SPECIAL LECTURE

**Factors Influencing the Secretion of Testosterone
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Testosterone and Δ^4 -androstenedione were first isolated and identified in spermatic vein blood of the dog in 1952 (1). Five years later our laboratory reported (2) that the concentration of these two androgens increased in spermatic vein blood of the anesthetized dog following the administration of human chorionic gonadotrophin (HCG). This effect of HCG on testosterone secretion *in vivo* has later been repeated by several investigators (3, 4, 5) and it also appears that HCG will promote the secretion of testosterone *in vitro* by testicular tissue (6, 7, 8). Mason and Samuels (9) demonstrated that the canine testis could secrete testosterone- C^{14} when infused with acetate- $l-C^{14}$ via the spermatic artery. When HCG was administered concomitant with acetate- $l-C^{14}$ via the spermatic artery the specific activity of biosynthesized testosterone- C^{14} increased. Thus, an effect of HCG on the *de novo* formation of testosterone was established. That the interstitial cell stimulating hormone (ICSH) also will promote *de novo* synthesis of testosterone *in vitro* (7) and *in vivo* (10) has been demonstrated by our group. This effect of ICSH on the testicular tissue is specific, since the administration of other pituitary hormones will not affect the secretion of testosterone *in vivo* (10). When large doses of follicle stimulating hormone (FSH) preparations are given to anesthetized dogs, the secretion of testosterone will, however, increase (10). This effect of FSH preparations is also seen *in vitro* (7). It appears possible that the small amount of ICSH activity found in the FSH preparations** used is unusually potent in promoting testosterone secretion. This puzzling property of FSH has recently been discussed by Eik-Nes (11).

Finally, our laboratory has demonstrated that pregnant mare serum gonadotrophin (PMS) will increase the synthesis *in vivo* and *in vitro* of total testosterone and of testosterone- C^{14} from acetate- $l-C^{14}$ in testicular tissue (12). If a mixture of PMS and puromycin is given via the spermatic artery (Fig. 1), the stimulatory effect of PMS is abo-

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** 1 mg FSH contained 6 μ g ICSH by bioassay.

Fig. 1. The effect of puromycin on PMS stimulated secretion of testosterone in an anesthetized dog (22 kg). During the 30-120 min period 210 I.U./kg of PMS were infused at a constant rate via the left spermatic artery. During this same period a mixture of 210 I.U./kg of PMS and 5 mg puromycin/kg was infused at a constant rate via the right spermatic artery. Arterial blood was infused via the left and right spermatic artery at a constant rate of 3.82 ml/min from 0-120 min. The animal preparation used is described in ref. no. 10 and the methods employed for testosterone and testosterone- C^{14} estimation in spermatic vein blood in all the work reported in this paper are described in detail in ref. 10.

lished. *De novo* synthesis of proteins may therefore be an important aspect in the mechanism of action of the gonadotrophins on the testis *in vivo* (7). Of considerable interest is the fact that many of the pituitary hormones may promote increased production of enzymatic proteins in order to affect their target cells (13, 14, 15). With regard to the gonadotrophins and their effects on the ovary and the testis, much has, however, to be learned about their site of action (16, 17).

In contrast to the published effects of the gonadotrophins on acetate metabolism in testicular tissue (6, 7, 9, 10, 12), reduced triphosphopyridine nucleotide (NADPH) will not promote the incorporation of acetate- C^{14} into testosterone- C^{14} by the canine testis *in vivo* (Fig. 2), but will increase the secretion of non-radioactive testosterone under the same experimental conditions. The data of Fig. 2 thus demonstrate that the canine testis *in vivo* behaves biosynthetically like the rabbit testis (7), and the bovine corpus luteum *in vitro* (18). It is currently the opinion of many investigators that the pituitary gonadotrophins increase steroid biosynthesis in the ovary and testis by activating the phosphorylase concentration in these tissues. An increased concentration of phosphorylase *a* should increase the tissue NADPH concentration and NADPH is needed for

Fig. 2. The effects of a NADPH generating system on the secretion of testosterone and on acetate-1-C¹⁴ metabolism in an anesthetized dog (19 kg). The animal was given 2000 I.U. HCG intramuscularly every second day for 10 consecutive days (cnf. ref. 9 and 10). On day 11 the animal was prepared as described (10) and a constant rate infusion of arterial blood containing acetate-1-C¹⁴ (total of 60 µc and 360 mg) was done via the left spermatic artery during time 0-180 min. During the last 120 min of infusion a NADPH generating system was also administered via the left spermatic artery (a total of 12 mg TPN, 12 mg glucose-6-phosphate and 0.7 mg glucose-6-phosphate dehydrogenase). Arterial blood was infused at a constant rate of 3.82 ml/min and the temperature of the infused testis was maintained at 36.6°C throughout the experiment.

○—○—○ concentration of testosterone.

•—•—• concentration of C¹⁴ in isolated testosterone.

steroid formation, i.e. in order to form one mole of testosterone from acetate at least 32 moles of NADPH are required. Gonadotrophic activation of ovarian or testicular phosphorylase has furthermore been presumed to take place through adenosine-3', 5'-monophosphate. This compound will, however, not stimulate the phosphorylase activity in the bovine corpus luteum *in vitro* (19) but will promote synthesis of progesterone-1-C¹⁴ in this tissue. Thus, the obligatory involvement of the pentose phosphate pathway in the mechanism of action of the gonadotrophins on the testis and on the ovary is not established. Recently we have, however, observed that dogs infused with adenosine-5'-triphosphate (ATP) via the spermatic artery will produce less testosterone than animals not infused with this compound when stimulated with PMS via the spermatic artery.

The enzyme phosphofructokinase could therefore be of importance for the mechanism of action of the gonadotrophins in the canine testis. Inhibition of phosphofructokinase by ATP can be relieved by adenosine-3', 5'-monophosphate.

Finally, over the past 3 years our group has investigated the metabolism of steroids by the canine testis *in vivo* (20, 21, 22, 23, 24, 25, 26, 27). In Fig. 3 we have summarized our findings after infusing radioactive steroids via the spermatic artery and measuring the concentration of radioactive metabolites of the infused compounds in the spermatic vein blood in anesthetized dogs. As can be seen, the conversion of Δ^5 -pregnenolone to 17α -hydroxypregnenolone is an important biosynthetic step in the canine testis. It should, however, be pointed out that the specific activity of the radioactive metabolites isolated and identified* in the spermatic vein blood has not been determined in our experiments *in vivo* since the concentration of non-radioactive 17α -hydroxypregnenolone, dehydroepiandrosterone, progesterone and 17α -hydroxyprogesterone in the spermatic vein blood is too low to be determined adequately by currently available techniques. This criticism can, however, also be leveled at most investigations of steroid metabolism *in vitro* using radioactive steroid substrates since few studies of this nature base their conclusions on the specific activity of the biotransformed radioactive material.

Fig. 3. Pathways used for the production of testosterone from Δ^5 -pregnenolone in the canine testis. Reservations as to the interpretations of this figure have been stated in the text of this paper. (From Hagen, A.A., and Eik-Nes, E.B., "In *vivo* testosterone biosynthesis in the canine following infusion of $[4-^{14}\text{C}]$ 17α -hydroxyprogesterone and $[7\alpha-^3\text{H}]$ dehydroepiandrosterone via the spermatic artery", *Biochim. Biophys. Acta*, in press, 1964.

* By the criterion of crystallization to constant specific activity after the addition of authentic material.

Recently our laboratory has developed a method for the quantitative estimation of steroids using gas-liquid chromatography with electron capture detection (28). This method will estimate $0.002 \mu\text{g}$ steroid-chloroacetate (Fig. 4). Experiments are now in progress determining the specific activity of many of the steroid intermediates (Fig. 3) which can be isolated in the spermatic vein blood of dogs infused with Δ^5 -pregnenolone- 7-H^3 via the spermatic artery. It is hoped that this investigation will demonstrate conclusively what pathway is the major one for the formation of testosterone by the canine testis *in vivo*. Considerable information has accumulated over the past decade on metabolism of steroid hormones *in vitro* (for review cfr. ref. 29, 30, 31, 32, 33). One should, however, remember that data from work *in vitro* will only indicate what the tissue *can* do *in vivo*.

Thus, we have shown that the canine testis *in situ* will produce testosterone from known precursors and that the metabolism of acetate to testosterone in this tissue is increased by the gonadotrophins (ICSH, PMS, HCG and possibly FSH). If no gonadotrophins are given to anesthetized dogs with a cannula in the left spermatic vein, the concentration of testosterone will fall in the spermatic vein blood over a 120 min observation period (10). We postulated that this fall in testosterone concentrations could be due to hypothalamic inhibition by sodium pentobarbital (34). Indeed, if extracts of dog brain tissue, prepared as described by our laboratory (35), are administered as a

Fig. 4. Calibration curve (cm^2 peak area vs μg) of pure testosterone chloroacetate. From Brownie, A.C., van der Molen, H.J., Nishizawa, E.E. and Eik-Nes, K.B., "Determination of testosterone in human peripheral blood using gas-liquid chromatography with electron capture detection." J. Clin. Endocrinol. and Metabol., in press, 1964.

constant 60 min infusion via a branch of the internal carotid artery, the extracts of some hypothalamic tissue seem to contain a factor which will prevent the fall in testosterone secretion seen in anesthetized dogs (Table 1). This factor is also present in hypothalamic tissue from the hog (Table 1). Considerable time has been spent on purifying this factor from the hog hypothalamic* tissue, but the factor is unstable when exposed to the chemical procedures employed for purification and work along these lines has now been terminated in our laboratory. The material in the crude homogenate of the hypothalamic tissue is neither ICSH nor FSH and current literature strongly indicates that the pituitary gonadotrophins may be released by hypothalamic factors of chemical nature in the female rat (36, 37, 38). This may also be the case for the male dog *in vivo*, but we have hitherto been unable to gain information as to the chemical nature of this hypothalamic ICSH releasor.

In the spring of 1963 an opportunity arose unexpectedly to measure the secretion of testosterone in a normal and in a cryptorchid testis in the same dog. Fig. 5 demonstrates that the ability of the cryptorchid testis to secrete testosterone and to increase its secretion of this androgen following PMS administration was diminished compared to the normal testis. Also, the flow of venous blood from the cryptorchid testis was low (Fig. 6). Since there seems to be little agreement in the literature about the capacity of the cryptorchid testis to secrete testosterone (39, 40, 41), experiments were conducted to examine the effects of changes in arterial blood flow and changes in testicular temperature on the production of testosterone by the normal canine testis *in vivo*.

The dogs used in these experiments weighed from 18-23 kg. Before operation each

Table 1. The effect of filtered homogenate of dog brain tissue on the secretion of testosterone in the anesthetized dog

Dog_Index	Tissue Used	Weight of Tissue (mg)	0-30	Testosterone $\mu\text{g}/\text{min}$ Time Sample 30-60	60-90
B _Z	0.9% NaCl only		.22	.10	.08
Z _Z	" "		.19	.15	.10
C _Z	Cortex cerebri	50	.31	.23	.16
D _Z	" "	72	.08	.05	.05
E _Z	N. supraopticus	42	.19	.23	.31
F _Z	" "	63	.08	.15	.17
G _Z	Median eminence	38	.05	.16	.21
H _Z	" "	63	.11	.21	.30
WA	" " (hog)	70	.07	.14	.20

The animal preparation used is described in ref. 10 of this paper. During time period 30-90 min each animal received as a constant rate infusion via a tributary of the internal carotid artery either 20 ml sterile 0.9% NaCl or a filtered homogenate of dog brain tissue diluted to 20 ml with sterile 0.9% NaCl. The preparation of the homogenate is described in ref. 35 of this paper.

* Eik-Nes, K.B. and White, W. : unpublished data, 1964.

Fig. 5. Secretion of testosterone by a cryptorchid and a eutopic testis in the same dog (animal LD). The animal preparation used is described in ref. no. 2 of this paper. At time 30 min the animal was given a rapid intravenous injection (10 sec) of PMS (190 I.U./kg). From Eik-Nes, K.B. and Hall, P.F., "The action of pregnant mare serum on the production of testosterone *in vivo* and *in vitro*". Endocrinology, in press, 1964.

Fig. 6. Venous blood flow in the left spermatic veins of 10 normal dogs (black area) and of a dog (LD) with one cryptorchid and one eutopic testis. Blood flow is expressed as ml spermatic vein plasma per 10 kg body weight per min. During the last 60 min of the experiment, PMS was administered intravenously to each dog. From Eik-Nes, K.B. and Hall, P.F., "The action of pregnant mare serum on the production of testosterone *in vivo* and *in vitro*". Endocrinology, in press, 1964.

animal was given a single intravenous injection of HCG (100 I.U./kg). The animal was then anesthetized by sodium pentobarbital intravenously (30 mg/kg). The left femoral artery was dissected and cannulated (10) and this cannula was connected to the inlet channel of a Harvard automatic withdrawal infusion pump (Model 600-950). The left spermatic artery and vein were reached through a flank incision and cannulated as described (10) and the cannula in the spermatic artery was attached to the outlet channel of the pump. This cannula was 20 feet long and was placed in a small waterbath. The temperature of the waterbath could be changed from 4°–40° in 10 minutes by a heater-cooler unit.

In the temperature experiments the left testis of the dog was infused with the animal's own arterial blood via the cannula in the spermatic artery at a constant rate of 3.82 ml/min, and blood was collected in a series of 30 min samples from the spermatic vein. A 22 gauge temperature probe (Yellow Springs Instrument Company, Yellow Springs, Ohio, U.S.A.) No. 547 was inserted into the center of the infused (left) testis. The probe was calibrated and connected with a Yellow Springs Model 41 TY Tele-Thermometer. The absolute accuracy of the temperature recordings by means of

this device was $\pm 3^{\circ}\text{C}$. The Tele-Thermometer was attached to a Yellow Springs Model 81 recorder so that continuous tracings of testicular temperature were obtained throughout the experiments.

In order to induce rapid changes in the temperature of the infused testes a small ice pack or a hot pad was applied to the scrotal region of the dog. When the desired temperature was reached it could in most instances be maintained by the temperature of the infused arterial blood. All animals received 0.9% sodium chloride as intravenous drip throughout the experiment at a rate of 4 ml/min.

In Table 2 are recorded testicular and abdominal temperatures in normal dogs 1-3 min after intravenous sodium pentobarbital. As has been known for a long time (42)

Table 2. Abdominal and testicular temperatures in the dog 1-3 minutes after the intravenous administration of 30 mg sodium pentobarbital per kg body weight.

Dog Index	testis °C	Abdomen
AA	34.8	37.8
AB	35.1	38.0
AC	34.2	37.8
AD	35.1	38.8
AF	35.0	38.9
AG	35.5	37.9
AH	34.8	37.4
AJ	34.9	37.1

The equipment used for measurement for testicular and abdominal temperature has been described in the text of this paper.

the testicular temperature is lower than the abdominal temperature and if the testicular temperature is lowered below the normal range of $35\text{--}35.5^{\circ}\text{C}$, the secretion of testosterone will decrease in HCG stimulated animals (Fig. 7). Still, some testosterone is being secreted at the low temperatures, confirming the data *in vitro* of Gospodarowicz and Legault-Démare (43). A 30 min exposure of the testis to an abnormally low temperature is not associated with permanent damage to the Leydig cells since a return to almost normal secre-

Fig. 7. The effects of decreasing testicular temperature on secretion of testosterone in anesthetized dogs. All animals were given 100 I.U. HCG/kg intravenously 60 min before the collection of the first blood sample. The conditions of these experiments are stated in the text of this paper.

tion of testosterone is seen in the ensuing 30 min if the temperature is elevated to the normal range in this time period (Fig. 8). In short term experiments (i.e. of 90-120 min duration) we have not been able to show that testicular temperatures between 36-39°C

Fig. 8. The effects of rapid changes in testicular temperatures on testosterone secretion in two anesthetized dogs. The animals were given 100 I.U. HCG/kg intravenously 60 min before the collection of the first blood sample. The conditions of these experiments are stated in the text of this paper.

will influence the ability of the canine testis to secrete testosterone *in vivo* (Fig. 9) and it is probable that the HCG stimulated testis can maintain normal production of testosterone at these high testicular temperatures for short periods of time. Only in one dog have we demonstrated that a testicular temperature of 39°C for 60 min will interfere with the secretion of testosterone (Fig. 10).

In the temperature experiments the left testes of animals weighing between 19-23 kg were infused with arterial blood at a rate of 3.82 ml per min. The reason for using this infusion rate

Fig. 9. The effects of increasing testicular temperature on the secretion of testosterone in anesthetized dogs. All animals were given 100 I.U. HCG/kg intravenously 60 min before the collection of the first blood sample. The conditions of these experiments are stated in the text of this paper.

Fig. 10. The effect of high testicular temperature for 60 min on the ability of the testis to secrete testosterone. The dog was given 100 I.U. HCG/kg intravenously 60 min before the collection of the first blood sample. The condition of this experiment is stated in the text of this paper.

was that the flow of venous blood in anesthetized dogs with a cannula in the left spermatic vein (2) will vary from 1.1–2.7 ml/10 kg body weight/min, the average flow in 70 dogs being 1.9 ml/10 kg/min. If the testicular temperature is maintained between 36.8–37.4° and arterial blood is infused at a rate of 3.82 ml/min in a 20 kg dog, the secretion of testosterone undergoes little variation (Table 3). Flow rates of arterial blood lower than 3.82 ml/min are associated with decreased secretion of testosterone which will increase when the arterial flow is increased (Table 3). Of considerable interest is the fact that testicular tissue receiving arterial blood at about 1/5 of its normal rate for

Table 3. Relationship between testicular blood flow and the secretion of testosterone in anesthetized dogs.

Dog Index	Testosterone secreted µg/min			Flow rates of Arterial Blood ml/min		
	0-30	Time Sample 30-60	60-90	0-30	Time Sample 30-60	60-90
A J	.35	.46	.44	3.82	3.82	3.82
AH	.33	.33	.38	3.82	3.82	3.82
AAK	.29	.31	.33	3.82	3.82	3.82
AC	.59	.38	.26	3.82	1.92	0.71
AN	.23	.11	.12	3.82	1.92	0.71
AK	.40	.08	.11	3.82	0.71	1.92
K _z	.32	.10	.16	3.82	0.71	1.92
AKH	.11	.12	.12	0.71	1.92	3.82
KR	.16	.15	.21	0.71	1.92	3.82
AP	.06	.07	.06	0.71	3.82	1.92
AKL	.44	.48	.19	1.91	3.82	0.71
AKO	.12	.10	.30	1.91	0.71	3.82

The animals were given 100 I.U. HCG/kg I.V. 60 min before the collection of the first blood sample (0-30 min sample). All infusions of arterial blood were done at testicular temperatures ranging from 36.8–37.4°C.

the first 30 min of experimentation shows a limited ability to regain its biosynthetic capacity in the ensuing 60 min even if the rate of arterial blood flow is increased to the normal (Table 3). One should thus question seriously how much of the physiological capacity the interstitial cell is able to retain after being severed from its blood supply even for a short period of time and then used for perfusion *in vitro* or as homogenates, minces or slices.

The secretion of testosterone into the left spermatic vein in anesthetized dogs stimulated with HCG before experimentation thus depends in part on the flow of arterial blood to the left testis. Why this relationship exists cannot be stated from the data, but probable factors are oxygen, cofactors, substrates and co-substrates for steroid biosynthesis and lack of removal of the different compounds formed in the metabolic processes of the testis. Both the testis and the ovary (44) appear to be most dependent on adequate arterial irrigation for normal production of steroid hormones. This fact should not be overlooked when working with endocrine organs *in vivo*; investigations *in vitro* should carefully consider if the *in vitro* systems employed compensate adequately for a complete lack of arterial irrigation.

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