Control of Growth and Development in Insects

Several growth hormones appear to be isoprenoid derivatives, and some may act upon the cell nucleus.

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The postembryonic growth of higher organisms is under hormonal control during most of the organism's life. The best-studied groups are the vertebrates and higher plants, but increasing attention is being paid to invertebrates, especially arthropods.

Insect endocrinology has made startling advances during the past decade. Three insect hormones have been crystallized: the molting hormone, by Butenandt and Karlson (*1*); the queen substance of bees, by Butler and his co-workers (*2*); and a substance with some of the properties of the brain hormone, by Kobayashi and his coworkers (*3*). Extracts of several other hormones have been prepared and partially purified: (i) a polypeptide or protein with the activities of the brain hormone, by Ichikawa and Ishizaki (*4*); (ii) the juvenile hormone, by Williams (*5*) and by others (*6*, *7*); (iii) the diapause hormone, by Hasegawa (*8*); (iv) a hormone regulating carbohydrate metabolism, by Steele (*9*); and a hormone regulating hardening and darkening of blowflies, by Cotrell and by Fraenkel and his associates (*10*). A year ago we knew of no chemicals which could mimic the action of insect growth hormones. We now know of more than a dozen chemicals which can simulate the effects of these hormones. In this discussion we summarize a few recent developments in the field of insect growth hormones which seem to suggest new approaches to comparative endocrinology and perhaps to developmental biology as a whole (*11*).

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The distinguishing feature of insect growth and development is the process of molting. The epidermis of an insect is bound to an outer layer of cuticle, and it cannot grow until it becomes detached from this cuticle. Periodically the epidermal cells of immature insects become detached from their cuticle, grow by cell division or cell enlargement, or both, and secrete a new and extensible cuticle with folds. When this new cuticle is nearly complete, the epidermal cells produce and release enzymes which digest the inner layers of the old cuticle, which they then absorb. Thereupon the epidermal cells waterproof the new cuticle, and the old cuticle is shed. An insect, such as a young cockroach, that has a rigid body fills itself with air immediately after the molt, when its cuticle is soft; when the cuticle hardens the insect has an enlarged, rigid skin which provides room for its internal organs to grow. A soft-bodied insect such as a caterpillar remains quite wrinkled after it molts, but gradually increases in size during the intermolt period until its cuticle is stretched and smooth.

Because of the process of molting, it is commonly claimed that insect growth is discontinuous. This is not strictly true in many insects: caterpillars, as we have just noted, increase in body weight steadily during their life. However, if we consider only one aspect of growth—namely, cell division—then indeed it may be said that the growth of many insect tissues is discontinuous. For example, in caterpillars and in many other insects the increase in size of the skin and of many internal organs between molts commonly occurs without an increase in cell number. Here, cell multiplication is periodic and is restricted to the period just prior to or just after shedding of the skin. Thus, unlike immature mammals, whose growth is essentially continuous, most immature insects have discontinuous growth, the visible expression of which is molting.

The periodic cell multiplication and molting of immature insects is brought about by two hormones, one produced by secretory cells in the insect's brain and the other by glands in the prothorax, the prothoracic glands. The "brain hormone" (*12*) acts by stimulating the prothoracic glands. The prothoracic glands respond to this stimulus by releasing prothoracic gland hormone, "ecdysone," which in turn acts on various cells and causes them to grow. In the case of the insect's epidermal cells, the prothoracic gland hormone sometimes stimulates cell division, but it always causes these cells to deposit a new cuticle and to molt. If a few micrograms of ecdysone are injected into an isolated fragment of an insect—for example, an isolated abdomen—the fragment will molt. The prothoracic gland hormone is thus a true growth and molting hormone for insects and perhaps for all arthropods.

A third hormone, the juvenile hormone, is secreted by endocrine glands known as the corpora allata, which are located near the brain of the insect. Nearly 30 years ago Wigglesworth showed that this hormone promotes larval development but prevents metamorphosis (*13*). The presence of juvenile hormone in the immature insect insures that when the larva molts it will retain its larval characters and not differentiate into an adult. The juvenile hormone is a remarkable agent which permits growth but prevents maturation.

When larval cells are stimulated to grow and molt by the prothoracic gland hormone, the presence of a high concentration of juvenile hormone causes them to use their synthesizing machinery to secrete larval cuticle. In the presence of a small amount of juvenile hormone, the same cells secrete pupal cuticle; in the absence of juvenile hormone, these cells may secrete adult cuticle directly and by-pass the pupal molt. Through regulation of the release of brain hormone, molting is controlled; through regulation of the release of juvenile hormone, maturation is controlled. Control of maturation by the juvenile hormone is also evident in many adult insects, where the juvenile hormone, or at least a secretion of the corpora allata, stimulates the matura-

tion of eggs in females and the development of accessory glands in males. These various endocrine events are summarized schematically in Fig. 1 (see *7*, *14–16*). With these basic endocrine events in mind, let us consider the hormones themselves.

"Brain Hormone"

The first extracts of "brain hormone" were prepared in 1958 by Kobayashi and Kirimura, who succeeded in extracting oily material from 8500 surgically isolated silkworm brains which had "brain hormone" activity (*3*). Injection of 0.1 milligram of extract into brainless silkworm pupae caused them to molt. The active principle appeared to be a lipid. Subsequently, water-soluble extracts with brain hormone activity were prepared by Ichikawa and Ishizaki (*4*), and these appear to be polypeptides.

Recently, Kobayashi and his coworkers reported the isolation and identification of an active principle in lipid extracts of insect brains (*3*). They painstakingly dissected out 220,000 brains from silkworm pupae, homogenized and extracted them with methanol, re-extracted the dried methanolic extract with ether, and then purified the extract, largely by countercurrent distribution in a variety of solvent systems. They reported that the crystallized brain hormone is probably identical with cholesterol. Brainless pupae molted when injected with the extract (0.02 μg in 10-percent ethanol). Kobayashi and his associates also reported that commercial preparations of cholestanol and 7-dehydrocholesterol had "brain hormone" activity.

This astonishing result must have prompted a number of laboratories to check their findings. John Edwards and Krishnakumaran at Western Reserve University and Gilbert at Northwestern University have confirmed a key result. Commercial preparations of cholesterol and of several other sterols in microgram amounts activate prothoracic glands and cause brainless pupae to molt. Table 1 gives data for a typical experiment. Hundreds of control preparations of brainless pupae have been examined, and none of these has ever molted, nor have isolated abdomens injected with these same sterols.

The reader may wonder how entomologists, who are traditionally accused of bombarding insects with every known chemical, could have failed to try cholesterol. The answer is that many did try cholesterol but apparently did not use the proper solvent system or the right concentrations. For reasons which are not yet clear, 9- or 10-percent ethanol is the solvent of choice.

Although it has been possible to confirm the effectiveness of cholesterol and other sterols as mimics of the brain hormone, the results in Table 1 are difficult to understand, for there appears to be no clear correlation between sterol concentration and brain hormone activity. The data suggest that some factor other than chemical structure may be responsible for the brain hormone activity of these alcoholic preparations. Perhaps surface activity or some other properties of the sterol solutions and suspensions are involved. There is also other evidence that brain hormone and cholesterol are not identical. For example, Goodfellow and Gilbert (*17*) have shown that cholesterol levels in the blood are quite high at all stages: female pupae of the cynthia moth contain 520 micrograms of sterols (largely cholesterol) per milliliter, and male pupae contain 300 micrograms per milliliter of blood. The pupa possesses about 1 milliliter of blood. Thus the amount of cholesterol which appears to cause molting is about 0.0001 the amount normally present in the blood (*18*)! Notwithstanding these curious observations, it seems clear that substances with brain hormone activity derived from insect brains have properties similar to cholesterol, and that several sterols have brain hormone activity. If this work is further confirmed and the active principle in the extracts should prove to be a steroid, it would be most exciting. For the present, the work stands as the first report of a steroid with hormonal effects on an insect.

Table 1. Effect of injecting sterols into brainless pupae of *Samia cynthia*. The brains of the pupae were removed 3 months prior to injection, and the pupae were inspected prior to assay to make sure they were not developing. All injections were in 9-percent ethanol; 50 to 250 microliters were injected into each pupa. No effects were observed when injections were made into isolated abdomens, nor were effects observed with injections of up to 5.0 μg/g (fresh weight) of stigmasterol, stigmasterol acetate, dehydrocholesterol, viosterol, or dihydroisoandrosterone. About nine pupae were tested at each concentration except when ethanol alone was used; in that case 50 pupae were tested.

Amount injected (μg/g, fresh weight)	Percentage completing adult development
Cholesterol	
0.005	40
.05	60
.5	20
5.0	15
Ergosterol	
0.005	0
.05	33
.5	0
5.0	48
Beta sitosterol	
0.005	33
.05	67
.5	0
5.0	0
Ethanol 9-percent only	
	0

How does the brain hormone act? The brain hormone has as its principal target the prothoracic glands. As Williams first showed in 1947, the brain hormone stimulates the prothoracic glands to secrete ecdysone (*19*). What is this "stimulation" in biochemical terms? To answer this question Oberlander (*20*) has studied, with autoradiographic techniques, the effects of brain hormone on the prothoracic glands of various wild silkworms. The first event of activation that he has observed is intense RNA synthesis within the nuclei of prothoracic gland cells, followed by the appearance of cytoplasmic RNA and by protein synthesis. Presumably these events reflect the synthesis of enzymes necessary for the production of ecdysone, and they are followed by the release of the hormone. From these observations it is clear that, as far as the prothoracic glands are concerned, brain hormone is a growth hormone and stimulates the prothoracic gland cells in much the same way that ecdysone stimulates other cells of the insect's body. Whether the brain hormone stimulates other cells besides those of the prothoracic gland remains to be demonstrated.

Ecdysone

Ecdysone was first crystallized by Karlson and Butenandt in 1954 (*1*). Its partial structure has just been published. From an analysis of 250 milligrams of crystals isolated from 1000 kilograms of dried pupae of *Bombyx mori* (the silkworm of commerce), Karlson and his co-workers (*21*) concluded that ecdysone was a steroid with the empirical formula $C_{27}H_{44}O_6$. The key to its identification as a steroid was an x-ray diffraction analysis of the crystals by a new "diffuse scattering" method, which permitted determination of the shape of the molecule and the molecular weight without the introduction of heavy atoms. From ultraviolet, infrared, and proton reso-

nance spectra it was concluded that ecdysone had a keto group at carbon atom 12 conjugated to a double bond between carbon atoms 9 and 11, a hydroxyl group on the side chain at carbon atom 25, and four other hydroxyl groups at undetermined locations. Its identification marks the culmination of two decades of painstaking work. Karlson is sensibly cautious in advancing his claim and states that "even the partial structure requires further confirmation" (*14*). However, it is clear that he is convinced that ecdysone is a steroid, a view which is shared by most organic chemists who have examined his data, and a view which we have adopted.

The discovery that ecdysone is a steroid is of uncommon interest. But equally remarkable are some recent reports on its mode of action, which appear to have important consequences for students of growth and suggest that this agent may act on the genes themselves or on the nuclear membrane. Now, it has long been assumed that the activity of genes varies in different tissues and at different developmental stages. We might expect to find different genic activity in an epidermal cell of a dormant pupa and in the same cell engaged in adult synthesis. Experimental support for this opinion comes from recent studies by Beermann and others, who have described characteristic puffs or swelling in certain segments of the giant chromosomes in the salivary glands of developing Diptera, as well as differences in giant chromosome morphology in different tissues (*22*). These giant chromosomes consist of hundreds of individual chromosomal threads and are found in several tissues. Chromosomal puffs indicate a local loosening of the bundles of threads and reflect enhanced metabolic activity in particular chromosomal regions which probably are the site of heightened gene activity (*23*). Studies with tritiated uridine and with labeled amino acids reveal that RNA synthesis and protein synthesis proceed at a rapid rate in puffed regions. These observations suggest that the changing biochemical activities of insect cells during development may be caused by the differential activation or suppression of different sets of genes (*24*). Since ecdysone initiates the postembryonic growth and development of insects, one is led to the view that ecdysone may have as a principal target the nucleus itself, where it activates particular chromosomal regions and brings about "the elaboration of specific substances (RNA?) that are destined to participate . . . in the cytoplasmic syntheses that characterize growth and molting" (*25*).

Recently, Clever and Karlson (*26*) have provided direct evidence to support this conjecture. They have shown that injection of pure ecdysone into *Chironomus* larvae causes prompt and characteristic changes in the puffing pattern of the chromosomes, changes identical to those that occur during pupation. In more recent experiments, Clever (*27*) has shown that injection of ecdysone causes new puffs to appear within 15 minutes of injection and that the size of the puffs is proportional

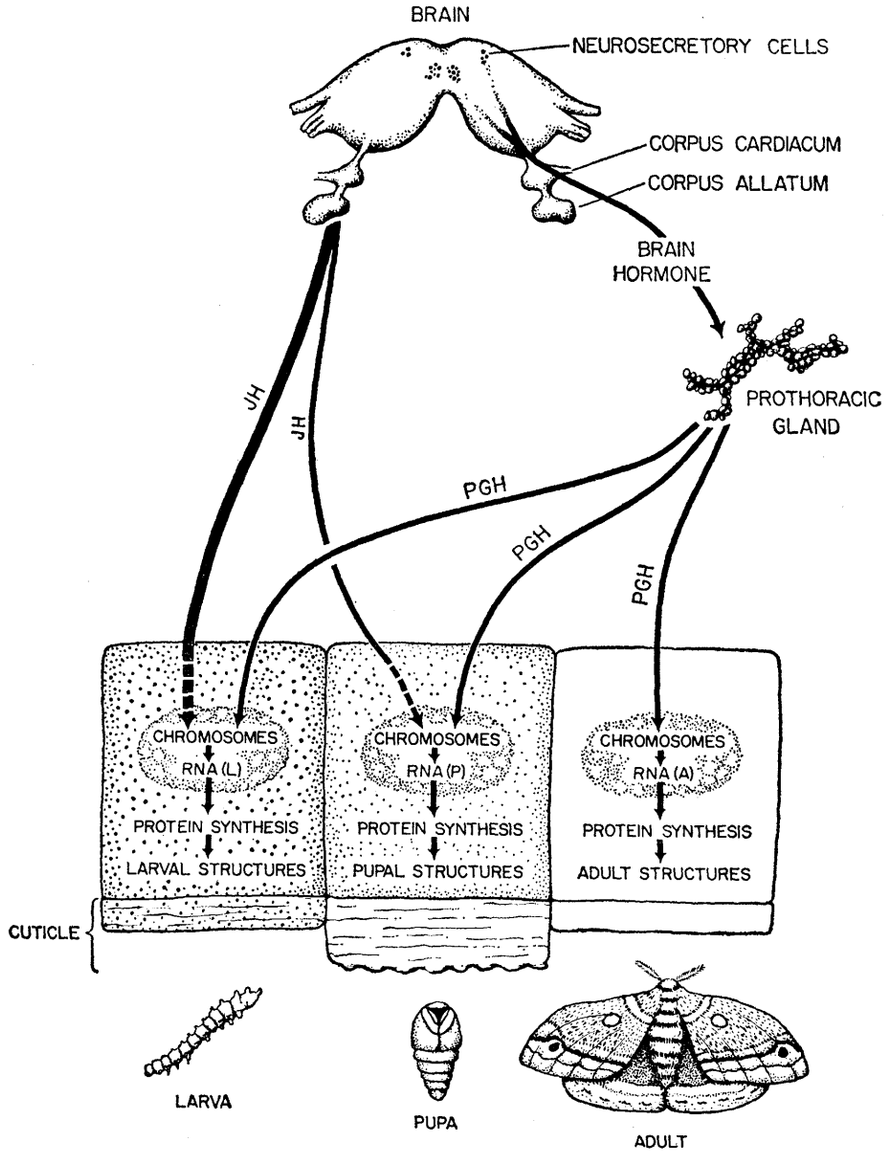

Fig. 1. Schematic diagram of the principal endocrine organs of the cecropia silkworm and the site of action of their hormones. The larva of this insect molts four (or occasionally five) times. These larval molts appear to be initiated by brain hormone, which stimulates the prothoracic glands to secrete prothoracic gland hormone. At the same time the corpora allata secrete juvenile hormone, which favors larval syntheses, so that when the larva molts in response to prothoracic gland hormone it molts into a larva. At the end of larval life the corpora allata cease secreting and thus the mature larva is left with a low concentration of juvenile hormone. At the next molt the epidermal cells respond to prothoracic gland hormone and to the low concentration of juvenile hormone by secreting pupal cuticle, and in response to these hormonal conditions other tissues within the insect either break down or transform into pupal structures. At the final molt, no juvenile hormone remains, and the epidermal cells respond to prothoracic gland hormone alone by secreting adult cuticle; the other pupal tissues either break down or develop into adult structures. The Lepidoptera, unlike many other insects, appear not to require the corpora allata for egg maturation.

to the dose. As little as 10^{-5} microgram is effective. One particular puff on one chromosome appears to be affected before any of the others. In these experiments ecdysone appears to act on the chromosomes and activates particular gene loci to bring about a variety of syntheses that culminate in molting.

In an equally impressive series of experiments, Kroeger (*28*) has demonstrated that a number of chemicals copy the effects of ecdysone on the puffing pattern of salivary chromosomes of *Chironomus*; zinc ions, cadmium ions, and narcotics cause a puffing pattern identical to that caused by ecdysone (although they do not cause pupation and molting). By surgically removing parts of the chromosome set, Kroeger was able to show that each puff responded independently of other parts of the genome. This finding suggests that puff activity patterns are not caused by interactions of various parts of the genome (for example, by one puff activating a second puff, and so on) but result from a changed intranuclear environment, perhaps from a change in the nuclear membrane (*28*). Kroeger believes (*29*) that these several agents, by acting on the nuclear membrane, affect the balance of sodium and potassium ions in the nucleus. He argues that it is changes in the sodium and potassium ions that cause changes in the puffing pattern of the chromosomes. To support this view he shows that by changing the potassium and sodium concentrations of the nucleus he can cause puffs to increase and decrease in size on demand. Presumably, in the normal situation ecdysone is responsible for the changes in the permeability of the nuclear membrane; in experimental situations other agents, such as zinc ions, produce similar permeability changes.

Perhaps the juvenile hormone, which can modify events initiated by ecdysone, has a similar site of action in the nucleus. Becker (*30*), in studies of salivary chromosomes in *Drosophila*, and Kroeger (*28*), in studies on *Chironomus*, have shown that wounding blocks and reverses puff patterns induced by ecdysone. Kroeger believes that this reversing of the puffing patterns is analogous to the action of juvenile hormone.

Recently Laufer and his co-workers (*31*) have succeeded in relating changes in the puffing pattern of chromosomes with changes in the synthesis of specific enzymes. They showed, for example, that actinomycin D, an antibiotic which inhibits DNA-dependent synthesis of RNA in many systems, inhibits both puffs and the synthesis of certain proteins in the salivary gland cells of *Chironomus thummi*.

Table 2. Incorporation of tritiated thymidine into pupae of *Hyalophora cecropia* at various times after pupation. The pupae were fixed 2 hours after a single injection (10 μc/g) (*66*).

Tissue	Days after pupation				
	0	2	5	10	20
Fat body	0	0	0	0	0
Epithelium	0	0	0	0	0
Muscle	0	0	0	0	0
Blood cells	+	+	+	0	0
Brain	+	+	+	0	0
Thoracic ganglion	0	0	0	0	0
Malpighian tubules	0	0	0	0	0

These experiments on the puffing of giant chromosomes promise to become landmarks in biology. They have removed insect hormones from the parochial field of entomology to the center of contemporary biological thought. For a central problem of biology today is: How are genes activated and what is gene activity? (*32*). With this evidence that the activity of certain genes may be under the control of a specific chemical agent (ecdysone), and because the chromosomes of Diptera are so large that the biochemical events of gene activity can be analyzed by fine-resolution autoradiography, it seems likely that within the next few years the study of insect chromosome metabolism will become a most active area of biological research. For the student of genetic mechanisms in multicellular organisms, insect chromosomes may again become strategic research objects and regain some of the esteem they lost in 1947 when Max Delbrück introduced geneticists and biochemists to the elegance and simplicity of bacteriophage as an object of research.

Table 3. Rate of uptake of tritiated uridine into pupae of *Hyalophora cecropia* at various times after pupation. The pupae were fixed 2 hours after a single injection (10 μc/g) (*67*).

Tissue	Days after pupation				
	0	2	5	10	20
Epithelium	++	++	+	0	0
Muscle	+	+	0	0	0
Fat body	+	+	+	+	0
Blood cells	++	++	++	++	++
Malpighian tubules	+++	+++	+++	++	++
Nerve tissue	+	+	+	+	+
Oenocytes	++	++	++	++	++
Connective tissue	+	+	+	0	0
Wing bud	+	+	+	+	0

Thus, ecdysone may have the chromosomes as a primary target, but how are its effects manifested in different cells and tissues? Do all cells require ecdysone for DNA synthesis and cell division, for RNA synthesis and protein synthesis? What synthetic activities remain possible in cells which have been deprived of ecdysone? The newly molted insect provides a perfect experimental situation for answering this question, because, after a molt, ecdysone secretion ceases. Krishnakumaran attempted to answer the question by measuring with autoradiography the ability of various tissues to synthesize DNA, RNA, and proteins (*33*). We studied, first, the larval-pupal molt of the cecropia silkworm. This is a special molt, for the pupa that is formed enters a prolonged period of developmental arrest, or pupal diapause, during which ecdysone secretion ceases. At various times after pupation the pupae were injected with tritiated thymidine to determine the rate of DNA synthesis, or with tritiated uridine to determine the rate of RNA synthesis. The results of a typical experiment are seen in Table 2. When the larva molts into a pupa destined to enter diapause, DNA synthesis ceases except in the blood cells and in parts of the brain that continue to make DNA for nearly a week. Table 3 indicates the rate of RNA synthesis in different tissues. The different tissues "shut down" their RNA synthesis at different times after pupation. Thus, 3 weeks after pupation, epithelium, muscle, fat body, and imaginal discs are inactive, while blood cells, malpighian tubules, nerve tissue, and oenocytes continue to synthesize RNA. Apparently, almost all tissues of the pupa require ecdysone for DNA synthesis. By contrast, RNA synthesis continues in many tissues when little or no ecdysone is present, while other tissues require ecdysone for RNA synthesis. These findings are being pursued in a variety of ways and promise a simple and direct approach to some of the problems of ecdysone action in insects.

Ecdysone profoundly affects the development of insects, but it must not be supposed that developmental processes cannot proceed without it. In many insects, fragments deprived of prothoracic glands or brains are still capable of considerable biosynthesis. Thus, when a large piece of the imag-

inal disc which is destined to give rise to the future pupal and adult wing is removed from the larva of the flour moth, *Ephestia*, the insect will regenerate the imaginal disc completely in the absence of both brain and prothoracic glands (*34*). A fragment of a larval genital disc of *Drosophila* will also regenerate and grow to full size when transplanted into an adult that has no prothoracic glands (*35*). Similarly, isolated fragments of cecropia pupae can heal large wounds and secrete a modest cuticle. Even adult insects whose prothoracic glands have degenerated during the course of adult development are capable of growth, wound healing, and tissue regeneration. Adult cockroaches, for example, during their long adult life, continue to produce spermatogonia, oogonia, ovarial sheath cells, and probably many other cells throughout life; they can also regenerate nerve fibers that have been surgically removed (*36*, *37*). In our preoccupation with the growth hormones of insects, we must not forget that the tissues of most insects, like those of most other metazoa, are capable of considerable regeneration whether or not hormonal conditions favor the continuation of development. A besetting problem in insect endocrinology is that of distinguishing the intrinsically controlled biosynthetic activities of wound healing and regeneration from the extrinsically controlled biosynthteic activities of molting and metamorphosis (*38*).

Juvenile Hormone

In 1956 Williams prepared the first extract of juvenile hormone from cecropia moths (*5*). When these extracts were injected into pupae, the pupae molted into second pupae: the epidermal cells, instead of secreting an adult cuticle, synthesized a second pupal cuticle. Injection of varying amounts of these extracts produced graded effects, thus providing the basis for a bioassay of the juvenile hormone. Later, we showed that extracts of bovine adrenal glands possessed juvenile hormone activity (*39*). Subsequently, juvenile hormone activity was found in extracts of the tissues of many invertebrates and vertebrates and in plants and microorganisms (*40*). These results indicate that we are dealing with a group of substances which are widespread in nature.

Table 4. Some chemicals with juvenile hormone activity (*68*).

Substance	Units of juvenile hormone activity per gram*
Cecropia oil	1000
Phytol	32
Isophytol	0
Farnesol (commercial)	16
All-*trans* farnesol	140
Farnesal	32
Farnesyl acetate	5.4
Farnesenic acid	7.8
Hexahydrofarnesol	0
Nerolidol	8.9
Linalool	0.08
Geraniol	0
Geranyl linalool	0.14
Solanesol	.05

*Different units are employed by different workers to assay juvenile hormone activity. The common feature of these assays is the use of male cecropia oil as a standard. The assay used in the experiments recorded in this table was the *Galleria* wax test (see text and *44*). We have arbitrarily established as 1 *Galleria* unit of juvenile hormone activity, the juvenile hormone activity shown in the wax test by 1 mg of oil extracted by ether from the abdomens of male cecropia moths which have been permitted to age for 3 days at 25°C. These cecropia oil preparations are remarkably uniform in activity, and we have kept standard extracts at –20°C for a number of years without detectable loss of activity.

Recently the active principle of one of these juvenilizing extracts was isolated by Schmialek (*41*) from mealworm feces and shown to consist of the open-chain terpene alcohol, farnesol, and its aldehyde, farnesal. Farnesol copies the action of the juvenile hormone and also copies the action of the gonadotropin secreted by the corpora allata in promoting egg development (*42*). It was the first substance of known structure to act like an insect hormone, and its isolation was a major achievement in comparative endocrinology. Unfortunately, farnesol does not appear to be the juvenile hormone, for in most test systems pure farnesol is required in milligram or, at best, tenths of a milligram amounts, while purified preparations of the juvenile hormone of insects are active in millimicrogram amounts.

Farnesol is a straight-chain terpene primary alcohol composed of three isoprene units, and, as a precursor of squalene and other polyisoprenoids and of sterols in yeast and liver, it has been of considerable interest. It has four possible steric isomers, the isomerism occurring at the Δ^2 and Δ^6 positions of the molecule. Recently Yamamoto and Jacobson (*43*) determined that only isomers with a *trans* configuration at the Δ^6 position were active. In our laboratory we discovered that hexahydrofarnesol was inactive, a finding which suggests that some unsaturation is necessary for activity. The fact that farnesol had some juvenile hormone activity led us to explore a number of other terpenes. During the past 2 years we have examined several hundred compounds in a variety of test systems. In the type of assay we used most often, substances to be tested were dissolved in mixtures of paraffin wax and peanut oil and applied to wounds made with hypodermic needles on the thorax of pupae of the moths *Antheraea polyphemus* and *Galleria mellonella* (*44*). If the substance was inactive, the cells of the healed wound secreted a thin, scaly adult cuticle which revealed itself at the pupal-adult molt. If the substance had juvenile hormone activity, the cells in the region of the wound secreted an island of thick, rough pupal cuticle which was surrounded by normal adult cuticle. This pupal cuticle is wholly different from incompletely developed adult cuticle and has an unmistakable morphology (*45*).

The first terpene that came to mind was phytol, the C_{20} mono-unsaturated alcohol derived from chlorophyll (Table 4). Like farnesol it is an allylic primary alcohol. It too had some juvenile hormone activity, but isophytol, a rearrangement of phytol with a tertiary hydroxyl group, had no activity. We then turned our attention to a variety of other, related compounds. As Table 4 shows, some had activity while others did not, and there appeared to be no clear pattern except that all were polyisoprenoid compounds (*46*). The smallest was a C_{10} tertiary alcohol, linalool. At this juncture we began carefully to examine compounds other than terpenes. We tested a variety of straight-chain saturated alcohols, from ethanol on up through compounds with 20 or more carbon atoms, as well as other compounds. One alcohol—1-heptanol—had some juvenile hormone activity in the wax test. Similarly, commercial preparations of a sterol, beta sitosterol, had some juvenile hormone activity when injected into pupae of *Antheraea polyphemus* (*47*). Recent studies by Wigglesworth and Schmialek (*48*) and by Karlson (*14*) have also uncovered terpene derivatives with juvenile hormone activity, including two with activity greater than farnesol—farnesyl-*o*-methyl ether and farnesyl diethylamine. Karlson reports that in his test for juvenile hormone activity these two compounds have more than 200 times the activity of farnesol or of cecropia

extracts. In our assay systems—the wax test and the polyphemus injection assay—these compounds appear to be only about ten times as active as purified all-*trans* farnesol (*49*).

The significance of the juvenile hormone activity of these diverse compounds deserves analysis. (i) None of the compounds seems to be the natural juvenile hormone; and in our assay none of the active pure compounds appears much more active than the crude oil extracted from adult cecropia moths, which has served as a starting material for most purifications of juvenile hormone, and none is nearly as active as partially purified preparations of juvenile hormone. In considering the data of Table 4, it is worth noting that the most highly purified preparations of juvenile hormone for which a description of purification was given contained 200,000 Galleria units per gram (*7*, and Table 4, footnote), while the most active compounds discovered thus far contained only 1000 Galleria units per gram. (ii) Since terpenes, sterols, and alcohols occur widely in nature, it seems likely that the juvenile hormone activity reported in extracts from vertebrates, invertebrates, plants, and microorganisms is due to compounds of this sort rather than to the juvenile hormone. (iii) The widespread occurrence of such juvenilizing compounds makes the isolation of the true active principle of the corpora allata somewhat more difficult (*50*), since several of these substances contaminate insect extracts. [For example, lipid extracts of male cecropia moths contain 5 to 15 μg of farnesol per gram and three times this amount of phytol (*17*).] Thus, the insect endocrinologist faces the same problem that the "bar substance" posed for the *Drosophila* geneticist and the "inducer" posed for the experimental embryologist—namely, that substances other than the natural one cause developmental effects (*42*, *51*). The possible relation of these substances to the true juvenile hormone deserves consideration. There are many possibilities, but two are particularly attractive.

The first is the possibility that the substances resemble the juvenile hormone in some structurally significant way and act at the same site in the cell. The second is the possibility (which does not exclude the first) that the various compounds are precursors of the juvenile hormone and may be converted within the insect's body. Certainly insects can synthesize higher alcohols, and probably terpenes such as geraniol (*52*). However, if these substances are precursors of the juvenile hormone, they must be transformed by the cells they affect and not elsewhere, since, in the wax-test assay and other topical assays, their juvenilizing action can be restricted to the area of application.

Table 5. Effects of injecting farnesol into brainless pupae of *Antheraea polyphemus*. The brains of the pupae were removed 3 months prior to injection. All injections of farnesol were at a dosage of 5 mg/g (live weight). All suspensions of farnesol contained 1 part of farnesol to 3 parts of peanut oil or water and were made up in a vortex mixer (*67*).

Solution injected	Percentage dead	Percentage completing adult development
Farnesol	100	0
Farnesol and peanut oil	100	0
Farnesol and water	17	50
Farnesol and 9-percent ethyl alcohol	33	33
Water	0	0
Ethyl alcohol (9-percent)	0	0
Uninjected controls	0	0

Another point worth mentioning is the fact that purified juvenile hormone preparations from cecropia have recently been shown to have gonadotropic activity (*53*), as do most chemicals which copy the juvenile hormone (*48*).

In addition to possessing juvenile hormone and gonadotropic activity, crude and purified juvenile hormone extracts from cecropia also activate the prothoracic glands (*54*). Injection of 50 milligrams of crude juvenile hormone extract or 50 micrograms of purified juvenile hormone extract caused molting in brainless pupae of the giant silkmoths, *Samia cynthia* and *Antheraea polyphemus* (*54*, *55*). Similarly, implantation of active corpora allata also causes molting in brainless pupae. It has been argued that the corpora allata are able to activate the prothoracic glands because they contain neurosecretion from the brain (*56*). This may be true, but there is convincing evidence that the juvenile hormone itself is the agent. Oil extracted from allatectomized male cecropia moths lacks both juvenile hormone activity and brain hormone activity, so the factor that activates the prothoracic glands must come from the corpora allata.

Table 6. Some notable endocrine relationships.

1. All known insect endocrine organs involved in growth derive from embryonic ectoderm. Prothoracic glands and corpora allata orginate in adjacent segments.
2. Ecdysone, brain hormone, juvenile hormone, cholesterol, and farnesol activate the prothoracic glands.
3. Isolated abdomens may molt when brains or corpora allata are implanted, as well as when the abdomens are injected with ecdysone.
4. Juvenile hormone extracts and terpene derivatives, such as farnesol, that stimulate the juvenile hormone have gonadotropic activity.
5. Many terpenes (sterol precursors in many organisms) have juvenile hormone activity.
6. Ecdysone is probably a steroid.

A more convincing argument comes from the recent discovery by Krishnakumaran that farnesol and farnesol derivatives can activate the prothoracic glands of brainless diapausing pupae and cause molting. Results of a typical experiment are shown in Table 5. Alcoholic solutions of farnesol, or aqueous suspensions, activate the prothoracic glands of brainless pupae of *Antheraea polyphemus* and cause the pupae to develop. These agents also activate the prothoracic glands of brainless cecropia pupae, which are ordinarily quite resistant to activation. Thus, farnesol and its derivatives copy not only juvenile hormone and gonadotropin but also the brain hormone.

Why should chemicals with juvenile hormone activity and, presumably, the juvenile hormone itself be able to activate the prothoracic glands? There appear to be four main possibilities.

1) It may be that activation of the prothoracic glands by these diverse compounds is a pharmacological rather than a physiological phenomenon (*15*) and represents no more than the nonspecific effect of comparatively large doses of physiologically active molecules on a "poised" target organ.

2) Perhaps brain hormone and juvenile hormone are chemically related, so that there is some overlap in the responses they can induce in target tissues.

3) It may be that the insect uses the corpus allatum and the juvenile hormone to activate the prothoracic glands during larval life, and uses the brain hormone to activate the prothoracic glands only during the larval-pupal and pupal-adult transformations.

4) Another possibility which seems particularly attractive is that the insect uses two hormones to control the prothoracic glands during larval life, one produced by the brain and the other

by the corpora allata. The argument runs as follows. During larval life the prothoracic glands are continuously active at a low level and produce and release prothoracic gland hormone continuously. This continued low level of ecdysone is necessary for the growth of many tissues, such as the imaginal discs, which grow continuously during larval life. The prothoracic glands are kept continuously active at a low level by the juvenile hormone of the corpora allata. Periodically the brain hormone "superactivates" the prothoracic glands, which respond by releasing a large amount of ecdysone. Then molting occurs.

There is considerable experimental evidence to support this last hypothesis. Three observations are particularly pertinent. (i) Ligature, implantation, and histological studies have shown that larval imaginal discs require a continuous supply of ecdysone for growth (*57*). (ii) The juvenile hormone is necessary for maintaining the integrity of the prothoracic glands at all stages (*54*). If molting occurs in the absence of the juvenile hormone, the prothoracic glands will break down. This ability of small amounts of juvenile hormone to preserve the prothoracic glands may be related to the ability of larger amounts of juvenile hormone to activate the prothoracic glands. (iii) Autoradiographic studies have revealed that the prothoracic glands are active throughout larval life and only get completely "turned off" at pupation (*20*).

Hence, it appears possible that the prothoracic glands have two activation mechanisms—one via the juvenile hormone of the corpora allata, which can be copied by farnesol, and the other via the true brain hormone, which is produced by neurosecretory cells in the brain.

Another substance which appears to activate the prothoracic glands is ecdysone itself (*58*). Inasmuch as ecdysone is a steroid, it may be that the "cholesterol" preparations of Kobayashi discussed earlier activate the prothoracic glands because they resemble ecdysone rather than because they resemble the true brain hormone. Alternatively, inasmuch as farnesol-like compounds and sterols are chemically related, these "cholesterol" preparations may activate the prothoracic glands not because they resemble brain hormone but because they resemble juvenile hormone.

A New Look at Insect Hormones

What do all these findings mean? Let us re-examine the basic endocrine picture of insects (Fig. 1). During the past few years a number of significant deviations from this pattern have emerged. In addition, there are a number of fundamental relationships that are not apparent in the picture. These are summarized in Table 6. It must be borne in mind that some of these relationships may be exceptional and do not hold true for all insects.

1) The three insect endocrine organs involved in growth all derive from embryonic ectoderm. The prothoracic glands and the corpora allata derive from adjacent body segments. Recent light- and electron-microscope studies of silk moths (*59*) have shown that the cells of pupal corpora allata and prothoracic glands are so similar in structure that they are virtually indistinguishable. Berta Scharrer (*60*) has reached a similar conclusion from her studies of the cockroach *Leucophaea* and reports that the endoplasmic reticulum in both glands is similar to that found in mammalian endocrine cells engaged in steroid metabolism. These observations are consistent with the view that the corpora allata and prothoracic glands and their hormone products are related.

2) The fact that ecdysone, brain hormone, juvenile hormone, farnesol, and cholesterol activate the prothoracic glands has already been discussed and supports the suggestion that the hormones may be related chemically.

3) Further evidence that the hormones are related comes from the remarkable finding of Ichikawa and Nishiitsutsuji-Uwo that, in certain insects, the brain hormone *alone* or the juvenile hormone *alone* may substitute for ecdysone and will cause molting in isolated abdomens which have no known endocrine organs (*61*). Similarly, Kobavashi and Burdette (*62*) have shown that cholesterol preparations with brain hormone activity act synergistically with ecdysone and cause molting in isolated abdomens of fly larvae which have received subthreshold injections of ecdysone. This observation suggests that in some situations the three hormones can replace one another. A similarity between brain hormone and ecdysone is also suggested by the observation of Dietrich Bodenstein (*63*) that brain hormone may substitute for ecdysone in cockroach nymphs which can be induced to molt by brain implantation after extirpation of their prothoracic glands.

4) The finding that purified extracts of juvenile hormone have gonadotropic activity and that farnesol, which mimics juvenile hormone, has gonadotropic effects has important implications. Apparently the juvenile hormone and the gonadotropin are so similar in structure that a single substance may act in either capacity or may serve as a precursor for both agents. Another implication is that in the adult insect, where there is little or no ecdysone, juvenile hormone or farnesol *alone* can act as a growth hormone for the ovaries. If juvenile hormone acts as a growth hormone at one stage, may it not act as a growth hormone at other stages?

Items 5 and 6 in Table 6 have already been discussed.

Taken as a whole these findings suggest the following: (i) ecdysone, juvenile hormone, gonadotropic hormone, and perhaps the brain hormone may be related chemically; (ii) these substances may all be polyisoprenoid derivatives; (iii) the juvenile hormone and the brain hormone may act directly on many tissues to cause growth *without* ecdysone; and (iv) the possibility remains that the mechanism whereby the juvenile hormone activates the prothoracic glands is totally different from the mechanism whereby the brain hormone activates the prothoracic glands; and the brain hormone may turn out to resemble the more conventional neurosecretory polypeptides (for example, oxytocin and vasopressin).

Should most of the insect growth hormones turn out to be polyisoprenoid compounds, it would be most exciting from a number of standpoints. First, insects cannot synthesize sterols from acetate, mevalonic acid, or any of the other nonsterolic compounds that have been tested (*64*). All insects that have been studied appear to have a dietary requirement for sterols (*65*). Since some insect hormones are steroids, then either they must be made by modifying a dietary sterol (*66*) or different pathways of sterol biosynthesis exist in insects than those reported in microorganisms and mammals.

More significant than this is the implication for comparative endocrinology of the presence of steroid hormones in insects. If groups as diverse as mammals and insects use steroids and polyisoprenoid compounds as hor-

mones, may not their neighbors on the phylogenetic tree, the echinoderms and the annelids, use them too? It is our belief that this important class of biologically active compounds plays a central role in the humoral control of growth in invertebrates as well as vertebrates. On another occasion we speculated (*39*) that, "rather than being a recent innovation of the vertebrates, steroid hormones may prove to have a far more ancient lineage." Studies in many laboratories during the past 5 years appear to have confirmed this conjecture.

References and Notes

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11. We sincerely thank Dr. Andre Meyer, senior biochemist at the Schering Corporation, Bloomfield, N.J., for many helpful discussions and suggestions and for penetrating criticism. We also thank Miss Irene Colvin, Miss Barbara Keelor, and Mrs. Frances Parson, who conducted thousands of bioassays with fastidious care, and Professor Dietrich Bodenstein and Drs. John S. Edwards, Hans Laufer, and J. A. L. Watson for their helpful comments on typescript. Experiments from our laboratories were supported in part by research grants from the U.S. Public Health Service and the National Science Foundation.
12. Following a common convention of insect endocrinologists, we use the term *brain hormone* in this article to refer specifically to the humoral agent which activates the prothoracic glands. The brain is the source of other humoral agents besides this one.
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38. The problem may be stated in another way. Most insect cells have a certain endogenous biosynthetic "noise level." The effects of hormones like ecdysone must be assessed against this "noise level." The insects of choice for analyzing the action of ecdysone are obviously insects with low endogenous biosynthesis. For this reason a great deal of effort has been spent on studying diapausing pupae of giant silkworms, which are about as biosynthetically "quiet" as organisms can be. See, for example, H. A. Schneiderman and C. M. Williams, *Biol. Bull.* **106**, 210 (1953); W. H. Telfer and C. M. Williams, *J. Insect Physiol.* **5**, 61 (1960); F. Stevenson and G. R. Wyatt, *Arch. Biochem. Biophys.* **99**, 65 (1962).
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45. Several authors have reported that a variety of toxic compounds such as colchicine produce "abnormal adults containing large areas of a thick rough cuticle without scales" which was "pupa-like" [K. Slama, *Casopis Ceskoslovenské Spolecnosti Entomol.* **58**, 117 (1961); **59**, 323 (1962)]. Such effects have been called "pseudojuvenilizing," and it has been claimed that the effects of juvenile hormone extracts are "pseudojuvenilizing" and not true juvenilizing effects. The available evidence hardly favors this view. An elegant study of the cuticle of *Galleria mellonella*, the insect upon which most of our juvenile hormone bioassays are based, has been conducted by Adelheid Heims [*Arch. Entwicklungsmech. Organ.* **148**, 538 (1956)]. Heims points out that the structure of adult cuticle is similar to certain types of pupal cuticle and to certain types of larval cuticle. Nonetheless, the cuticles can be distinguished by their surface architecture, by their thickness, and by the structures they bear (scales, setae, and so on). We have made approximately 40,000 wax tests in *Galleria* and in other Lepidoptera. In fewer than two cases per 1000 were we unable to decide whether a juvenile hormone effect was positive (and in all such cases the experiments were repeated). Thus, there is no difficulty in distinguishing "pupa-like" cuticle induced by colchicine and other toxic agents from true pupal cuticle. True pupal cuticle and the second pupal cuticle which is secreted in response to juvenile hormone compounds are identical. Pupal cuticle has a characteristic surface architecture and pigmentation and bears pupal setae of characteristic structure. Pupa-like cuticle is abnormal adult cuticle; it does not have this surface architecture and it does not bear pupal setae. Indeed, the reason we have chosen the pupal cuticle of Lepidoptera for our hormone test system is that it provides a clear, unequivocal, highly sensitive yet highly specific end point in the biological assay for juvenile hormone, an end point which cannot be confused and which is not subjective. Furthermore, we routinely run parallel assays in which the agents to be tested are injected as well as applied topically. In such assays many structures besides the cuticle (for example, the genitalia) are affected by juvenile hormone [see L. I. Gilbert and H. A. Schneiderman (*44*)].
46. The following compounds were inactive in all of our test systems—that is, they had less than 5×10^{-5} times the activity of cecropia oil and less than 5×10^{-4} times the activity of purified all-*trans* farnesol: abietic acid, absynthin, allyl alcohol, ambrettolide, aritason, ascaridole, batyl alcohol, bixin, camphene, carotene, D-carvone, L-carvone, cedrene, cedrol, chimyl alcohol, cholesterol, cholesteryl chloride, cineole, citral, citronellal, citronellol, 1-decanol, *n*-dibutyl phthalate, digitoxin, *n*-dodecyl alcohol, ergosterol, estradiol benzoate, fenchone, fenchyl alcohol, geraniol, glutathione, 1-heptadecanol, 1-hexacosanol, δ-hex-α-ene lectone, 1-hexanol, hydrocortisone alcohol, α-ionone, β-ionone, iridodial, isodibutyl phthalate, isoiridomyrmecin, lanosterol, lignoceryl alcohol, D-limonene, linalyl acetate, lumisterol, lycopene, menthone, monobutyl phthalate, myrocene, nerol, 1-nonadecanol, 1-nonanol, octadecanol, 1-octanol, oleic acid, phthalic anhydride, pinene, 2-propyn-1-ol, pulegone, pyrethin I, α:β-santalol, selachyl alcohol, stearyl alcohol, tetradecanol, α-thujone, β-thujone, undecanol, Δ^{10}-undecenol, vitamin A, vitamin A acetate, vitamin A alcohol, vitamin D, vitamin D-3, and vitamin K.
47. A few commercial preparations of other compounds (for example, hexadecanol) showed slight juvenile hormone activity, but gas chromatographic analysis revealed that these preparations were contaminated with other compounds. When preparations of these compounds were obtained which appeared pure when analyzed by gas chromatography, they proved to be inactive. We continue to be puzzled by the effect of 1-heptanol; this is the only pure substance which is not a polyisoprenoid derivative in which we have been able to detect juvenile hormone activity. So far as we can determine, 1-heptanol has slight but real juvenile hormone activity.
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49. The mode of applying these hormones is crucial, and it is difficult to compare in detail the results of different bioassays. For example, when farnesol is injected either as a pure substance or in peanut oil, it is toxic to polyphemus pupae at doses of 5 mg per gram, live weight. By contrast, if it is suspended in distilled water or made up in a solution of 10 percent ethanol, injections of 5 mg per gram are tolerated and have

hormonal effects. Similar problems arise in the topical application of farnesol. In a typical experiment, solutions of farnesol were mixed with wax and applied to *Galleria* pupae in a wax test. In one series the farnesol was dissolved in peanut oil and mixed with wax; in a second series the farnesol was dissolved in an inactive oil derived from allatectomized male cecropia moths. The farnesol showed ten times as much activity when it was dissolved in inactive insect oil as when it was dissolved in peanut oil, although the inactive insect oil alone had no juvenile hormone activity. Formulation thus appears to be all-important in the bioassays. Possible reasons for these differences have been discussed elsewhere [see L. I. Gilbert and H. A. Schneiderman (*44*); V. B. Wigglesworth (*48*)]; they include differences in rates of inactivation or conversion of the active principle in different insects, the presence of inhibitors, the presence of substances which facilitate penetration of the active principle, and so on.

The lack of comparability of the several bioassays now used demands that extreme care be taken in making quantitative comparisons of activity when different bioassays are used. Indeed, different bioassays give different relative activities. Thus, in the *Tenebrio* assay of Karlson [Karlson and Nachtigall, *J. Insect Physiol.* **7**, 210 (1961)], farnesol is about as active as cecropia oil, and farnesyl methyl ether and farnesyl diethylamine are 100 times more active [see P. Karlson (*14*)]. In our wax test, even highly purified farnesol is less than 15 percent as active as active male cecropia oil, and in both the *Galleria* wax test and the polyphemus injection assay, farnesyl methyl ether and farnesyl diethylamine are about as active as cecropia oil.

A final problem in assaying terpene derivatives and related compounds is that many of them isomerize readily and are somewhat unstable. Indeed, there is considerable opportunity for change, both in the insect after injection or topical application and in the bottle prior to testing. The possiblity that the isomers or decomposition products may interfere with the action or metabolism of the active principle must also be reckoned with.

50. Because of the widespread distribution of these juvenile-hormone-like compounds, some workers have questioned whether there is any true juvenile hormone in the ether extracts which Williams (*5*) first prepared from male cecropia moths and which are now used in many laboratories as a starting material for juvenile hormone purification [see, for example, J. Beetsma, L. de Ruiter, J. deWilde, *J. Insect Physiol.* **8**, 251 (1962); H. E. Hinton, *Sci. Progr. London* **51**, 306 (1963); K. Slama, *Casopis Ceskoslovenské Spolecnosti Entomol.* **58**, 117 (1961); ———, *ibid.* **59**, 323 (1962)]. For example, they point out that these extracts do not affect lepidopterous larvae. Recently we have conducted experiments which strongly support the view that the material found in male cecropia moths is the juvenile hormone. In one experiment [H. A. Schneiderman and I. Colvin, unpublished] a group of ten male cecropia pupae were allatectomized. Special care was taken to insure that the corpora allata were completely removed and that no small fragments remained. These animals were allowed to develop into moths, and 3 days after they had emerged each was extracted with ether and the extracts were tested for juvenile hormone activity. We used the *Galleria* wax test, which is sensitive and permits detection of juvenile hormone activity in extracts which contain about 10^{-5} as much of the activity as ether extracts of male cecropia moths. None of these extracts from allatectomized animals had any juvenile hormone activity. Hence, it appears that the juvenilizing principle in the male cecropia extracts is produced by the adult corpora allata. In a second experiment we attempted to determine whether the adult corpora allata produce a substance which has juvenilizing effects on larvae—in other words, juvenile hormone (L. Fuchsman, J. S. Edwards, H. A. Schneiderman, unpublished). We implanted adult corpora allata into last-instar larvae of *Galleria*. The donors were *Samia cynthia* moths, close relatives of cecropia; from *S. cynthia* active juvenile hormone extracts are obtained which are very similar to cecropia extracts. In a number of cases implantation of three pairs of adult corpora allata caused the larvae to molt into larval-pupal intermediates in which the only pupal characteristics were a mid-dorsal suture of pupal cuticle in the prothoracic segment and the beginnings of pupal antennae. In a similar experiment, S. Fukuda [*Annotationes Zool. Japon.* **35**, 199 (1962)] demonstrated that adult corpora allata of the silkworm, *Bombyx mori*, produce a substance which causes larval molting in allatectomized fourth-stage larvae. Thus, the corpora allata of adult moths produce an agent which has all of the characteristics of the juvenile hormone, and can cause supernumerary larval molts in Lepidoptera and replace extirpated larval corpora allata. This agent is not found in allatectomized animals. For these reasons, and in the absence of contrary evidence, we believe that the active principle in male moths is the juvenile hormone, although we recognize that this has not been proved beyond the shadow of a doubt.

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65. H. House, *Ann. Rev. Biochem.* **31**, 653 (1962). There have been two recent claims that certain insects are capable of sterol biosynthesis from C^{14} acetate [R. B. Clayton, A. M. Edward, K. Bloch, *Nature* **195**, 1125 (1962); M. Saito, M. Yamazaki, M. Kobayashi, *ibid.* **198**, 1324 (1963)]. In both cases the sterol-synthesizing activity of microbial and fungal contaminants was not excluded, hence the results are not convincing. However, the possibility still exists that certain insect tissues are capable of some sterol synthesis.
66. On the basis of nutritional studies, a number of workers have been led to the view that insects use sterols for hormone synthesis. M. G. Horning [in *Cholesterol*, R. P. Cook, Ed. (Academic Press, New York, 1958), p. 451] wrote: "It seems likely that the sterol acts as a source of steroidal substances which probably fill hormonal functions in insects." Similarly, Z. H. Levinson [*Proc. Intern. Congr. Entomol. 11th, Vienna, 1958* (1960), vol. 3B] speculated that "cholesterol may act as a precursor of steroid hormones, in addition to its structural role. . . ." Very recently P. Karlson and H. Hoffmeister [*Z. Physiol. Chem.* **331**, 298 (1963)] have shown that tritium-labeled cholesterol is converted by fly larvae into ecdysone, and that cholesterol is a precursor of ecdysone.
67. A. Krishnakumaran and H. A. Schneiderman, unpublished observations.
68. Highly purified *trans-trans* farnesol used in these experiments was generously provided by Givaudan and Company, Geneva, Switzerland, and was prepared from all-*trans* nerolidol. Hexahydrofarnesol and farnesyl acetate were prepared by Dr. John Pollard and his associates at the California Biochemical Corporation and were assayed as 99.5 percent pure. Other preparations were obtained from diverse commercial sources and were said to be at least 90 percent pure. Different batches of these commercial substances varied somewhat in activity. The values given in Table 4 are typical. Hoffmann LaRoche, Inc., of Basel, Switzerland, generously provided samples of farnesyl methyl ether and samples of farnesyl diethylamine and gave us permission to publish the results of experiments made with these compounds.