Studies on Hormonal Regulation of Squalene Synthesis in Preputial Gland and Skin of the Rat.* (28479)

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(Introduced by M. D. Siperstein)
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There is now convincing evidence that the synthesis of squalene in several tissues is under hormonal regulation. André and Canal originally demonstrated that the content of shark liver squalene rises with age(1), and Nicolaides and Rothman subsequently observed that the squalene concentration of human hair fat rises following puberty(2). Finally, a previous study from this laboratory established that squalene synthesis in the rat preputial gland and skin is enhanced by administration of either testosterone or progesterone(3). However, since the sebaceous glands are the principal source for skin squalene(4) and since both testosterone and progesterone exert marked trophic effects on the preputial glands and on the skin sebaceous glands of the rat(5), it seemed likely that the increase in sebaceous gland squalene following administration of these hormones was simply the passive consequence of an increase in gland size.

In this communication are described further studies on hormonal regulation of squalene synthesis and content in rat skin and preputial gland. Administration of either testosterone or progesterone clearly results in an increase in content of rat preputial gland squalene out of all proportion to the trophic effects on this sebaceous gland. Evidence is also presented that this hormonal effect is not specific for squalene synthesis; indeed, the increased rate of fatty acid synthesis, together with the increase in squalene and cholesterol synthesis, suggests that these hormones act in some general way to enhance lipid synthesis in the sebaceous glands.

Experimental. All animals used in these studies (male, Long-Evans rats, weighing 50-75 g) were castrated under ether anesthesia 6 days prior to death. One group of castrated animals served as controls. The remainder of the castrated rats were given by intramuscular injection one milligram of either testosterone-propionate, progesterone, or estradiol 6, 4 and 2 days before *in vitro* studies. Both the hormone-treated group and the castrated controls were allowed free access to food. At the end of the treatment period the animals were killed by decapitation, and the tissues were quickly removed and chilled in ice-cold Krebs-Ringer-bicarbonate buffer, pH 7.4. For each experiment the preputial glands from 25-50 animals were pooled.

The preputial glands were dissected free of fat, and the skin was shaved prior to slicing. Slices of preputial gland and skin, approximately 1-2 mm in thickness, were prepared by hand. The slices were blotted, weighed, and transferred to flasks containing Krebs-Ringer-bicarbonate buffer, pH 7.4, glucose, and radioactive substrate. The flasks were gassed with 95% oxygen - 5% carbon dioxide, capped, and incubated at 37°C with shaking for 1½ hours. At the end of incubation period the reaction was stopped, and the contents were washed into 250 ml flasks with chloroform-methanol and evaporated to dryness.

Either fresh tissue samples or the incubation mixture was saponified at 80°C for 30 minutes in 1 N KOH and extracted with petroleum ether. The non-saponifiable fraction was dried with heating under nitrogen and dissolved in benzene. The benzene solutions were placed in columns (20 × 1 cm) of silicic acid-celite (1:1) as described by Frantz, *et al.*(6); the columns were eluted first with 30 ml of benzene and then with 30 ml of 20% ethyl ether in benzene. The benzene solutions (containing squalene) and the ether-benzene solutions (containing sterols) were then analyzed for C^{14} or by gas chromatographic techniques.

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In the incubation experiments, carrier cholesterol (1 mg) was added to the benzene-ether fraction; digitonides were formed and washed by the method of Sperry and Webb (7), dissolved in methanol, added to 0.4% diphenyloxazole in toluene, and assayed for C^{14} in a liquid scintillation spectrometer. The sterol fractions from rat skin and preputial gland contain, in addition to cholesterol, large quantities of other sterols(6); consequently, this fraction is subsequently referred to as "sterols." Carrier squalene (50 mg) was added to the benzene eluate, and squalene hexachlorides were formed, washed, and assayed for C^{14} by a previously described modification of the method of Heilbron and his associates(8). The alkaline water solution which remained following the petroleum ether extraction was acidified, and fatty acids were extracted with petroleum ether, back-washed with water, taken to dryness, dissolved in 0.4% diphenyloxazole in toluene, and assayed for C^{14} content in a liquid scintillation counter.

Gas chromatographic analyses were performed on a Research Specialties Co. gas-liquid chromatography assembly in which the entire gas-flow circuit had been siliconized. Six-feet × 4 mm columns packed with 3% neopentylglycol succinate columns on Gas Chrom P, 100-140 mesh(9), were used in these experiments with operating temperatures as follows: column, 200°; detector, 250°; outlet 300°; and vaporizer 310°. The argon inlet pressure was 30 psi, and the monitored outflow was 100 ml/min. A new series of standards was run for each day of the chromatographic analyses. The areas recorded under the various peaks were estimated by the method of Bartlet and Smith(10). Under these conditions the peak appearance time for squalene was 15 minutes. Quantities of squalene less than 10 μg per 100 mg tissue could not be reliably analyzed.

Results. Quantitation of squalene. The neopentylglycol succinate - Gas Chrom P column as described by VandenHeuvel, *et al.* (9) was found to provide a satisfactory means for chromatographing squalene. The operating conditions used were chosen because they provided convenient and clear-cut separation of squalene from the contaminating materials in the biological extracts used here. Under these circumstances, a linear relationship was demonstrated between amount of squalene injected and area recorded under the curve for quantities ranging from 1-9 μg (Fig. 1).

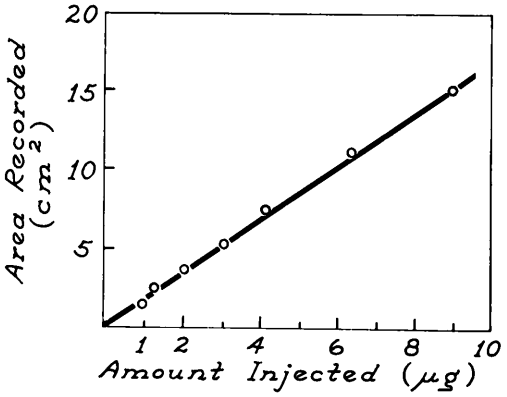

FIG. 1. Relation of varying quantities of squalene to area recorded. Varying amounts of squalene in 5 μl chloroform were injected into a gas-liquid chromatography assembly. Conditions as described in the text.

Squalene content of skin and preputial gland. In Table 1 the influence of castration alone and castration plus hormonal administration on squalene content of skin preputial gland is compared. Each of these hormones produced an increase in average preputial gland weight during the 6 day treatment period. Although long-term estradiol treatment results in shrinkage of the sebaceous gland volume, the effect of estradiol is actually a 2-phase phenomenon — an early increase in the mitotic rate followed by atrophy(11); the increase in sebaceous gland size following estradiol treatment in these experiments can consequently be explained as the result of the short duration of treatment.

Even when these changes in weight are taken into consideration, however, the alterations in squalene concentration are very striking; testosterone resulted in a 7-9 fold increase and progesterone in a 2-3 fold increase, whereas estradiol administration resulted in a slight decrease in preputial gland squalene concentration. In the one experiment in which squalene concentration was

TABLE I. Influence of Testosterone-Propionate, Progesterone, and Estradiol on Squalene Content of Skin and Preputial Gland of Castrated Rats.

	Exp 1				Exp 2		
	Preputial gland			Skin	Preputial gland		
Treatment	No. of samples pooled	Avg wt (mg/gland)	Squalene content (μg/100 mg)	squalene content (μg/100 mg)	No. of samples pooled	Avg wt (mg/gland)	Squalene content (μg/100 mg)
Castration	20	8	102	10	20	11	122
Testosterone	8	22	944	14	10	44	840
Progesterone	8	17	210	27	10	30	383
Estradiol	8	14	90	<10	10	23	59

Male rats (50-75 g) were castrated 6 days prior to death and given by intramuscular injection one milligram of either estradiol, progesterone, or testosterone propionate 6, 4, and 2 days prior to death. Exp 1 and 2 represent separate pooled groups.

measured in skin estradiol, progesterone, and testosterone produced similar directional changes, although not quantitatively as marked as those demonstrated in the preputial gland.

Squalene synthesis in skin and preputial gland. Since the administration of testosterone produced the most marked effect on the preputial gland squalene, the influence of this hormone on squalene and sterol synthesis from acetate-2-C^{14} and mevalonate-2-C^{14} was studied in slices of preputial gland and skin (Table II). In the preputial gland, testosterone pre-treatment resulted in a marked increase in the synthesis of both squalene and sterols; this enhancement was observed when either acetate-2-C^{14} or mevalonate-2-C^{14} was used as substrate. In skin a similar pattern was seen, although in this tissue mevalonate-2-C^{14} does not appear to be as active a sterol presursor as does acetate-2-C^{14}; the reason for this discrepancy, which has also previously been noted for the intestine(8), is unclear.

To determine whether this hormonal enhancement is specific for squalene and sterol synthesis the influence of testosterone administration on fatty acid synthesis by preputial gland was then investigated (Table III). In 2 experiments squalene synthesis in the slice preparation was enhanced 2-4 fold, while in

TABLE III. Influence of Testosterone-Propionate on Conversion of Acetate-2-C^{14} to Fatty Acids, Squalene, and Sterols by Slices of Rat Preputial Gland.

Exp No.	Treatment	No. of samples pooled	Acetate-2-C^{14} converted to: Fatty acids	Squalene	Sterols
			(cpm)	(cpm)	(cpm)
1	Castration	20	5,858	2,919	226
	Testosterone	10	9,806	11,861	552
2	Castration	20	7,277	3,027	339
	Testosterone	10	15,358	6,862	1,666

Treatment of animals as in Table I. Each incubation flask contained slices (100 mg), Krebs-Ringer-bicarbonate buffer, pH 7.4, glucose (9×10^{-3} M), and acetate-2-C^{14} (1.2×10^6 cpm) to make a final volume of 1.0 ml. Incubated at 37° with shaking for 90 min.

TABLE II. Influence of Testosterone-Propionate on Sterol and Squalene Synthesis from Acetate-2-C^{14} and Mevalonate-2-C^{14} by Slices of Rat Skin and Preputial Gland.

Tissue	Treatment	No. of samples pooled	Acetate-2-C^{14} converted to: Squalene	Sterols	Mevalonate-2-C^{14} converted to: Squalene	Sterols
			(cpm)	(cpm)	(cpm)	(cpm)
Preputial gland	Castration	20	1,130	130	1,520	110
	Testosterone	10	5,960	580	2,530	440
Skin	Castration	2	380	1,880	80	370
	Testosterone	2	3,380	6,190	250	400

Treatment of animals as in Table I.

Each incubation flask contained slices (100 mg), Krebs-Ringer-bicarbonate buffer, pH 7.4, glucose (9×10^{-3}M), and either acetate-2-C^{14} (1.2×10^6 cpm) or mevalonate-2-C^{14} (5.3×10^5 cpm) to make a final volume of 1.0 ml. Incubated at 37° with shaking for 1½ hr.

the same preparations sterol synthesis and fatty acid synthesis were also enhanced about 2 fold by testosterone treatment. The parallel enhancement of the synthesis of fatty acid and of squalene and sterols suggests that the accelerated squalene synthesis is due, not to a specific hormonal effect, but is rather secondary to some general influence on lipid synthesis.

Discussion. Squalene is a major lipid component of the sebaceous gland secretions of man(12). Furthermore, it has long been known that the amount of lipid excreted to the skin is small in childhood, increases rapidly with onset of puberty, then levels out to fairly constant values in young adults(13). It has been suggested that this increase of sebaceous gland excretion with oncoming puberty in humans is accompanied, and possibly caused, by an increase in sebaceous gland volume; in the male the role of testosterone in initiating this pubertal event is well established, while in the female the hormone responsible for these effects may be progesterone(5).

The findings reported here strongly suggest that the increase in lipid excretion by the skin with onset of puberty may not be simply the passive consequence of an increase in gland size. Indeed, even when changes in gland size are taken into account, administration of testosterone still results in striking increases in the content and synthesis of squalene and sterols and synthesis of fatty acids. It is concluded that testosterone and progesterone, in addition to promoting sebaceous gland growth, must enhance over-all lipid synthesis in some general way. Such a conclusion fits well with the histological evidence that the sebaceous gland secretion actually represents the conversion (and subsequent metamorphosis) of the entire sebaceous cell into a globule of fat which is eventually extruded to the skin surfaces(13).

Summary. Utilizing gas-liquid chromatographic techniques, the influence of administration of testosterone, progesterone, and estradiol on the content of skin and preputial gland squalene has been studied in the castrated rat. Testosterone and, to a lesser extent, progesterone markedly enhance the squalene content of the preputial gland. Testosterone also enhances the synthesis of fatty acids from acetate-2-C^{14} in this tissue, suggesting that it may act in a general way to enhance fat synthesis.

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