then represent a merozoite stage, since they form in the host by endodyogeny (*3*), and give rise directly to sexual stages in cell culture.

RONALD FAYER
National Animal Parasite Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

References and Notes

1. R. Fayer, *Science* **168**, 1104 (1970); *J. Parasitol.* **56** (2nd Int. Congr. Parasitol., section II, part 1), 98 (1970).
2. J. M. Vetterling and D. E. Thompson, a polychrome stain for parasitology (personal communication).
3. J. Senaud, *Protistologica* **3**, 167 (1967).
4. I thank R. M. Kocan for supplying the grackles and D. E. Thompson for technical assistance.

13 August 1971

Mechanism of Gonadotropin Action in Amphibia: Involvement of Mitochondria

Abstract. *Luteinizing hormone (a pituitary gonadotropic hormone) stimulates Δ^5-3β-hydroxysteroid dehydrogenase activity in the microsomal fraction of frog testes when incubated together with the mitochondria; incubation together with the nuclei instead of the mitochondria does not result in increased Δ^5-3β-hydroxysteroid dehydrogenase activity. The increase is not induced by adenosine triphosphate, it appears to be hormone-specific, and it is sensitive to puromycin and actinomycin D. These data suggest that the mitochondrial DNA may be involved in mediating the action of luteinizing hormone in amphibian steroidogenesis.*

The controlling effect of pituitary gonadotropic hormones on steroid production in the vertebrate testis has long been recognized (*1*). Biosynthesis of gonadal steroid hormones is accelerated when gonadotropins are administered in vivo and when added to incubating tissue slices (*2*). Moreover, histochemical and biochemical data indicate that certain enzymes (the hydroxysteroid dehydrogenases) involved in steroid hormone biosynthesis show increased activity after in vivo or in vitro administration of gonadotropins (*3*, *4*). One such enzyme is the Δ^5-3β-hydroxysteroid dehydrogenase complex (3β-HSD). Testicular, ovarian, adrenal, and placental tissues are known biochemically to contain this enzyme, which irreversibly converts Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids (*5*). Its presence has been demonstrated histochemically in all classes of vertebrates and it plays a cardinal role in the biosynthesis of many steroid hormones (*3*).

The activity of testicular 3β-HSD in amphibians is markedly increased by injections of mammalian gonadotropic hormones (*4*). Recently it also has been demonstrated that a homogenate of frog (*Rana pipiens*) testes incubated for several hours together with mammalian luteinizing hormone (LH) will show greater 3β-HSD activity in the microsome fraction than one incubated without the hormone (*6*); this demonstrates that the cells do not need to be intact in order for gonadotropins to cause increased steroid hormone biosynthesis.

One important aspect of gonadotropin-stimulated steroidogenesis which has remained largely unstudied is the site of action within the target cells, that is, whether the primary controlling site of action of gonadotropins within the steroid-producing cells resides in the cell membrane, the cytoplasmic organelles, or the nucleus. Below I present evidence —based on various recombinations of cell organelles—that LH will give an increased 3β-HSD activity in the microsomal fraction of frog testes in the presence of the mitochondria but not the nuclei. Evidence is further presented which idicates that this increase in 3β-HSD activity involves protein synthesis (puromycin-sensitive) and may be mediated via the mitochondrial DNA (actinomycin-sensitive).

Testes from grass frogs (*Rana pipiens*) were excised and weighed. The gonads from about 10 to 15 individuals were pooled over ice and then homogenized at 0°C with a motor-driven glass-Teflon tissue homogenizer. The homogenizing medium consisted of 0.02*M* phosphate buffer (*p*H 7.6) and 0.25*M* sucrose, and sometimes contained mercaptoethanol (0.001*M*); the final tissue concentration was usually 5 percent (wt./vol.). Standard differential centrifugation procedures were used to separate the organelles: pellet from 10 minutes at 200 to 800*g* = nuclei; pellets from 10 minutes each at 800 to 5,000*g* and 5,000 to 24,000*g* = mitochondria; supernatant from 10 minutes at 24,000*g* = microsomes and dissolved substances. Organelles were resuspended in homogenization medium, recombined (see Figs. 1 and 2 and Table 1), and incubated in a medium consisting of homogenizing solution (90 percent) and calf serum (10 percent) and the various test substances. Incubation was carried out in 25-ml erlenmeyer flasks in a Dubnoff incubator (25 oscillations per minute) at 28° to 30°C for 4 to 6 hours under air. The contents were then centrifuged at 24,000*g* for 10 minutes and the supernatant was assayed for 3β-HSD activity as described (*4*).

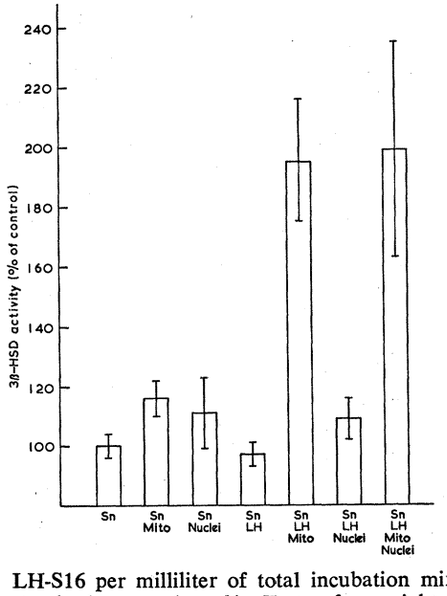

Fig. 1. The role of mitochondria and nuclei in mediating the stimulatory effect of LH on 3β-HSD activity in *Rana* testicular microsomes. Testes were homogenized (5 percent, wt./vol.) in the following medium: 0.02*M* potassium phosphate buffer (*p*H 7.4), 0.25*M* sucrose, and 0.001*M* mercaptoethanol. Organelles were isolated by differential centrifugation. The microsomal fraction was recombined with the mitochondria or nuclei (each equivalent to an amount from 100 mg of fresh tissue) and then incubated in homogenization medium containing calf serum (10 percent by volume). Final total incubation volume was 3.0 ml. All operations except incubation and final assay were at 0° to 4°C. Incubation in a Dubnoff incubator was at 29°C for 6 hours. The 3β-HSD activity was determined on the 24,000*g* supernatant by the spectrophotometric technique described previously (*4*). Sn = supernatant, 24,000*g* for 10 minutes. Mito = mitochondria. LH = 65 μg of ovine NIH-LH-S16 per milliliter of total incubation mixture. Bars and lines represent means and standard errors ($n = 3$). Testes from eight animals were pooled and each assay was run three times. One hundred percent is equal to approximately 2.5 nmole of the reduced form of nicotinamide adenine dinucleotide ($NADH_2$) per gram per minute formed between the second and twentieth minutes.

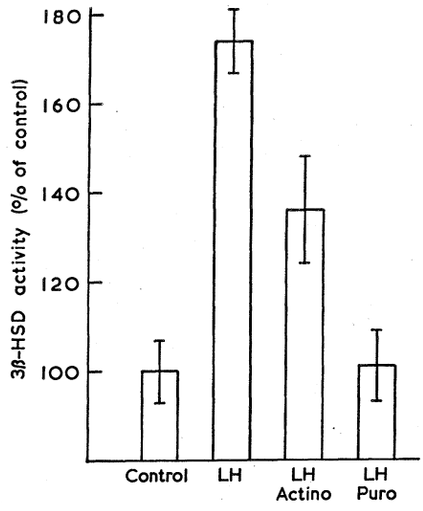

Fig. 2. Effect of actinomycin D and puromycin on LH-stimulated 3β-HSD activity in the microsome/mitochondria fraction of *Rana* testes. Procedures were similar to those described in Fig. 1. Fresh tissue equivalent was 100 mg, final total incubation volume was 4.0 ml, and incubation time was 4 hours. LH = 50 μg of ovine NIH-LH-S16 per milliliter of total incubation medium. Actino = 3 μg of actinomycin D per milliliter of total incubation medium. Puro = 3 μg of puromycin per milliliter of total incubation medium. Bars and lines represent means and standard errors, and $n = 3$.

Results of a typical experiment are shown in Fig. 1. The activity of 3β-HSD remains unaltered in the microsomal fraction which has been incubated with either LH, or nuclei, or mitochondria. However, a significant increase in enzyme activity results when the microsomes are incubated with LH in the presence of mitochondria; no such increased activity is observed when the nuclei are present instead of the mitochondria. Also, no further increase is observed when nuclei are included, in addition to the mitochondria, in the incubation mixture.

One immediate hypothesis to explain the above results is that an energy source is required for 3β-HSD activity —either to stabilize the enzyme, to prevent its breakdown, or to enhance its synthesis—and that the system in the mitochondria for producing energy-rich intermediates is stimulated by gonadotropins. However, in experiments in which microsomes in recombination with mitochondria were incubated together with either LH, growth hormone, thyroid-stimulating hormone, or adenosine triphosphate, only LH resulted in increased 3β-HSD activity (Table 1). These results suggest a hormonally specific reaction in which ATP is not the mediator. However, this does not rule out entirely the possibility that other energy-rich intermediates (that is, the so-called nonphosphorylated energy-rich compounds) are involved.

Table 1. The effect of adenosine triphosphate (ATP), LH, growth hormone (STH), and thyroid-stimulating hormone (TSH) on 3β-HSD activity in *Rana* testicular microsome fraction incubated together with mitochondria. Procedures were as in Fig. 1 but only the equivalent of 80 mg of fresh tissue was used for each incubation, and the final total incubation volume was 3.1 ml. Testes from seven animals were pooled and each assay was run twice ($n = 2$).

Treatment	3β-HSD activity (% of control, mean ± S.E.)
Sn (supernatant)	100 ± 7
Sn + mitochondria	97 ± 9
Sn + mitochondria + ATP*	92 ± 5
Sn + mitochondria + LH†	166 ± 7
Sn + mitochondria + STH†	101 ± 10
Sn + mitochondria + TSH†	84 ± 8

* Ten nanomoles per milliliter final total incubation volume. † Sixty-five micrograms per milliliter final total volume (ovine NIH-LH-S16; bovine NIH-TSH-B5; porcine STH, No. S-1501, Sigma).

In recent years the antibiotics actinomycin D and puromycin have frequently served as tools for investigating phenomena of hormone action on target cells. In order to determine whether the LH-induced increase in 3β-HSD activity involves transcription by the mitochondrial genome, organelles from *Rana* testes were recombined as described above and then incubated with either one of these antibiotics (Fig. 2). Both actinomycin D and puromycin resulted in decreased 3β-HSD activity. However, only puromycin caused a statistically significant (at $P < .05$) suppression of the hormone-stimulated enzyme activity. Actinomycin D is generally believed to act as a specific inhibitor of DNA-dependent RNA polymerase. DNA-dependent RNA polymerase has been reported in mitochondria, and like nuclear RNA polymerase it is sensitive to actinomycin D. However, there are reports that inhibition is not observed if intact mitochondria are used (*7*), and this may be the explanation for the incomplete suppression observed here. In vivo treatment with actinomycin D results in suppression of the gonadotropin-induced 3β-HSD activity in *Xenopus* testes (*4*), and in vitro experiments using whole homogenate of *Rana* testes also show a similar suppression (*6*). The fact that puromycin results in complete inhibition of the LH-induced increases in 3β-HSD supports the suggestion that protein synthesis is involved in this aspect of gonadotropin action (*4, 8*).

A mitochondrial ribosome has been isolated from the ovary of *Xenopus laevis* which is active in a submitochondrial protein synthesizing system (*9*). It contains two species of RNA which have sequences complementary to mitochondrial DNA and which apparently share no sequence homology with cytoplasmic ribosomal RNA. If we suppose that mitochondrial DNA may be able to code for the synthesis of other than mitochondrial protein via mitochondrial RNA, several possible mechanisms involving the mitochondrial genome may be forwarded to explain the present findings. On the one hand, it may be suggested that gonadotropins stimulate 3β-HSD activity in mature and/or maturing amphibian testes by acting on the mitochondrial DNA (possibly via a second messenger), resulting in increased synthesis of RNA, and, finally, more 3β-HSD. A second possibility is that gonadotropin action on the mitochondrial genome results finally in the production of a specific substance which protects the steroid dehydrogenase. Puromycin then may be assumed to inhibit the synthesis of proteinaceous end products in either situation.

JOHN P. WIEBE

Department of Biology,
Texas A&M University,
College Station 77843

References and Notes

1. J. M. Dodd, in *Marshall's Physiology of Reproduction*, A. S. Parkes, Ed. (Longmans, Green, London, 1960), pp. 417–582; W. S. Hoar, in *Fish Physiology*, W. S. Hoar and D. J. Randall, Eds. (Academic Press, New York, 1969), pp. 1–72.
2. R. I. Dorfman, in *Actions of Hormones on Molecular Processes*, A. Litwack and D. Kritchevsky, Eds. (Wiley, New York, 1964), pp. 470–481; W. A. Moyle and D. T. Armstrong, *Steroids* **15**, 681 (1970).
3. A. H. Baillie, M. M. Ferguson, D. McK. Hart, *Developments in Steroid Histochemistry* (Academic Press, London, 1966).
4. J. P. Wiebe, *J. Endocrinol.* **47**, 439 (1970).
5. L. T. Samuels, M. L. Helmreich, M. B. Lasater, H. Reich, *Science* **113**, 490 (1951).
6. J. P. Wiebe, in preparation.
7. D. E. Roodyn and D. Wilkie, *The Biogenesis of Mitochondria* (Methuen, London, 1968), pp. 31–52
8. J. P. Wiebe and V. F. Thornton, in preparation.
9. R. F. Swanson and I. B. David, *Proc. Nat. Acad. Sci. U.S.* **66**, 117 (1970).
10. Supported in part by the Texas A&M University Fund for Organized Research. The assistance of Miss Janice Larner is acknowledged. I thank the Endocrinology Study Section of N.I.H. for the LH and the thyroid-stimulating hormone.

13 August 1971