

THE CHEMISTRY OF INSECT HORMONES AND INSECT PHEROMONES

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I. INSECT HORMONES

1. Introduction

While the hormones of vertebrates have been known to biologists for more than a century, and to chemists for well over half a century, the existence of insect hormones was recognized only thirty years ago, when Wigglesworth published his first work on *Rhodnius* and Bounhiol and Kühn repeated and confirmed the older, nearly forgotten work of Stefan Kopeč. (For a review of the earlier literature see references 1-6.)

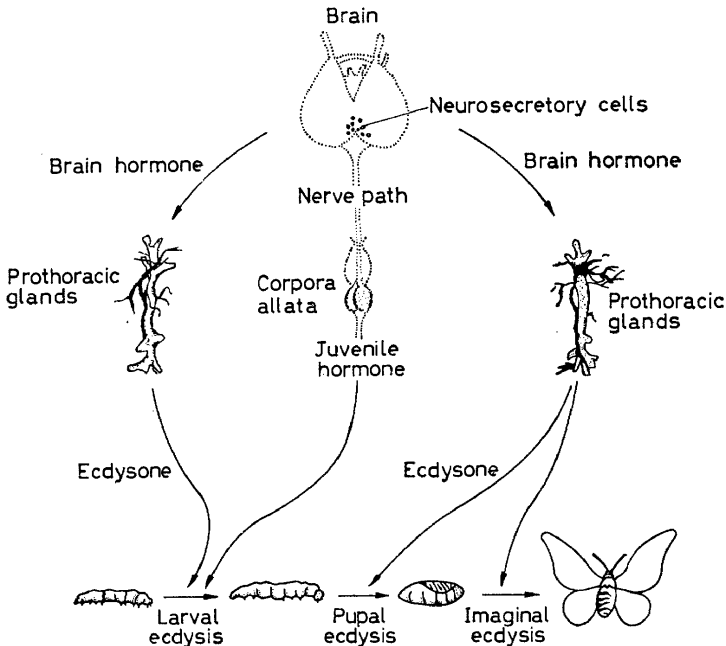


Figure 1. Hormonal control of insect development. Three hormones are involved: the brain hormone, acting on the prothoracic glands, the juvenile hormone, secreted by the corpora allata, and ecdysone, secreted by the prothoracic glands. The bottom row shows the development from the caterpillar through the pupa to the moth. The larval ecdyses are controlled by ecdysone and juvenile hormone, but the pupal and imaginal ecdyses are induced only by ecdysone

Insect hormones are primarily involved in the regulation of post-embryonic development, which is characterized by moulting and metamorphosis. Larval moults are initiated by a secretion from the neurosecretory

cells of the brain; this hormone stimulates the prothoracic glands to produce another hormone, now known as ecdysone, which acts on the periphery and causes moulting. Simultaneously, the corpora allata secrete the so-called "juvenile hormone" which guarantees the larval character of the moult. If juvenile hormone is lacking, the larvae will undergo a pupal or imaginal moult. *Figure 1* summarizes the hormonal control of development for the lepidoptera.

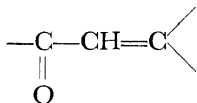
2. Chemistry of ecdysone

Our own work has been concerned with the prothoracic gland hormone, ecdysone⁷. Preliminary work on the purification of this hormone was done by Becker⁸ in the laboratory of A. Kühn. We continued this work, using the *Calliphora* bioassay of Fraenkel⁹ for tracing the biological activity, and used pupae of the commercial silkworm, *Bombyx mori*, for our extractions. It soon became clear that a large amount of starting material would be needed to obtain the pure hormone, and in 1953 the institute bought about 1200 kg of silk cocoons for the work on the prothoracic gland hormone as well as for the sex attractant to be discussed later. The male pupae served as starting material for our extraction, the first steps of which were done in the factory of Hoffmann-La Roche and Co. The concentrate was further purified in our laboratory, and in the spring of 1954 we isolated 25 mg of the crystalline hormone from the 500 kg pupae extracted¹⁰. This means a 20 million-fold purification. We can now estimate that the yield was not so bad; we got about 50 per cent of the hormone originally present in the extract. (The estimate is based on determinations of ecdysone content in *Bombyx* by Shaaya and Karlson¹¹.)

Soon after the isolation of ecdysone, we detected in the extracts a second substance with biological activity. This substance was isolated and provisionally termed " β -ecdysone"¹²; the amount available was so small (2.5 mg), that it could only be characterized spectroscopically². Recently, my coworker Hoffmeister¹³ has described the isolation of an active substance, which he termed ecdysterone, and which is more polar than ecdysone. Apparently the same substance has been isolated by Hocks and Wiechert¹⁴ in the laboratory of Schering AG, Berlin. Finally, mention should be made of the moulting hormone of the crustaceans. Extracts from crustacea are active in the *Calliphora* bioassay¹²; this was not too surprising, since moulting of crustacea is physiologically very similar to those in insects. The moulting hormone of crayfish has been obtained in nearly pure form by Hampshire and Horn¹⁵. There are indications that all these substances are identical with our old β -ecdysone¹⁶.

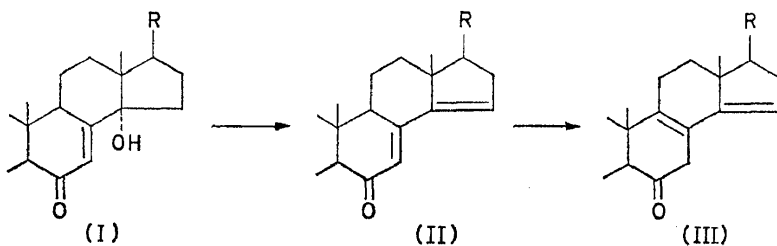
The elucidation of the chemical structure of ecdysone was especially difficult, since only very little material was available for chemical studies. Analysis gave a composition of $C_{7.7}H_{4.4}O_1$, which with the actual molecular weight, gave a formula of $C_{27}H_{44}O_6$. Due to an error in molecular weight determination, we believed for several years in a formula $C_{18}H_{30}O_4$. Mainly through x-ray evidence, this was later corrected to $C_{27}H_{44}O_6$, a formula which requires four rings, taking into account that ecdysone is an α,β -unsaturated ketone. A four ring structure immediately pointed to a

possible steroid nature; this was confirmed by a dehydrogenation experiment, which yielded methyl-cyclopentenophenanthrene¹⁷. Further information was obtained through the n.m.r. spectrum; it showed two angular methyl groups, thus confirming the steroid nature, and several hydroxyl groups, one of which must be located at C-25. The double bond had only one hydrogen, thus giving the structure



The assignment of this structure to a position in the ring system posed some difficulties¹⁸; finally, we identified it as the Δ^7 -6-ketone.

Of special importance for the structure of ecdysone was its decomposition in acid solution, yielding two substances: (i) a ketone with two double bonds in conjugation, (ii) a ketone with two conjugated double bonds no longer conjugated to the carbonyl group. The latter is the more stable product. This transformation could only be explained by a hydroxyl group in C-14, which in acid medium would be split off as water:

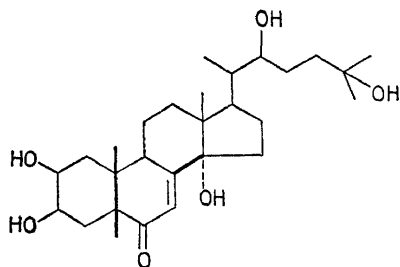


The compound (II) would rearrange to compound (III) which in this case is the more stable one, since there is less strain in ring B/C.

Thus, one hydroxyl group was assigned to C-14; a second one was located in the side chain, *viz.* at C-25, as was evident from the n.m.r. spectrum: there was no hydrogen beneath the two terminal methyl groups. Position 3 could be expected to bear an oxygen function, in this case hydroxyl, and since there were indications of a glycol grouping, another hydroxyl was tentatively assigned to either C-2 or C-4. For the hydroxyl unaccounted for, a position in the side chain was discussed¹⁹. The full structure, including stereochemistry, was finally elucidated by the x-ray work of Huber and Hoppe²⁰. We had first tried to provide them with a heavy atom derivative of ecdysone; unfortunately, these derivatives did not crystallize well enough, and lack of material prevented further studies in this direction. From ecdysone crystals with the dimensions of $0.45 \times 0.35 \times 0.15$ mm (weighing about $30 \mu\text{g}$), Huber and Hoppe determined by x-ray-scattering 3400 structural parameters. Using a new technique^{20,21}, they were able to calculate therefrom a complete Fourier synthesis of the ecdysone molecule, showing all carbon and oxygen atoms and about half of the hydrogens²². The structure thus determined can be described as $2\beta,3\beta,14\alpha,22R,25$ -pentahydroxy- Δ^7 -5 β -cholesten-6-one (IV): This structural formula, published a year ago, has in the meantime been confirmed by two independent syntheses in the

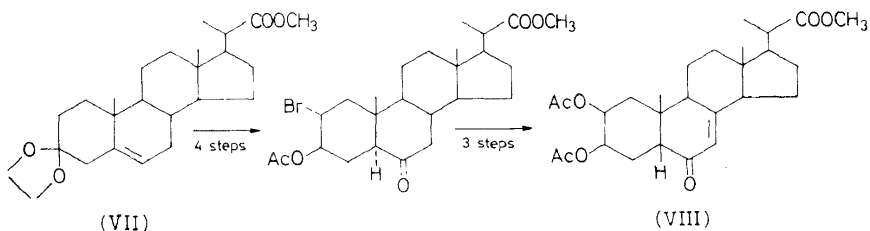
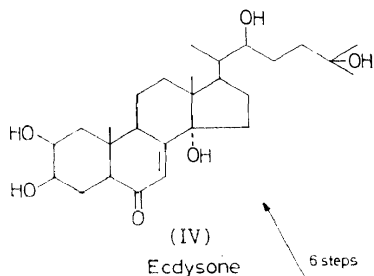
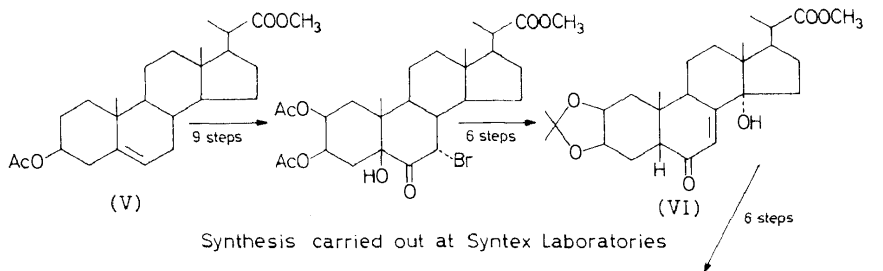
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laboratories of Schering/Hoffmann-La Roche²³ and in the Syntex labora-



(IV)

tories^{24,25}. Both groups started out from a derivative of bisnorcholeic acid (V) and (VII) respectively. The Schering-Roche group obtained by 7 steps the key intermediate, 2 β ,3 β -diacetoxy-6-keto- Δ^7 -5 β -bisorcholeic acid (VIII). After reduction of the carboxyl group to the aldehyde group, the

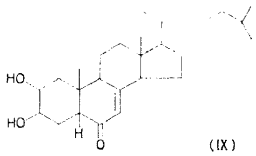
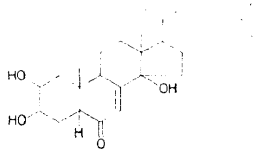
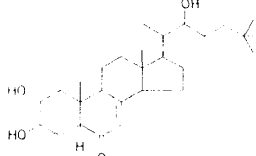
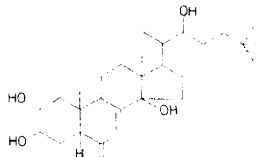
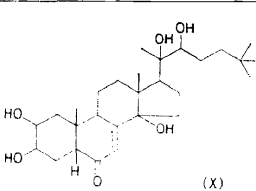


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latter was reacted with a 5-carbon fragment to yield the side-chain; as the last step, the hydroxyl group at C-14 was introduced by direct oxidation with SeO_2 . The syntex group introduced the 14α -hydroxyl earlier and obtained through 15 steps the 6-keto- 5β -cholenic acid (VI) with three hydroxyl functions; also, the side chain was introduced by a different route. Both syntheses yielded a product identical with natural ecdysone in its physical, chemical, and biological properties.

Another interesting question is the specificity of the structure in respect to the biological activity. The chemical syntheses have made available a certain number of analogues which have been assayed in the *Calliphora*

Table 1. Activities of the analogues of ecdysone.
(The values given are the average of several determinations)

Compound	WD ₅₀	Relative activity to ecdysone	Modification of natural ecdysone
 <p>(IX)</p>	4	1/80	Minus three OH-groups
	2.5	1/50	Minus two OH-groups
	0.75	1/15	Minus one OH-group
	0.05	1	No change
 <p>(X)</p>	0.04	1.25	Plus one OH-group

bioassay. Table 1²⁶ lists the compounds that are active. The last compound listed is the natural 20-hydroxyecdysone (X) isolated by Hocks and Wiechert¹⁴, presumably identical with ecdysterone¹³, crustecdysone, and β -ecdysone¹⁶. It should be mentioned that the products of the 5 α -series (with a *trans* junction of rings A/B) are inactive. Likewise, compounds with the α,β -unsaturated ketone in Ring C (synthesized in our laboratory¹⁷) are inactive.

It is somewhat surprising that only two hydroxyl groups (compound IX) are necessary for biological activity. However, the number of substances assayed is so small that any generalizations seem premature.

3. Biochemistry of ecdysone

For a biochemist, the biochemistry of a compound, *i.e.*, the route of biosynthesis and the enzymatic degradation, is of main interest. In the case of a hormone, its biochemical mechanism of action seems to be even more important.

Studies on the biosynthesis of ecdysone have revealed that ecdysone is derived from cholesterol; the detailed biochemical pathway of its biosynthesis remains to be elucidated. This is a very difficult task, since the enzyme(s) will be found predominantly in the corresponding gland, the prothoracic gland, which in most species is a tiny, delicate structure. It seems virtually impossible to get even milligram quantities of this tissue for enzymatic studies.

However, it is not too difficult to study the precursor-product relationship with radioisotopes. We have used tritium-labelled cholesterol; this was injected into 1000 mature *Calliphora* larvae, the animals killed 36 hours later and extracted for ecdysone. Purification of this extract by solvent fractionation and paper chromatography yielded a radioactive fraction with the same R_f -value as ecdysone. The eluate of this chromatogram was mixed with non-radioactive ecdysone and crystallized to constant specific activity, demonstrating that the ecdysone derived from the cholesterol-treated larvae was indeed radioactive²⁷.

It was to be expected that ecdysone is formed from cholesterol, since insects are unable to form sterols from mevalonate or squalene; they rely on dietary sources for their needs²⁸. Thus, cholesterol is an essential nutrient for most, if not all insects; it can be replaced to a certain extent by other plant sterols. In the beetle *Dermestes vulpinus*, up to 95 per cent of the sterol may be sitosterol; but 5 per cent has to be cholesterol. This result led Clark and Bloch to interesting speculations about steroid hormones in insects, which turned out to be correct.

As for the route of ecdysone biosynthesis, it can be expected that hydroxylations by oxygen and NADPH play a major role. It has recently been shown²⁹ that insects are capable of converting cholesterol to 7-dehydrocholesterol; this may be the first step towards the Δ^7 -6-ketone.

Turning to the metabolism of ecdysone, it is to be expected that there is an enzymatic mechanism for the inactivation, though part of the hormone is excreted with the faeces in an apparently unchanged form³⁰. Recent experiments have shown that ecdysone is indeed degraded *in vivo* as well as *in vitro*. Further work on this subject is in progress.

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The mechanism of action of ecdysone has been studied in great detail in the last three years in our laboratory, mainly in collaboration with my coworker, Dr. Sekeris. It would be a special lecture in itself, if I were to present here all the data obtained. In brief, the mechanism of action can be represented by the scheme given in *Figure 2*.

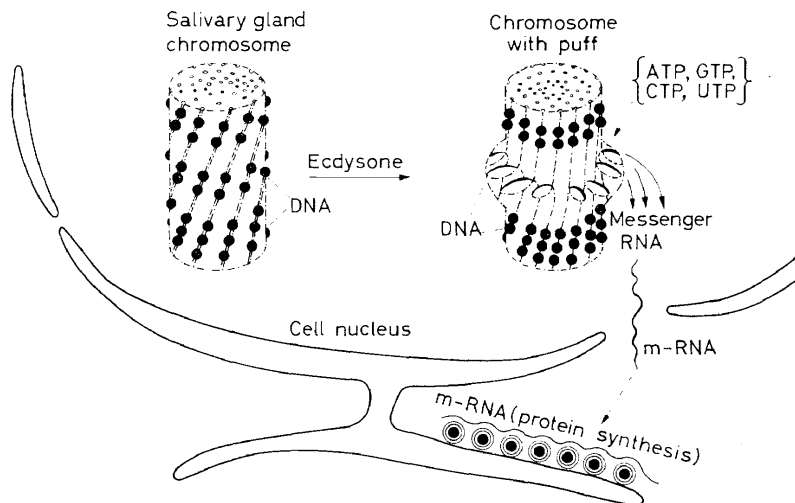


Figure 2. Mechanism of action of ecdysone

The site of action is the cell nucleus. The earliest effect observed *in vivo* is the induction of puffs in giant chromosomes³¹; they become visible 15 min after ecdysone injection into the whole animal. The dose needed to give a positive response is extremely small, $2 \times 10^{-6} \mu\text{g}$ per animal of 20 mg weight. Puff induction means the activation of genes; it is known that the puffs are sites of active RNA synthesis, and this RNA is presumably messenger RNA, carrying the genetic information into the cytoplasm³². Increase of RNA formation can indeed be measured after ecdysone injection into *Calliphora* larvae³³; this RNA has all the characteristics of messenger RNA³⁴. Even isolated nuclei respond to ecdysone with enhanced RNA synthesis³⁵. The messenger RNA is believed to combine with ribosomes and direct protein synthesis; in case of an enzyme protein, this process will be termed "enzyme induction". Ecdysone induces the synthesis of the enzyme dopa decarboxylase^{36,37}. It has even been possible to demonstrate that ecdysone stimulates the production of the messenger RNA carrying the information for this enzyme. When the messenger RNA fraction from hormone-induced animals is incubated with an *in vitro* system of protein synthesis, dopa decarboxylase is formed³⁸. Thus, all essential steps for the mechanism given above are well documented. For a detailed discussion of this work, the reader is referred to recent reviews^{39,40}.

4. The juvenile hormone

As mentioned in the introduction, the juvenile hormone is produced in the corpora allata. Its biological function is the determination of larval characters at the moult, so that moulting to the next larval instar occurs.

Very little can be said about the chemical nature of the juvenile hormone except that it is highly controversial⁴¹. Substances with biological activity are apparently widespread in nature, they have been obtained from microorganisms, plants, invertebrate, and vertebrate animals⁶ and even from newspapers⁴². We found⁴³ that excreta from the mealworm, *Tenebrio molitor*, yield active extracts, and my former coworker Schmialek⁴⁴ isolated therefrom farnesol and farnesal, both being active in the bioassay. However, the activity is rather small compared with the original natural source, an extract from male *Hyalophora cecropia* moths⁴⁵. Though derivatives from farnesol, like the methyl ether or the corresponding amine, are more active than the parent substance, most workers regard it as unlikely that the natural hormone is one of these compounds.

Recently, the purification of the "natural" juvenile hormone has been described by several groups. Williams and Law⁴⁶ isolated a crystalline material, identified as methyl-9,10-epoxy-hexadecanoate, but this carried the true hormone as an impurity, since a synthetic sample was devoid of activity. Meyer *et al.*⁴⁷ described a 300 000 fold purification through a number of counter-current distributions, adsorption chromatography and gas-liquid chromatography (GLC). However, the yield was very small and the active principle proved to be unstable. The molecular weight was estimated around 300. Röller *et al.*⁴⁸ reported a similar purification method, based mainly on thin layer chromatography and GLC, and isolated a single peak from GLC in which the activity was concentrated. The amount obtained was again very small (microgramm quantities), and chemical identification was not yet possible. The molecule is considerably larger than the farnesol derivatives used as reference.

It will be of special interest to see if the juvenile hormone is also a steroid, or if it is a terpenoid, as might be implied by the activity of farnesol derivatives.

The chemical investigations of other insect hormones^{49,50} has not grown much beyond preliminary extractions and crude preparations, so that they shall not be covered in this lecture.

II. INSECT PHEROMONES

1. Terminology

The term "pheromone" has been introduced by Lüscher and myself in 1959⁵¹. It embraces chemical substances acting as messengers *between individuals* of the same species. In this respect they differ from hormones which correlate certain tissues or organs *within* the individual^{52,53}. In a sense, pheromones create a "chemical language" for communication or rather signalization.

The classical example of a pheromone is the sex attractant of a butterfly or moth⁵⁴⁻⁵⁶. It is produced by the female in special glands at the tip of the abdomen and attracts the male moth over considerable distance. It is perceived by the antennae, *i.e.*, through the "chemical sense", and elicits a characteristic behavioural response: the male becomes excited, flutters its wings, approaches the female and finally copulates. In the field, the sex attractants are presumed to play an important role in the assembly of the sexes in efficient mating. The scent substances are carried with the wind, and

the migration of the male is directed against the wind rather than by a chemical gradient. Thus, an exact measurement of the concentration of the substance in the air is unnecessary for the animal^{57,58}.

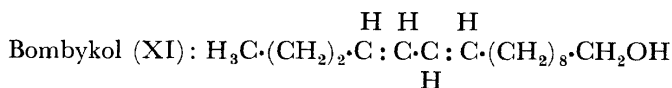
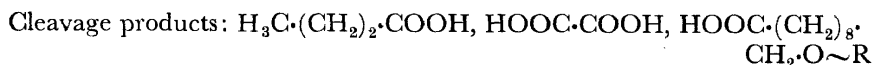
A large number of species have been shown to produce sex attractants (see the list in ref. 56). Only the few that have been isolated and characterized will be dealt with here.

2. Bombykol, the sex attractant of the silkworm

Pioneering chemical work on the sex attractants has been done by Butenandt and coworkers. They used the commercial silkworm, *Bombyx*, and extracted the active substance from the abdomen tips of virgin females. Several hundred thousand females were raised and processed in that way. Since only females could be used, and the males had to be sorted out to avoid copulation, it was fortunate that the work described above on the isolation of ecdysone could make use of the male pupae; thus, both programmes were carried forward with the same crop of cocoons.

The purification of the substance proved to be very difficult. An important step forward was the esterification of the pheromone (which is an alcohol) with *p*-nitroazobenzene-carboxylic acid. This derivative, a coloured substance, was easier to handle and purify. For the determination of the biological activity it had to be saponified, since the esters are inactive.

After many years of study, Butenandt's group finally succeeded in obtaining the pure ester of the *Bombyx* sex attractant⁵⁹. About 12 mg were obtained in crystalline, pure form. The empirical formula of the parent alcohol termed "Bombykol" was $C_{16}H_{30}O$; two conjugated double bonds are present in the molecule. The elucidation of the structure⁶⁰ was done (after elaborate studies of the method with model substances) by oxidative cleavage at the double bonds with less than one milligram of the isolated ester; it can be regarded as a masterpiece of classical microchemistry. The cleavage products were butyric acid, oxalic acid, and the *p*-nitroazobenzoate of ω -hydroxy-decanic acid. From the fragments, the structure of bombykol was easily reconstructed:



The stereochemistry is Δ^{10} -*trans*- Δ^{12} -*cis*. The formula was confirmed by synthesis, which also made available the other three stereoisomers⁶¹. The natural isomer is by far the most active.

A few words should be said about the threshold values. During the purification, Butenandt *et al.*⁶² used the behaviour bioassay carried out in the following manner: Male moths are kept in individual cages. A glass rod is dipped in a very dilute petroleum ether solution of the attractant and held in front of the moth. In case of a positive response, the male flutters its wings and begins a whirling dance, eventually trying to copulate with the glass rod. This assay is not very accurate, even when large numbers of animals

(up to 60) are used per dilution tested. Only differences in concentrations of 1:10 can be detected with certainty.

A more elaborate assay method is the recording of electroantennograms. As mentioned above, the attractant is perceived by the male through the chemoreceptors of the antennae. It is possible to insert micro-electrodes into the antennae and record the stimulation of the receptor cell; this recording is known as an "electroantennogramm"⁶³.

From the electrical response under standardized conditions, Boeckh *et al.*⁶⁴ determined the threshold value necessary to stimulate single chemoreceptors. It turned out that about one hundred molecules per cell second suffice to elicit an electrical response. He also studied the behaviour reaction† in comparison with the electrophysiological data. For a positive behaviour response, only 200 bombykol molecules per cm³ air are needed. In this threshold situation, 40 out of 40 000 receptor cells specialized for the sex pheromone are stimulated per second. The chemoreceptors thus function as a "molecule counting device".

3. Other sex attractants

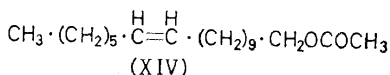
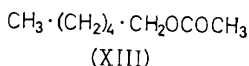
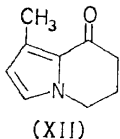
Bombykol was the first sex pheromone of the lepidoptera to be isolated and identified. The choice of *Bombyx* was for practical reasons—*Bombyx* is a highly domesticated animal well suited for laboratory work. In other laboratories, species of economic importance have been predominantly studied, since sex pheromones may become important for controlling insect pests. The sex attractant of the gypsy moth, *Porthetria dispar*, has been extracted from moths collected in the field. Jacobson and Beroza reported the isolation of the pheromone in pure form and its identification as 10-acetoxy-*cis*- Δ^7 -hexadecanol-1⁶⁵; a synthetic sample prepared by Jacobson *et al.*⁶⁶ was found identical in physical properties and biological activity with the natural substance, while the *trans*-isomer was not an attractant. However, the same compound, 10-acetoxy-*cis*- Δ^7 -hexadecanol-1, has been synthesized by other laboratories, and though the physical data were confirmed, these preparations were inactive in the behavioural as well as the electrophysiological assay⁶⁷. The discrepancy has not yet been resolved.

Also controversial is the isolation of the sex pheromone of *Periplaneta*, the American cockroach. The substance is produced by virgin females and can be extracted from filter-paper on which the females have been kept. Wharton *et al.*⁶⁸ reported the purification of this extract and the isolation of the pure attractant therefrom in microgram quantities. Jacobson *et al.*⁶⁹ using a different method of collection and purification, obtained 12 mg of a substance to which a cyclopropyl structure was assigned. This structure, however, proved to be incorrect⁷⁰, and the chemical nature of the cockroach pheromone is still open⁷¹.

Sex attractants are not only produced by the female, but also by the male. In males of the tropical butterfly, *Lycora ceres ceres*, a peculiar pair of glands,

†According to these studies, the threshold determined by Butenandt *et al.* in the routine bioassay has to be corrected. It is still not clear for what reason the old, low values have been found in so many experiments.

the so-called hairpencils, are found. They can be protruded from the tip of the abdomen and are rich in a secretion. Extraction of this material and analysis with gas liquid chromatography yielded three fractions, which were identified⁷² as 7-methyl-2,3-dihydropyrrolizidin-1-one (XII), cetyl acetate (XIII) and Δ^{11} *cis*-vaccenyl acetate (XIV).



It is remarkable that two of the components bear striking similarities to bombykol and the gypsy moth attractant. Will the sex pheromones of the lepidoptera all belong to this class of straight-chain, more-or-less unsaturated aliphatic alcohols or esters? This remains to be seen. As for the heterocyclic compound, it is not clear whether it serves as part of the pheromone or rather as a defence substance.

Another male sex pheromone is found in the Indian water bug, *Belostoma indica* (= *Lethocerus indicus*). It was analysed by Butenandt and Tam⁷³ in 1957 and found to be Δ^2 -hexenyl acetate. This paper can indeed be regarded as the first chemical identification of a pheromone in insects. Besides the acetate, the butyrate is also present as a minor component in the secretion⁷⁴. Though field observations on the role of this substance have not been made, there is little doubt that they act as pheromones.

Finally, mention should be made of a pheromone attracting both sexes. It can thus be classified as an "assembling scent". However, it is very likely that it facilitates mating, and that the evolutionary value of the substance is due to this fact rather than that it accounts for mass attacks of some trees by this pest.

The pheromone is produced in the hind gut of the male Bark beetle, *Ips confusus*, and is secreted with the faeces. It is only produced by animals feeding in a suitable tree. Laboratory rearing of the beetles in mass cultures provided the starting material for a chemical investigation⁷⁵ of the substance responsible for the attracting activity. Through solvent fractionation and gas-liquid chromatography, a substance was obtained which travelled in GLC between nonanal and geranyl acetate, and was highly attractive in the bioassay.

4. Pheromones of the social insects

The phenomenon of social organizations in insects has fascinated not only biologists. In our modern technical language, we can state that the community of, for instance, a bee hive or an ant's nest, must rely on a suitable system of communication between the members in order to cope with the needs. A large part of this communication uses the chemical language of pheromones⁷⁶. Space does not allow me to review the large body of chemical evidence on the nature of these pheromones, many of which are related to terpenoids⁷⁷.

Most of the substances analysed so far are derived from ants or bees. The pheromones of the termites have found only little attention. In collaboration with Prof. Lüscher, Bern, we have begun a chemical investigation of the trail pheromone of a termite, *Zootermopsis nevadensis*. A large number of termites of this species were collected in California. The animals were washed with ether and the solvent evaporated, leaving a greasy residue which is highly attractive to workers of this species. Amounts of μg per cm are sufficient to lay down a trail which is confidently followed in the behaviour assay⁷⁸. The active principle is steam-volatile. Actually, it is rather difficult to concentrate solutions (even if the solvent is as volatile as pentane) without losses. Preliminary studies with gas-liquid chromatography shows that the substance probably has a rather small molecular weight the order of 100. However, it is premature to draw any conclusions on the chemical nature of this substance.

[*Note added in proof.*—Juvenile hormone has been identified as methyl ester of 7-ethyl-3,11-dimethyl-10-epoxy-2,6-tridecanoic acid [H. Röller, K. H. Dahm, C. C. Sweeley, and B. M. Trost. *Angew. Chem.* **79**, 190 (1967)].

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