}\alpha_T)$$

Application of these equations to interpret the data in Table 2 indicates that practically all the metabolism of T occurs via T in the immature rat testis while in mature tissue T is mostly converted to other products through the intermediacy of A.

Note also that the fraction of superfused A metabolized to products other than T (γ_{AX}) is larger in mature than in immature animals. Consistent with this observation is the increase in the net uptake of A ($\alpha_A - \beta_A$) which is observed with testicular tissue from rats which have passed puberty:

$$(\alpha_A - \beta_A)_{mat} = 0{\cdot}19 > (\alpha_A - \beta_A)_{immat} = 0{\cdot}10$$

The net uptake of A is even larger than the net uptake of T ($\alpha_T - \beta_T$) in mature rats even though the opposite was true in immature testes:

$$(\alpha_A - \beta_A)_{mat} = 0{\cdot}19 > (\alpha_T - \beta_T)_{mat} = 0{\cdot}09$$

$$(\alpha_A - \beta_A)_{immat} = 0{\cdot}10 < (\alpha_T - \beta_T)_{immat} = 0{\cdot}21$$

Also note that the fraction of A released to the medium (β_A/α_A) declines after puberty (Table 2).

Rates of de novo synthesis of testosterone

Table 3 shows the results of calculations based on the intracellular levels of T found in superfused mature and immature tissue. By subtracting from the total concentration of T (c_T) the portion derived from superfused T and A ($c_{T,exo}$) the levels of T due to endogenous synthesis ($c_{T,endo}$) can be estimated. The concentration $c_{T,exo}$ is calculated as follows:

$$c_{T,exo} = \frac{c_T^{3H} \text{ in tissue}}{\text{sp. act } [^3H]\text{-T superfused}} + \frac{c_T^{14C} \text{ in tissue}}{\text{sp. act } [^{14}C]\text{-T superfused}}$$

The intracellular clearance of T (IC_T) which corresponds to the ratio of the rate of *de novo* production of T per gram of tissue and the concentration resulting

Table 3. Rates of "*de novo*" synthesis and secretion of T

Parameter	Estimation	Values Immature	Mature
Intracellular concentration			
after superfusion (ng/g)	c_T by RIA	126	287
from superfused T and A	$c_{T,exo}$ from isotopic data	96	171
from endogenous sources	$c_{T,endo} = c_T - c_{T,exo}$	30	116
Intracellular clearance (h^{-1})	$IC_T = \frac{\phi\alpha_T}{W(Tiss/Med)_T}$	19	7·7
Endogenous rate of production (ng/g × h)	$PR_{T,endo} = IC_T \times c_{T,endo}$	570	890
Endogenous secretion rate (ng/g × h)	$SR_{T,endo} = PR_{T,endo}\frac{\beta_T}{\alpha_T}$	120	490
(ng/testis × h)		30	1470

from that production at the steady state[18], is shown in the fourth line of Table 3. As a result of the extensive 5α reduction of T in immature testes, IC_T is larger before puberty.

The product of the intracellular clearance and the concentration of T from endogenous origin indicates the rate at which T is synthesized *de novo* by the superfused tissue. This rate of endogenous production does not distinguish between the various sources of the hormone. Only a fraction of the T formed is released to the medium as T. Isotopic data obtained by superfusing labeled T and A reveals the value of this fraction (β_T/α_T). Therefore, the rate of secretion of T during the superfusion, expressed as ng of T/h and per g tissue or per testis, can be estimated. Note that the large increase in the output of T after puberty is not due to a large increase in the rate of synthesis of the hormone per unit of tissue weight but rather to an increase in the fraction released to the medium and, of course, to the augmentation of the testicular weight.

DISCUSSION

An obvious extension of this work requires the separation and testing of the various cell types present in the testis. Leydig cells, which contribute only about 6% of the testicular mass[10], are considered to be the main elements involved in the synthesis of androgens from cholesterol. Although Leydig cell-enriched fractions can be easily obtained mechanically[10] or by collagenase treatment[15], better methods are needed to obtain homogeneous cell preparations in good yield.

Superfusion of seminiferous tubules from one immature and one mature rat, prepared by dissection in the presence of collagenase, showed extensive interconversion between T and A and the presence of 5α reductase before puberty. As seen when whole testes were superfused, 3α,17β androstanediol and DHT were present in the tissue from the immature rat and had similar $^3H/^{14}C$ ratio as T. These preliminary experiments indicate that the tubules concentrate T, but not A, to a much larger extent than whole testicular tissue does. Also in both immature and mature rats, strikingly smaller fractions of T and A are released back to the medium by tubules than by whole testis preparations. It can then be expected that the rates of release of T and A by Leydig cells may be higher than the values estimated by superfusion of whole testes. Work in progress in our laboratories aims to investigate the synthesis of T in the tubules from endogenous precursors[10, 24] by measuring the specific activity of the hormone in tissue at the steady state. Simultaneous determinations of the specific activities of A will yield further information on the sources of the hormone, both in tubules and in whole testes.

The anatomical relationship between Leydig cells and tubules necessarily affect the output of the hormone since the secretory product of the interstitial cells can be picked up and metabolized by the Sertoli and germ cells. The existence of this situation must be considered in the interpretation of results obtained during incubation of whole testes since the binding of the steroids secreted by the Leydig cells can be affected by the composition of the extracellular fluid.

It should be emphasized that the technical difficulties or effort involved in tracer studies by superfusion or in batch-incubations in which tissue and medium are analyzed separately, are essentially equal. The superfusion apparatus is very simple and the calculations are trivial. We hope to have shown that tracer superfusion experiments can yield greater quantitative information than usual batch-incubations.

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DISCUSSION

Lindner:

How can you be sure that the change in the ratio of androstenedione to testosterone at puberty is due to changes in the 5α-reductase activity, since this enzyme really accepts both androstenedione and testosterone as a substrate? Couldn't a change in the 17β-dehydrogenase activity be responsible? I'm aware there is a marked change also in the reductase activity, but I don't quite see how this explains the change-over in the ratio between androstenedione and testosterone. Do you know where this reductase is located? If it is mainly located in the tubular tissue, then again it may not explain the change-over you get in the relative output of the two steroids into the spermatic vein blood.

Gurpide:

Since we have observed extensive interconversion between testosterone and androsterone both in mature and immature testes, we believe that this enzymatic activity is not the critical factor in determining the ratio of the secreted androgens. On the other hand, the identity of the isotope ratios in testosterone and the 5α reduced, 17β-hydroxy metabolites is experimental proof that testosterone is the preferred substrate for the 5α reductase. Other studies showing that labeled androstenedione can also be converted to androstanediol do not reveal the pathway by which the conversion occurs.

Isotopic data from superfusion experiments indicate that androstenedione is also metabolized without the intermediary of testosterone, particularly in the mature rat testes, but we have not searched for those metabolites as yet.

Reduction of testosterone in the tubules can be expected to affect the testicular output of the hormone produced in the Leydig cells. As I already mentioned, the isotope ratio in 5α-androstenediol isolated from tubules was very different from the ratio in androstenedione, but equal to the ratio in testosterone.

Cooke:

We've been very much concerned with the amount of testosterone which is formed by interstitial tissue isolated by wet dissection compared with the interstitial tissue in the intact total testis. The Figure 1 shows the formation of cyclic AMP and testosterone in the medium plus tissue in various testis preparations. The results are calculated per mg of interstitial tissue protein which is present. The formation of cAMP is the same in all the tissues containing interstitial tissue but there is a loss in total capacity of the interstitial tissue to produce testosterone. If seminiferous tubules are added to form a "reconstituted testis", then it does not make any difference in the total testosterone that is produced. So it seems that when you isolate the interstitial tissue by wet dissection you do lose some capacity to produce testosterone. This may be caused by cell damage.

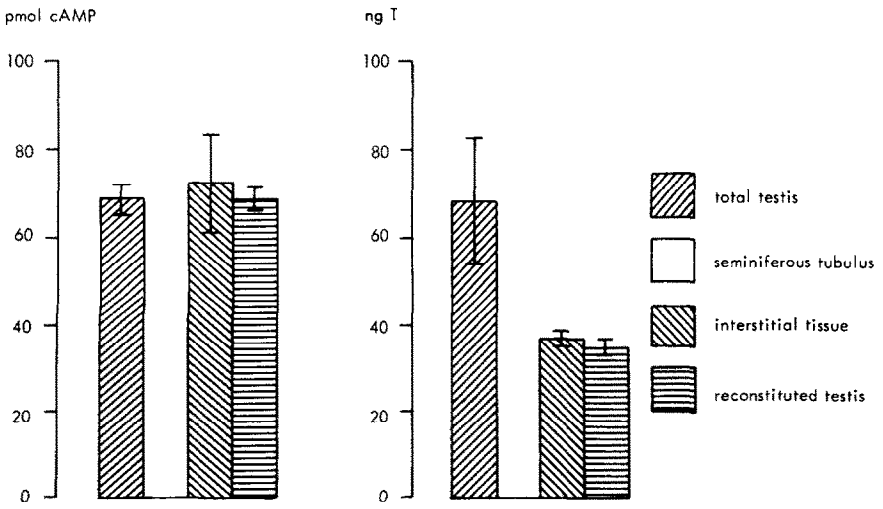

Fig. 1. (Cooke).