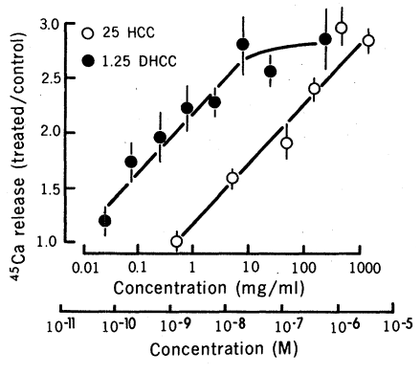

Fig. 1. Comparison of response to 1,25-dihydroxycholecalciferol (DHCC) and 25-hydroxycholecalciferol (HCC). Bones were first cultured for 24 hours, and then paired cultures were treated with or without 1,25-DHCC or 25-HCC at the indicated doses. Points indicate the means and vertical lines one standard error for the ratio of treated to control cultures of 48-hour ^{45}Ca release in 4 to 16 pairs of cultures. Data are pooled from five culture experiments.

in vivo, but is not concentrated by bone in culture (*10*). The specific activity of the 1,25-DHCC used in our experiments was sufficient to have detected a tenfold or greater concentrative uptake in bone cells. However, the radioactivity in chloroform-methanol extracts of bones treated with 1,25-DHCC was no greater than would be expected for an equivalent amount of medium. We cannot rule out a higher concentrative uptake in a small proportion of the bone cells.

The increased potency of 1,25-DHCC is specific to the extent that 21,25-DHCC, the only other dihydroxy metabolite tested in this system, is considerably less potent than 25-HCC (*11*). Moreover, the second hydroxylation does not appear to be required for 25-HCC to act. When labeled 25-HCC is incubated with fetal bones, there is no 1,25-DHCC detectable in the tissue or the medium (*12*).

Our studies do not tell us whether 1,25-DHCC acts on bone in vivo. Although the final level of mobilization achieved is no greater, 1,25-DHCC acts more rapidly than 25-HCC in mobilizing bone mineral in vivo in vitamin D–deficient rats (*13*). Because 25-HCC is present in plasma at much higher concentrations than 1,25-DHCC and probably has a longer half-life, it is possible that the physiologic or pathologic effects of vitamin D on bone resorption are actually mediated by the less potent metabolite. A low concentration in serum or short half-life could explain the low potency of 1,25-DHCC in curing rickets or mobilizing calcium in vitamin D–deficient rats. Whatever physiological role of 1,25-DHCC, the discovery of such a potent stimulator of bone resorption is of great interest.

L. G. RAISZ, C. L. TRUMMEL
Departments of Pharmacology and Toxicology and *Dentistry and Dental Research, University of Rochester, Rochester, New York 14642*
M. F. HOLICK, H. F. DELUCA
Department of Biochemistry, University of Wisconsin, Madison 53706

References and Notes

1. H. F. DeLuca, in *The Fat Soluble Vitamins*, H. F. DeLuca and J. W. Suttie, Eds. (Univ. of Wisconsin Press, Madison, 1970), p. 3.
2. C. L. Trummel, L. G. Raisz, J. W. Blunt, H. F. DeLuca, *Science* **163**, 1450 (1969); E. B. Olson and H. F. DeLuca, *ibid.* **165**, 405 (1969); L. G. Raisz, C. L. Trummel, in *The Fat Soluble Vitamins*, H. F. DeLuca and J. W. Suttie, Eds. (Univ. of Wisconsin Press, Madison, 1970), p. 93.
3. M. F. Holick, H. K. Schnoes, H. F. DeLuca, *Proc. Nat. Acad. Sci. U.S.* **68**, 803 (1971).
4. D. R. Fraser and E. Kodicek, *Nature* **288**, 764 (1970); R. Gray, I. Boyle, H. F. DeLuca, *Science* **172**, 1232 (1971).
5. M. R. Hausler, J. F. Myrtle, A. W. Norman, *J. Biol. Chem.* **243**, 405 (1968); R. J. Cousins, H. F. DeLuca, T. Suda, T. Chen, Y. Tanaka, *Biochemistry* **9**, 1453 (1970); D. E. Lawson, P. W. Wilson, E. Kodicek, *Biochem. J.* **150**, 269 (1969); M. R. Hausler, D. W. Boyce, E. T. Littledyke, H. Rasmussen, *Proc. Nat. Acad. Sci. U.S.* **68**, 177 (1971).
6. L. G. Raisz and I. Niemann, *Endocrinology* **85**, 446 (1969).
7. M. F. Holick, H. K. Schnoes, H. F. DeLuca, T. Suda, R. J. Cousins, *Biochemistry*, in press; J. Omdahl, M. F. Holick, T. Suda, H. F. DeLuca, Y. Tanaka, *ibid.*, in press; M. F. Holick and H. F. DeLuca, *J. Lipid Res.*, in press.
8. J. W. Blunt and H. F. DeLuca, *Biochemistry* **8**, 671 (1969).
9. L. G. Raisz and C. L. Trummel, in *Cellular Mechanisms for Calcium Transfer in Homeostasis*, G. Nichols, Jr., and R. H. Wasserman, Eds. (Academic Press, New York, 1971).
10. ———, H. Simmons, *Endocrinology*, in press.
11. C. L. Trummel, unpublished observations.
12. ——— and M. F. Holick, unpublished observations.
13. Y. Tanaka and H. F. DeLuca, *Arch. Biochem. Biophys.*, in press.
14. J. W. McArthur, H. Ulfelder, D. J. Finney, *J. Pharmacol. Exp. Ther.* **153**, 573 (1966).
15. Supported by PHS grants 5T1-DE3-15, AM-06205, and AM-14881 and the Harry Steenbock fund for the Wisconsin Alumni Research Foundation.

28 July 1971; revised 24 September 1971 ■

Spermatogenesis in Cultured Testes of the Cynthia Silkworm: Effects of Ecdysone and of Prothoracic Glands

Abstract. *In vitro spermatogenesis takes place when intact testes are cultured in blood plasma containing ecdysone or certain other steroids possessing ecdysone activity. The ecdysone requirement can be satisfied by culturing the testes in the presence of living, active prothoracic glands. The most likely explanation of these results is that the prothoracic glands constitute the principal source of ecdysone.*

Insect molting and metamorphosis depend on a hormone secreted by the prothoracic glands (*1*). In the absence of this hormone virtually all aspects of postembryonic development come to an abrupt halt. The hormone in question is thought to be ecdysone—a polyhydroxylated steroid first isolated in 1954 (*2*). By the injection of ecdysone, one can provoke all the developmental reactions that would otherwise require the presence of active prothoracic glands (*3*).

On the basis of this convincing but nonetheless circumstantial evidence, a long-standing principle of insect endocrinology is that ecdysone is synthesized and secreted by the prothoracic glands when the latter are activated by brain hormone (*4*). Manifestly one must allow for more complicated possibilities. For example, the very same results would be observed if the prothoracic glands secrete a hormone that promotes the release of ecdysone from some other endocrine organ. A further possibility is that the prothoracic glands secrete an inactive precursor of ecdysone, and that the precursor is transformed into active hormone elsewhere in the insect body. These prospects

Table 1. Spermatogenesis in vitro in intact testes of diapausing cynthia pupae cultured in blood plasma plus graded concentrations of α-ecdysone. After 7 days, the testes were torn open, and the percentages of developing germinal cysts were ascertained.

α-Ecdysone (μg/100 μl)	Testis cultures (No.)	Developed cultures (No.)	Developing cysts in responding testes (%)
8	2	2	60–90
4	4	4	30–70
2	4	4	40–60
0.4	2	2	15–50
0.16	2	2	25–40
0.08	3	3	15–40
0.04	2	2	15–40
0.01	4	4	10–40
0.005	4	2	10–30
0.001	4	0	0

Table 2. Spermatogenesis in vitro in intact testes of diapausing cynthia pupae cultured in blood plasma: effects of simultaneously cultured brains or prothoracic glands (or both). After 3 to 6 days the testes were torn open and the percentages of developing germinal cysts were ascertained.

Endocrine organs	Cultures (No.)	Developed cultures (No.)	Developing cysts in responding testes (%)
Controls			
None	15	0	0
Larvae (1st day of spinning)			
Active prothoracic glands	10	9	30–80
Active prothoracic glands (homogenized)	6	0	0
Pupae (immediately after pupation)			
Inactive prothoracic glands	9	0	0
Active brain*	9	0	0
Inactive prothoracic glands plus inactive brain†	6	0	0
Inactive prothoracic glands plus active brain*	6	4	10–40

* Brains from nondiapausing individuals reared to pupation under long-day conditions (17 hours light : 7 hours darkness). † Brains from diapausing individuals reared to pupation under short-day conditions (12 hours light : 12 hours darkness).

merit serious attention because, insofar as we are aware, no one has demonstrated any trace of ecdysone activity in extracts or homogenates of prothoracic glands.

On the basis of electron microscopic studies of the cells of the lepidopteran *Calpodes ethlius*, Locke (*5*) has suggested that ecdysone is secreted by the oenocytes within the abdomen and that these cells are activated by a nonsteroid hormone secreted by the prothoracic glands. This same hypothesis is favored by Weir (*6*) on the basis of ligation experiments performed on *C. ethlius*. Even more surprising is the report of the recovery of traces of labeled α- and β-ecdysone 24 hours after the injection of [4-^{14}C]cholesterol into isolated abdomens of larvae of the commercial silkworm *Bombyx mori* (*7*). For these several reasons the source of ecdysone within the living insect has become a matter of increasing concern (*8*).

In our experiments we used an in vitro system to examine the problem. Testes of diapausing pupae of the cynthia silkworm, *Samia cynthia*, were excised and cultured in depression slides containing 100 μl of medium, which consisted of cell-free blood plasma derived from diapausing cynthia pupae 5 to 6 months after pupation. As already described (*9*), plasma of this type contains a high titer of a "macromolecular factor" which is necessary for the transformation of spermatocytes into spermatozoa. After 3 to 7 days of culture at 25°C, each testis was torn open and the developmental status of the germinal cysts was determined by microscopic examination.

We found that spermatogenesis occurred within the cultured testes only when ecdysone was added to the medium. Significant development invariably took place when the 100 μl of medium contained not less than 0.01 μg of α-ecdysone (Table 1). The lower dose of 0.005 μg provoked significant development in two of four cultured testes, whereas the still lower dose of 0.001 μg was completely ineffective. The critical dose of 0.01 μg corresponds to a final concentration of 1 part of α-ecdysone per 10 million parts of medium ($2 \times 10^{-7}M$).

In similar experiments we tested the effectiveness of a number of other steroids, including cholesterol and five different mammalian steroid hormones (*10*). The effects of α-ecdysone were duplicated by β-ecdysone and by a series of phytoecdysones (*11*), all of which are highly active in provoking adult development when injected into diapausing cynthia pupae (*12*). By contrast, the other steroids lacking ecdysone activity in vivo were also inactive in vitro.

Having established that spermatogenesis requires catalytic amounts of ecdysone or ecdysonelike materials, we tested the ability of prothoracic glands to satisfy this requirement by culturing them along with the testes. The results (Table 2) were unambiguous. Prothoracic glands, which were known to be endocrinologically active on the basis of in vivo experiments (*4*), were able to provide the necessary ecdysone. So also, prothoracic glands known to be endocrinologically inactive were inactive in vitro. However, it is of particular interest (Table 2) that glands of this sort could be "turned on" when active brains were also placed in the cultures. Essentially the same results were observed in 75 additional preparations in which the testes of *Samia cynthia* were cultured in the presence of active or inactive organs of *Antheraea polyphemus* or *A. pernyi*. Oenocytes dissected from the abdomens of cynthia larvae during the first day of spinning showed no activity when tested in three cultures.

In summary, we find that a critical level of ecdysone activity can be generated within an in vitro system containing a minimum of components: cell-free hemolymph, a diapausing testis, and living, endocrinologically competent prothoracic glands. Since this activity failed to appear in the absence of the prothoracic glands, our experiments support the view that the prothoracic glands synthesize and secrete one or more materials with ecdysone activity. The only other reasonable alternative is that the prothoracic glands secrete an ecdysone precursor that can be converted into active hormone by the plasma or the reacting tissues—in this case the testis itself.

MICHAEL P. KAMBYSELLIS*
CARROLL M. WILLIAMS
Biological Laboratories,
Harvard University,
Cambridge, Massachusetts 02138

References and Notes

1. S. Fukuda, *J. Fac. Sci. Univ. Tokyo Sect. IV* **6**, 477 (1944); C. M. Williams, *Biol. Bull.* **93**, 89 (1947).
2. A. Butenandt and P. Karlson, *Z. Naturforsch.* **9b**, 389 (1954).
3. C. M. Williams, *Anat. Rec.* **120**, 743 (1954); V. B. Wigglesworth, *J. Exp. Biol.* **32**, 649 (1955).
4. C. M. Williams, *Biol. Bull.* **103**, 120 (1952).
5. M. Locke, *Tissue Cell* **1**, 103 (1969).
6. S. B. Weir, *Nature* **228**, 580 (1970).
7. K. Nakanishi, *Proc. Int. Union Pure Appl. Chem. Congr. 23rd*, in press; K. Nakanishi, H. Moriyama, T. Okauchi, S. Fujioka, M. Koreeda, *Chem. Commun.*, in press.
8. After we submitted our report, F. Romer [*Naturwissenschaften* **58**, 324 (1971)] briefly reported ecdysone activity (as determined by the *Calliphora* assay) in extracts prepared from oenocytes, prothoracic glands, and fat body of "prepupae" of the beetle *Tenebrio molitor*. We are unable to evaluate these experiments in the form in which they were presented.
9. M. P. Kambysellis and C. M. Williams, *Biol. Bull.*, in press.
10. The mammalian hormones included 17β-estradiol, estriol, progesterone, aldostosterone, and deoxycorticosterone.
11. The phytoecdysones included cyasterone, inokosterone, ponasterone A, and ponasterone C.
12. C. M. Williams, *Biol. Bull.* **134**, 344 (1967).
13. For helpful discussions and critical reading of the manuscript we thank Professor Lynn M. Riddiford and Dr. James Truman. Supported by the Rockefeller Foundation and NSF grant GB-26539.
* Present address: Department of Biology, New York University, University Heights, Bronx 10453.

5 August 1971; revised 13 September 1971