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Effect of Heat Acclimatization on Testicular Enzymes Involved in Androgen Biosynthesis via the 5-ene Pathway

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Abstract

The metabolism of 3β-hydroxy-5-ene steroids by testicular homogenates of heat-acclimatized and control mice was investigated *in vitro*. Acclimatization was achieved by keeping the animals in a hot room (33–35°C, 25–40% R.H.) for 5 weeks. The control animals were kept in a temperate environment (20–22°C, 30–50% R.H.). Some of the heat-acclimatized animals were supplied with additional water in a trough placed inside each cage (HAII mice). This source of water was used by the mice mainly for body cooling. A pronounced decrease in body weight and testis weight, and a smaller decrease in the weight of seminal vesicles which was associated with atrophy of the seminiferous tubules and hyperplasia of the Leydig cells was characteristic of heat-acclimatized mice with only drinking water available (HAI), but not of HAII mice. Although body cooling abolished the adverse physiological response described, it did not prevent specific changes in enzyme activity associated with androgen production. There was increased activity of 3β-hydroxysteroid dehydrogenase and isomerase, and of 5-ene-17β-hydroxysteroid dehydrogenase. These data offer partial explanation for the lower peripheral blood testosterone level observed in some species of heat-acclimatized mammals.

Introduction

Testicular function changes adversely in mammals exposed to an ambient temperature higher than that of the scrotum. The most pronounced effects—arrested spermatogenesis, severe hypogonadism and decrease in weight and secretion of accessory reproductive organs (Clegg 1965)—occur in cryptorchid animals. These phenomena are characterized by a decrease in the testicular function involving testosterone† secretion (Eik-Nes 1966; Amatayakul *et al.* 1971). These observations are supported by the *in vitro* studies of Llaurado and Dominguez (1963) and Inano and Tamaoki (1968) who demonstrated a significant decrease in testosterone production via the 4-ene and 5-ene pathways, respectively.

In rats and mice continuous exposure of the intact animal to a high environmental temperature results in decreased body, testis and seminal vesicle weight (Bedrak *et al.* 1971*a*, 1971*b*, 1973; Slonim and Bedrak 1974) and in increased scrotal temperature (Sod-Moriah *et al.* 1974). Since body temperature also rises, the gradient of temperature between body and scrotum is maintained.

Generally, the activity of most of the testicular enzymes involved in the metabolism of pregnenolone via the 4-ene and 5-ene routes is enhanced, except for that of 4-ene-17β-hydroxysteroid dehydrogenase (EC 1.1.1.64) (Bedrak *et al.* 1973). Histological sections indicate that male mice are probably more sensitive to high environmental temperature than male rats—exposure of male mice to a hot environment (35°C) for

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† See p. 92 for nomenclature of steroids.

5 weeks has been found to result in total atrophy of the seminiferous tubules and inhibition of spermatogenesis (Slonim and Bedrak 1974); on the other hand, only small changes of this kind are noted in male rats exposed to a high environmental temperature, and their reproductive capacity is not critically affected (Bedrak *et al.* 1973; Sod-Moriah *et al.* 1974).

The present experiments were designed to study the effects of continuous exposure of the intact animal to an ambient temperature of 35°C for 5 weeks on the *in vitro* metabolism of 3β-hydroxy-5-ene steroids in the testes of mice. The data presented herein suggest that acclimatization of male mice to a hot environment is coupled with a significant rise in activity of 3β-hydroxysteriod dehydrogenase and isomerase resulting in increased production of androgens via the 4-ene pathway.

Materials and Methods

Animals

Newly weaned male mice (C57/BL/6, Weizmann Institute strain) were kept in a control (C) environment (20–22°C, 30–50% R.H.) up to the age of 8 weeks, and then randomly divided into four experimental groups. Two groups were kept in the C environment. The other two groups were heat acclimatized (HA) by being kept in a hot room (33–35°C, 25–40% R.H.) for 5 weeks. Tap water and rat chow were freely available to all mice throughout the experiment. Lighting regimen consisted of 14 h daylight and 10 h darkness. The water supply of one HA group and one C group was augmented by troughs of water placed inside their cages. The groups without supplementary water supply are designated as CI and HAI, and those with supplementary water supply as CII and HAII. The HAII animals were observed to use their supplementary water for cooling by squatting against the troughs with their tails dipped in the water. All groups of animals were weighed each week during the experiment.

Preparation of Tissues

The animals were killed by severing the spinal cord. The testes were removed immediately and placed in ice-cold 0·25 M sucrose. Generally 10 animals from each C group and a greater number of HA animals were used in each experiment. (The number of animals killed depended on testis weight: the heavier the animal, the heavier the testes and, consequently, the fewer the number of animals killed). When an adequate quantity (2–3 g) of testicular tissue had been obtained, the testes were weighed (as a group), then stripped of their tunica, and used for preparation of 5% (w/v) homogenates. The homogenates were centrifuged at 800 g, and the supernatant was used in the experiments. The seminal vesicles were emptied of their content and weighed (as a group).

Nomenclature of Steroids

Trivial and systematic names used in this paper are shown in the following tabulation:

Trivial name	Systematic name
androstenediol	androst-5-ene-3β,17β-diol
androstenedione	androst-4-ene-3,17-dione
dehydroepiandrosterone	3β-hydroxyandrost-5-en-17-one
deoxycorticosterone	21-hydroxypregn-4-ene-3,20-dione
dihydroxyprogesterone	17α,20α-dihydroxypregn-4-en-3-one
hydroxypregnenolone	3β,17α-dihydroxypregn-5-en-20-one
hydroxyprogesterone	17α-hydroxypregn-4-ene-3,20-dione
pregnenolone	3β-hydroxypregn-5-en-20-one
progesterone	pregn-4-ene-3,20-dione
testosterone	17β-hydroxyandrost-4-en-3-one

Radioactive Steroids and Incubation Procedures

The radioactive steroids (obtained from the Radiochemical Centre, Amersham, England) were checked for purity by thin layer chromatography in benzene–acetone (4 : 1 v/v). [^{14}C]Androstenediol

was prepared by reduction of [^{14}C]dehydroepiandrosterone with sodium borohydride in darkness for 4 h. The reduction was stopped by addition of a few drops of acetic acid dissolved in H_2O; [^{14}C]androstenediol was then extracted four times in dichloromethane and purified by chromatography in hexane–benzene (1 : 1 v/v)–formamide. The ^{14}C-labelled compounds used as substrates were diluted with corresponding non-radioactive steroids (Ikapharm Ramat Gan, Israel) to a specific activity of 5·0 μCi/μmol and the ^{3}H-labelled compound to a specific activity of 25 μCi/μmol. Details of the procedure and conditions of the incubation have been reported previously (Bedrak 1974). The time of incubation varied and is indicated in the text.

Extraction, Isolation and Identification of Products

At the end of incubations, the mixtures were acidified with 0·05 ml of 1 M HCl, the tubes were placed in an ice bath and non-radioactive steroids (reference standards), at the level of 50–100 μg, were added as described in Table 1. The contents of the tubes were extracted four times with 5 ml of ether–chloroform (4 : 1 v/v). Recovery at this stage was more than 90%.

Table 1. Reference standards added at the end of incubation

1, pregnenolone; 2, hydroxypregnenolone; 3, dehydroepiandrosterone; 4, androstenediol; 5, progesterone; 6, hydroxyprogesterone; 7, androstenedione; 8, testosterone. +, Addition of the steroid to the tube

Substrate	Reference standard (50–100 μg/tube)							
	1	2	3	4	5	6	7	8
[^{14}C]Pregnenolone	+	+	+	+	+	+	+	+
[^{3}H]Hydroxypregnenolone		+	+	+		+	+	+
[^{14}C]Dehydroepiandrosterone			+	+			+	+
[^{14}C]Androstenediol				+				+

The extracts of [^{14}C]pregnenolone and [^{14}C]dehydroepiandrosterone incubations were chromatographed first by thin layer chromatography (silica gel GF_{254}, Merck) in benzene–acetone (4 : 1 v/v). After development, steroids of the 4-ene configuration were located by viewing the chromatogram under a u.v. lamp, and those of the 5-ene configuration by staining with iodine. The plates were scanned through a windowless radiochromatogram scanner. The detected spots were scraped off the thin layer plates and eluted three times with 3 ml methanol. The extracts of [^{3}H]hydroxypregnenolone incubations were first chromatographed on Whatman No. 1 chromatography paper in hexane–formamide, followed by hexane–benzene (1 : 1 v/v)–formamide, according to Zaffaroni and Burton (1951). After location of the reference standards and scanning, the radioactive areas were eluted twice with 10 ml methanol. The various metabolites contained in the extracts were separated by various chromatographic procedured. Those metabolites having the same R_F values in the various chromatographic systems employed were separated by formation of acetates, followed by chromatography (Dominguez *et al.* 1963). The steroids were finally identified by recrystallization to a constant specific activity with 20–30 mg of authentic non-radioactive steroids from different solvents (Axelrod *et al.* 1965). Representative data are shown in Table 2. Protein was determined by the method of Lowry *et al.* (1951). Yields of individual compounds were calculated as described previously (Bedrak and Samuels 1969). Enzymic activity was estimated by the amount and nature of the end product formed upon incubation of individual substrates with appropriate cofactors. Under these conditions, where more than 50% of the substrate remained unmetabolized, the activity calculated was shown to be appropriate (Samuels *et al.* 1964).

Histological Examination

Specimens of testes were fixed in Bouin solution and sections 4 μm thick were cut. The sections were stained with haematoxylin, eosin and light green.

Results

Effects of Exposure to 35°C on Growth Rate, Testis and Seminal Vesicle Weight and Protein Content

The growth rates of control and heat-acclimatized mice are shown in Fig. 1. Since the animals were weighed by group, the mean value for each group is shown. In mice with no supplementary water supply, body weight decreased sharply during the first 2 weeks of exposure to the hot environment. After the third week, however, it

Table 2. Recrystallization of steroids to constant specific activity (dpm/mg)

Steroid	Original solution	No. of crystallizations				
		1	2	3	4	5
Pregnenolone-3-acetate	1181	958	950	942	972	957
Hydroxypregnenolone-3-acetate	624	595	704	615	619	689
Dehydroepiandrosterone-acetate	1584	1438	1426	1405	1384	1378
Androstenediol-diacetate		378	389	362	372	361
Progesterone	1262	1221	1297	1259	1266	1256
Hydroxyprogesterone	720	698	682	700	708	690
Androstenedione	1206	1218	1188	1156	1178	1164
Testosterone-acetate	915	894	845	908	874	887

increased in a manner similar to that of the CI group, but did not reach the level of the CI animals during the 5 weeks of the experiment (Fig. 1*a*). The decrease in body weight of the HAI group was accompanied initially by a high level of mortality—30% up to the end of the second week. Deaths continued, although at a much lower rate,

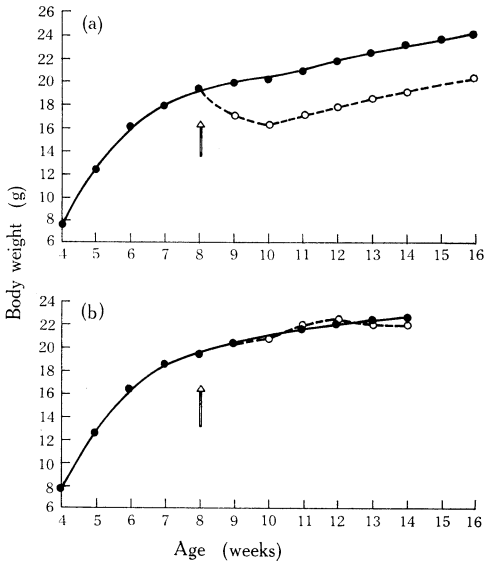


Fig. 1. Growth of male mice kept in the control (●) and the hot (○) environments for 5 weeks. (*a*) Mice without augmented water supply inside the cages (groups CI, HAI). (*b*) Mice with augmented water supply (groups CII, HAII). Mean of 30–50 animals per group. Arrows point to the beginning of exposure of the HA animals to the hot environment.

during the remaining weeks of the experiment. Decrease in body weight and mortality did not occur in the group given supplementary water (HAII, Fig. 1*b*). Table 3 shows body weight, testis and seminal vesicle weight, and protein content of testicular homogenates for the four groups of mice. HAI animals had a lower body weight

compared to the CI animals, a marked decrease (78·3%) in testis weight and a small decrease (20·0%) in seminal vesicle weight. When supplementary water was available, testis weight was the same in both CII and HAII animals (0·86% of body weight) although there was a decrease (10·0%) in seminal vesicle weight. Only small changes occurred in the protein content of testicular homogenates in heat-acclimatized animals compared to control animals.

Table 3. Body weight, testis and seminal vesicle weights, and protein content of testicular homogenates

Since animals and tissues were weighed by group, mean values are recorded. No statistics are available. Protein content was determined using 5% testicular homogenates (see Methods)

Group	Body weight (g)	Testis weight (% of body wt)	Seminal vesicle weight[A] (% of body wt)	Protein content (mg/0·5 ml)
CI	22·4	0·83	0·20	1·19
HAI	18·5	0·18	0·16	1·22
CII	22·1	0·86	0·30	1·24
HAII	21·8	0·86	0·27	1·20

[A] Seminal vesicles were emptied prior to weighing.

Metablism of [^{14}C]Pregnenolone by Testicular Homogenates of CI and HAI Mice

The utilization of pregnenolone by testicular preparations of the HAI mice was higher than that of the CI mice. The percentages of unmetabolized substrate during 20 min incubation were 40·5 and 63·0% for the HAI and CI groups respectively

Table 4. Metabolism of [^{14}C]pregnenolone by testicular homogenates

The substrate was incubated for 20 min in the 800-*g* supernatant of 5% testicular homogenates with NADPH generating system. Values given are means of two experiments

Steroid	Amount formed (nmol/mg protein) in group:			
	CI	HAI	CII	HAII
Pregnenolone[A]	(63·0%)	(40·5%)	(66·9%)	(65·6%)
Dehydroepiandrosterone	0·34	0·51	0·09	0·03
Androstenediol	0·29	0·15	0·07	—
Progesterone	2·63	4·03	1·36	2·78
Hydroxyprogesterone	0·36	1·68	0·44	0·38
Androstenedione	0·28	0·86	0·19	0·16
Testosterone	2·34	2·51	0·99	0·97
Hydroxypregnenolone			0·68	0·72
A			0·68	0·38
B			0·76	0·35

[A] Values in parentheses are the percentages of unmetabolized substrate.

(Table 4). In both groups the major product formed during incubation was progesterone, although its production was greater in the HAI group. The amounts isolated after 20 min incubation were 4·03 and 2·63 nmol/mg protein in the HAI and CI groups respectively (Table 4). The amounts of hydroxyprogesterone and androstenedione

isolated were greater in the HAI mice, which suggests greater activity of 3β-hydroxysteroid dehydrogenase and isomerase in the HAI group.

Metabolism of 3β-Hydroxy-5-ene Steroids by Testicular Homogenates of CII and HAII Mice

(i) *Metabolism of* [^{14}C]*pregnenolone*

The utilization of pregnenolone was similar in the two groups (Table 4). In both groups, pregnenolone was metabolized to testosterone primarily via the 4-ene route. The amounts of 3-oxo-4-ene steroids (progesterone, hydroxyprogesterone and androstenedione) isolated were larger than the amounts of 3β-hydroxy-5-ene steroids (hydroxypregnenolone, dehydroepiandrosterone and androstenediol) in the two groups. After 20 min incubation the amounts of 3-oxo-4-ene steroids isolated were 1·99 and 3·32 nmol/mg protein in the CII and HAII groups respectively; the amounts of 3β-hydroxy-5-ene steroids were 0·84 and 0·75 nmol/mg protein (Table 4). The relative distribution of the major metabolites isolated differs in these two groups and it is evident that heat acclimatization stimulated the activity of 3β-hydroxysteroid dehydrogenase and isomerase (as judged by the production of progesterone in these groups). However, there were similar amounts of testosterone isolated from the two groups; after 20 min incubation the amounts isolated were 0·99 and 0·97 nmol/mg protein in CII and HAII mice respectively. There is possibly a higher activity of certain enzymes of the 5-ene route in the CII animals. Two unknown compounds (A and B, see Table 4) were isolated by chromatography and acetylated. One of them (A) appeared to be very polar when chromatographed in benzene–ethyl acetate (5 : 1 v/v). The other compound (B) had an R_F value similar to that of deoxycorticosterone, but on crystallization appeared to be a different compound. No further attempts were made to identify these two metabolites.

(ii) *Metabolism of* [^{3}H]*hydroxypregnenolone*

Continuous exposure of mice to heat for 5 weeks stimulated the conversion of hydroxypregnenolone to 3-oxo-4-ene steroids (Table 5). After 40 min incubation the amounts of hydroxyprogesterone isolated were 0·74 and 1·43 nmol/mg protein and the amounts of androstenedione were 0·56 and 1·01 nmol/mg protein in CII and HAII mice respectively. The amounts of testosterone produced after 40 min incubation were 4·70 and 4·11 nmol/mg protein in CII and HAII mice respectively. The amount of dehydroepiandrosterone isolated was about 50% higher in the CII group than in the HAII group.

(ii) *Metabolism of* [^{14}C]*dehydroepiandrosterone*

Table 5 indicates that dehydroepiandrosterone is converted to testosterone primarily via androstenediol. However, the formation of androsterediol from this substrate is enhanced by heat exposure, suggesting increased activity of the 5-ene-17β-hydroxysteroid dehydrogenase.

(iv) *Metabolism of* [^{14}C]*androstenediol*

The rate of mobilization of androstenediol to testosterone was slightly higher in HAII than in CII mice. The amounts of testosterone produced after 30 min incubation

were 5·25 and 4·45 nmol/mg protein in HAII and CII mice respectively. These results suggest greater activity of 3β-hydroxysteroid dehydrogenase and isomerase in heat-acclimatized mice.

(v) *Histological examination*

Histological sections of testes in control mice (Fig. 2*a*) show active spermatogenesis, and Leydig cells are observed in the small interstitial spaces among the seminiferous tubules. Testes from mice exposed for 5 weeks at 35°C (HAI) show marked atrophy of the seminiferous tubules, lack of spermatogenesis and hyperplasia of interstitial cells (Fig. 2*b*). The testes of heat-acclimatized mice with a supplementary water supply (HAII) resemble those of control mice, although the diameter of the tubules is somewhat smaller (Fig. 2*c*).

Table 5. Metabolism of [^{3}H]hydroxypregnenolone and [^{14}C]dehydroepiandrosterone by testicular homogenates

The former substrate was incubated for 40 min and the latter for 15 min in the 800-*g* supernatant of 5% testicular homogenates with NADPH generating system. Values given are means of two experiments

Steroid	Amount of steroid formed (nmol/mg protein) from:			
	[^{3}H]Hydroxypregnenolone		[^{14}C]Dehydroepiandrosterone	
	CII	HAII	CII	HAII
Hydroxypregnenolone[A]	(60·1%)	(60·1%)		
Dehydroepiandrosterone[A]	0·24	0·16	(70·0%)	(63·4%)
Androstenediol	—	—	3·70	5·03
Hydroxyprogesterone	0·74	1·43		
Androstenedione	0·56	1·10	0·32	0·42
Testosterone	4·70	4·11	0·67	0·74

[A] Values in parentheses are the percentages of unmetabolized substrates.

Discussion

The results of these experiments indicate that continuous (5 weeks) exposure to heat of the intact male mouse stimulates the activity of known 3β-hydroxysteroid dehydrogenases and isomerases which catalyse the conversion of 3β-hydroxy 5-ene steroids (pregnenolone, hydroxypregnenolone and androstenediol) to their 3-oxo-4-ene steroidal homologues. The activity of these enzymes increased irrespective of augmentation of the animal's water supply. Nevertheless, those animals with a higher heat load (HAI) utilized more of the substrate for conversion to the 3-oxo-4-ene steroids. This suggests that although the heat load was eased by body cooling effected by the supplementary water supply, it did not change the pattern of enzyme activity induced by the process of heat acclimatization. This pattern is probably related to the hypothalamic–hypophyseal adaptation of the body in adjusting to the new physiological environment to which it is exposed. The 5-ene 17α-hydroxylase and 17,20-lyase activities were not influenced by the process of heat acclimatization. It has been assumed (Hall 1970) that parallel steps in the 5-ene and 4-ene pathways are catalysed

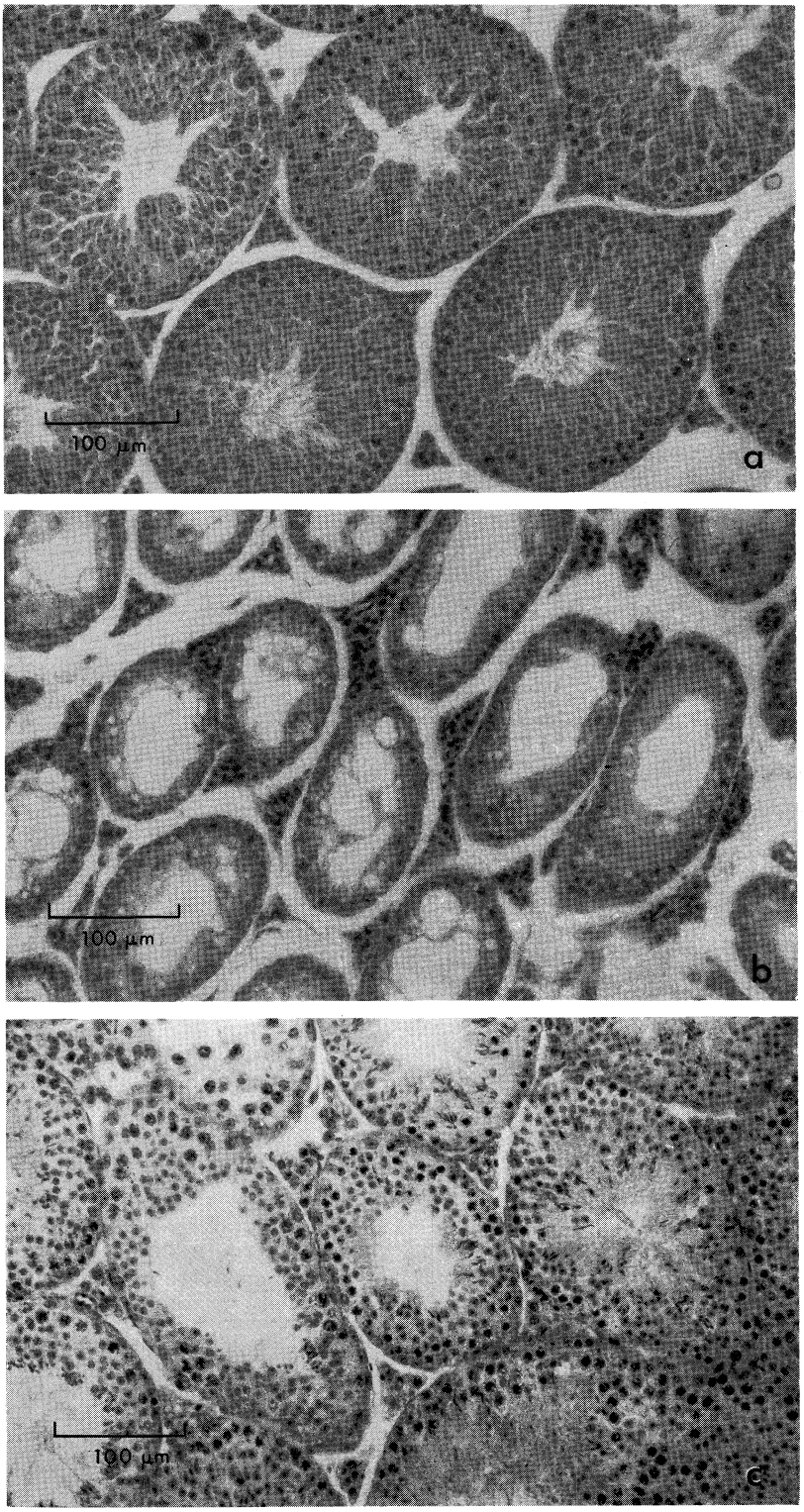
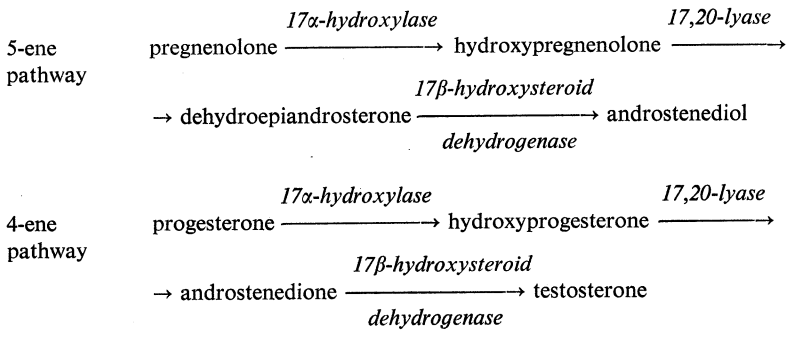


Fig. 2. Light micrographs of testes from (*a*) control, (*b*) HAI, and (*c*) HAII mice.

by the same enzymes, as shown below:



In the present experiments the enzymes responded differently to exposure to heat—heat acclimatization resulted in stimulation of almost all the enzymes associated with the production of testosterone via the 4-ene pathway. The one exception was 17β-hydroxysteroid dehydrogenase, the activity of which decreased sharply (Bedrak *et al.* 1971*a*; Slonim and Bedrak 1974). The difference in enzyme response between the two pathways supports the hypothesis that the enzymes catalysing the reaction steps in the 5-ene pathway are different from those enzymes catalysing the corresponding steps in the 4-ene pathway. The data presented here demonstrate that while heat acclimatization induced an increase in the activity of 5-ene-17β-hydroxysteroid dehydrogenase, it did not affect the activity of 5-ene-17β-hydroxylase or of 5-ene-17,20-lyase.

These findings, together with data presented previously (Bedrak 1974; Slonim and Bedrak 1974), support the hypothesis that under the present experimental conditions testosterone is primarily synthesized via the 4-ene pathway. As reported for the rat (Bedrak *et al.* 1973), the 5-ene route possesses a rate-limiting step—that catalysed by 17,20-lyase. The data presented also partially explain the lower peripheral blood testosterone level observed in heat-acclimatized male rats (Bedrak *et al.* 1973) and in heat-acclimatized bulls (Rhynes and Ewing 1973); i.e. heat acclimatization accelerates the conversion of pregnenolone to progesterone, which further metabolizes at an increased rate to deoxycorticosterone, dihydroxyprogesterone and its 20β epimer, hydroxyprogesterone, and further to androstenedione. However, the last step, namely the conversion of androstenedione to testosterone which is catalysed by 4-ene-17β-hydroxysteroid dehydrogenase, occurs at a reduced rate in heat-acclimatized mice (Bedrak 1972; Slonim and Bedrak 1974).

The conflict between the above statement and the similarity in the production of testosterone for each group (Tables 4 and 5) is explained as follows. Although the the activity of 4-ene-17β-hydroxysteroid dehydrogenase is decreased, the level of testosterone produced *in vitro* is similar because there is an increased amount of androstenedione due to activation of the 4-ene pathway, and this is coupled with a higher conversion rate of dehydroepiandrosterone via the 5-ene route in the heat-acclimatized mice. Since the concentration of testosterone in peripheral blood is a function of synthesis and catabolism, it is suggested that in heat-acclimatized animals the catabolism of testosterone is greater than in control animals. This suggestion is supported by the fact that the daily urinary excretion rate of 17-ketosteroids is significantly higher in heat-acclimatized rats (Sod-Moriah and Bedrak 1975), whereas the concentration of testosterone in blood plasma is decreased (Bedrak *et al.* 1973).

If the male mouse responds to heat acclimatization in the same way as the male rat (Bedrak *et al.* 1973) and the bull (Rhynes and Ewing 1973), then a lower level of testosterone in peripheral blood would be expected. No determinations of blood testosterone level were made in the present investigation.

Spermatogenesis appeared normal in the HAII mice (Fig. 2*c*) and the decrease in testosterone did not impede the reproductive capacity of these male mice since mating them with control females led to the birth of normal numbers of viable pups. This was not the case with the male mouse (HAI, Fig. 2*b*) which exhibited complete atrophy of the seminiferous tubules and hyperplasia of the interstitial cells (Chap, personal observation). The data presented here show only small changes in the protein content of testicular tissue from the various groups, and such changes cannot indicate any significant differences in enzymic content. On the other hand, heat acclimatization caused hyperplasia of interstitial cells which contain the enzymes involved in the biosynthesis of androgens. It is tentatively suggested that this hyperplasia may differentially alter rates of steroidogenesis.

When exposure to heat was coupled with the provision of a supplementary water supply, the pronounced changes in activity of some of the enzymes involved in *in vitro* androgen production persisted in a very definite pattern which is characteristic of desert mice (Bedrak *et al.* 1971*b*; Bedrak 1972). These data suggest that the responses to heat acclimatization of steroidogenic enzyme activity and/or synthesis precede processes leading to decreased body and organ weight and to changes in organ structure. While indispensible to a knowledge of the subject, the data from *in vitro* studies contribute little to the understanding of *in vivo* steroid production. However, this limitation is accompanied by the very tangible advantage that those factors which cannot be controlled *in vivo* can be controlled in enzyme investigations *in vitro*. The data presented here indicate the producing capacity of endocrine tissue and we can only postulate what happens in the intact animal where the complex hypothalamo–hypophyseal system controls gonadal response. The role of the gonadotropin- releasing hormone and of gonadotropins during heat acclimatization must still be determined.

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