SECTION 2: STRUCTURAL ELUCIDATION OF NATURAL PRODUCTS OTHER THAN MACROMOLECULAR COMPOUNDS

INTRODUCTORY REMARKS BY THE HONORARY PRESIDENT OF THE SECTION

ROGER ADAMS

It was eight to ten years ago when initial steps were taken to organize a symposium on the chemistry of natural products. In the interim three successful symposia have been held with the fourth one now under way. During these years an ever increasing number of communications, describing the elucidation of the structure and syntheses of many complex products occurring in plant and animal life, have appeared. I intend to reminisce a bit this afternoon and to note the basic contributions of the last sixty or seventy years that have made possible the current brilliant successes.

But first I must cite conditions that existed in earlier times and a few examples of the achievements. As a student I had the privilege to study in 1912–1913 in the laboratory of Emil Fischer and later in that of Richard Willstaetter. Consequently I became acquainted with natural product researches of those days and of the preceding two decades. It is difficult for chemists trained after World War II to imagine how the structure of complex molecules could be solved almost exclusively by chemical methods. Yet at the turn of the century they were solved in that way, if you are willing to overlook absolute configuration. It often required years of painstaking research by large groups of chemists to complete the study of a single natural product.

An investigation was always started by detection of the presence of the functional groups by means of various reagents. Degradation experiments followed and the fragments separated. Each fragment was then studied chemically until it was identified or degraded further into identifiable products. With this information, assuming rearrangements during degradation did not distort the picture, the original molecule could usually be constructed. One of the handicaps during that period was the requirement of relatively large amounts of material for the numerous reactions and degradations; and in addition 0·1 to 0·2 grams were needed for each elemental analysis. This made it imperative to select for study readily available products, such as quinine, morphine, cocaine, strychnine, and brucine.

Yet without the many advantages of today, the investigations by the leaders in organic chemistry were noteworthy. I cite, for example, the determination of structure of many of the tropin alkaloids and subsequent synthesis by Willstaetter and assistants. Their publications on the anthocyanins, the colouring matter of flowers and fruits, represent some of the finest aspects of chemistry found anywhere in the literature. I recall that just adjacent to the laboratory there were acres of red geraniums and of cornflowers from which each morning the dieners would pluck fresh petals and promptly bring them to the laboratory for immediate extraction. These investigations extended over many years. I have often wondered how long it would take the clever chemist of today with modern facilities to realize the same goals. And this applies to the natural product studies of the following three decades.

Molecules which contained many different types of rings presented much more difficulty to the investigator who had to rely merely on chemical methods. Satisfactory degradation reactions were more difficult to discover, for frequently they involved rearrangements or often complete destruction; this was especially true of molecules composed exclusively of heterocyclic rings. For example, I may refer to the many dozens of papers published by Leuchs and his coworkers on the structure of brucine and strychnine as well as a long list on the same alkaloids by Robinson and associates before structural formulae were proposed adequate to explain the chemical reactions. Even so, the structures have been modified more recently when modern techniques made more detailed investigation possible.

It must not be overlooked also that in those early days no satisfactory procedure was available for purification of natural products contaminated with materials closely related physically and chemically. Chromatography had not been developed. Months were often consumed in a preliminary investigation to isolate a substance of adequate purity for structural study. Physical tools were not available excepting polarimeters and refractometers. Equipment for determining absorption spectra in the visible and ultraviolet was primitive and rarely used by the organic chemist. It was primarily of interest to the physical chemist who measured and reported oscillation frequencies and absorption wavelengths usually of impure samples of material. It was years afterwards before ultraviolet absorption equipment was developed suitable for effective utilization by the organic chemist.

The first important contribution which aided the natural product chemist was the introduction of microanalyses during the 1920s. This made it possible to function without the amount of material previously considered essential. The development of laboratory apparatus suitable for running reactions with small quantities of reagents followed.

The second contribution came in the late 1920s and 1930s with the gradual perfection of chromatographic procedures for separation of mixtures of compounds closely related physically and chemically. To be sure, these methods were not what they are today but they were very serviceable. Satisfactory ultraviolet spectrophotometers also appeared during this period and proved of real significance to the chemist particularly when aromatic nuclei and unsaturated groups were present in the molecule.

But the most salient breakthrough for the natural product investigator

came after World War II with the perfection of infrared spectrophotometers which furnished precise information concerning functional groups. Then came nuclear magnetic resonance equipment which made possible further deductions concerning atoms and groups and, more importantly, their relative configurations. With n.m.r. to supplement i.r., particularly since i.r. sometimes fails to identify functional groups in complicated structures, the chemist was supplied with a spectacular means for accurate insight into the constitution of a molecule. Simultaneously, x-ray crystal structure techniques proved their value, and unique chromatographic and counter-current procedures for separation of molecules resistant to orthodox methods were added to the list. Then came equipment for optical rotatory dispersion, electron spin resonance, and finally for analytical and mass spectrometry to aid further in solving the chemist's problems.

The natural product investigator, however, must have also a comprehensive knowledge of synthesis. The chemist primarily interested in synthesis now has a reservoir of techniques and reagents not available in earlier times. Many new named reactions of general applicability as well as a host of others of more specific utility have been discovered in the last thirty to forty years. The number of organic chemicals and solvents commercially available has multiplied manifold. Dozens of reagents have been uncovered for modifying and protecting functional groups, many suitable for reactions in molecules containing other sensitive points of attack. The present knowledge of rearrangements prone to certain types of molecules often prevents the chemist from being misled. New catalysts with very specific action have been placed at the disposal of the investigator. Almost any complex natural product, excluding macromolecules, may now be synthesized. However, what is needed for success is a genius to conduct the research, a man who is omniscient in chemistry, who has absorbed and digested a library of information on reactions, reagents, and techniques and who can skillfully apply this knowledge.

The natural product chemist in earliest days required positive identification of small degradation fragments to solve his problems. In later years he was able to accelerate his progress by the use of certain physical methods. Today, the time has been reached when the elucidation of structure is usually initiated by physical methods and supplemented by chemical studies. Indeed, when very small quantities of material are available, confirmation of the structure established through physical methods is often attempted by synthesis. And today the chemist does not consider his problem completed without the knowledge of the absolute configuration of the atoms and groups.

I have cited previously a few of the researches of former chemists. Now I must mention one or two of the outstanding researches of the recent past to exemplify what may now be accomplished.

The colouring matter in black tea formed probably by an enzyme-catalyzed process during the fermentation of green to black tea is known as theaflavin. Initial chemical studies by Takino, followed by an examination of the u.v., i.r., n.m.r., analytical and mass spectra by Gianturco, Takino, and associates, led to a tentative structure consisting of two flavinyl residues attached to a benzotropolone nucleus. They then proceeded by a neat synthesis, involving

only a very few steps, to obtain the natural product. A few months later Ollis and his associates, following an extensive study of theaflavin, established that the tentative structure proposed by the other workers was incorrect only in the configuration of the bonds attaching the flavinyl nuclei to the benzotropolone.

For a most elegant synthesis reported recently I refer to the work on cephalosporin by Woodward and associates in the CIBA laboratories in Basle. By a short series of most ingeniously devised reactions the natural product was obtained.

The structural information gained from various spectra is so revealing that a new vista is opened to the natural product chemist. He is now able with extremely small quantities of material, usually obtained by one of the invaluable chromatographic procedures, to characterize almost any substance.

Results in the natural product field have already changed the pattern of thinking of the biochemist, physiologist, botanist, zoologist, entomologist, and microbiologist since there is now the possibility of isolating and identifying the critical chemical contributors to life processes.

Looking into the future, I can see only unlimited potentialities in the study of natural products. My view may be expressed by a taxi cab driver's homely but incisive reply to his customer. A visitor to Washington was riding in this man's cab. As they passed by the government building on which is found the inscription, "The past is prologue", the visitor turned to the driver and asked, "What does that mean?" The taxi driver replied, "Oh, that just means, you ain't seen nothing yet."

And now I have another illustration of recent brilliant experimentation. It involves the contributions of our speaker, Professor Karlson. While working as an assistant to Professor Butenandt in the Max Planck Physiological Institute in München he isolated the insect moulting hormone, ecdysone. He continued his researches at the same institute until recently, when he became professor at the Physiological Chemical Institute in Marburg. He has also found the time in recent years to contribute a superb text book on biochemistry which has been very widely accepted.

During the last twelve years, following the discovery of ecdysone, his researches have involved the biochemistry and physiology of the compound and a comprehensive chemical and physicochemical study that resulted in the establishment of its constitution. Only an investigator with a very astute mind, indefatigable patience and determination could undertake a programme of this kind and bring it to a successful conclusion. The reported structure has indeed now been confirmed by synthesis in two independent laboratories. Professor Karlson has pioneered the way for future investigations in this fascinating and intriguing field of insect hormones.

This afternoon his address will be on the "Chemistry of Insect Hormones and Insect Pheromones". It is a privilege to present Professor Karlson.

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.)

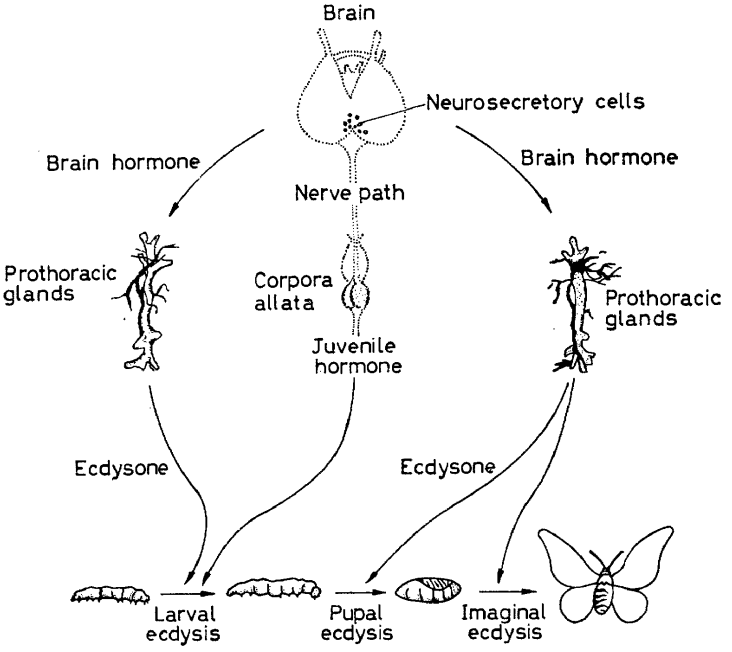


Figure 1. Hormonal control of insect development. Three hormones are involved: the brain hormone, acting on the prothoracic glands, the juvenile hormone, secreted by the corpora allata, and ecdysone, secreted by the prothoracic glands. The bottom row shows the development from the caterpillar through 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.

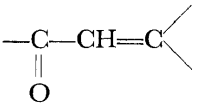
2. Chemistry of ecdysone

Our own work has been concerned with the prothoracic gland hormone, ecdysone[7]. Preliminary work on the purification of this hormone was done by Becker[8] in the laboratory of A. Kühn. We continued this work, using the *Calliphora* bioassay of Fraenkel[9] 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 extracted[10]. 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 Karlson[11].)

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 spectroscopically[2]. Recently, my coworker Hoffmeister[13] 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 Wiechert[14] 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* bioassay[12]; 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 Horn[15]. There are indications that all these substances are identical with our old β-ecdysone[16].

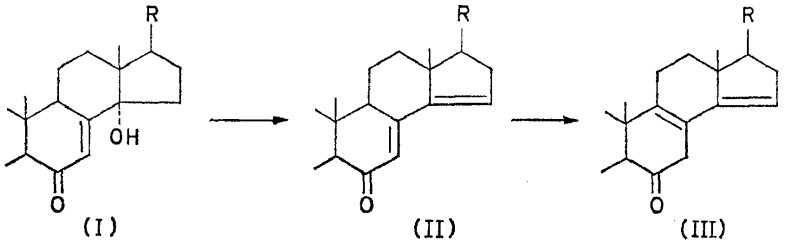
The elucidation of the chemical structure of ecdysone was especially difficult, since only very little material was available for chemical studies. Analysis gave a composition of $C_{7.7}H_{4.4}O_1$, which with the actual molecular weight, gave a formula of $C_{27}H_{44}O_6$. Due to an error in molecular 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-cyclopentenophenanthrene[17]. 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 difficulties[18]; finally, we identified it as the Δ^7-6-ketone.

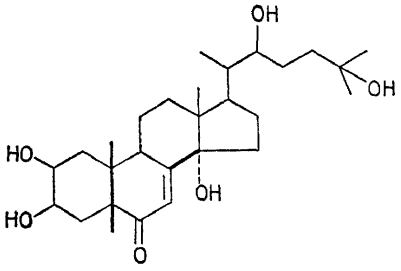
Of special importance for the structure of ecdysone was its decomposition in acid solution, yielding two substances: (*i*) a ketone with two double bonds in conjugation, (*ii*) a ketone with two conjugated double bonds no longer conjugated to the carbonyl group. The latter is the more stable product. 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.

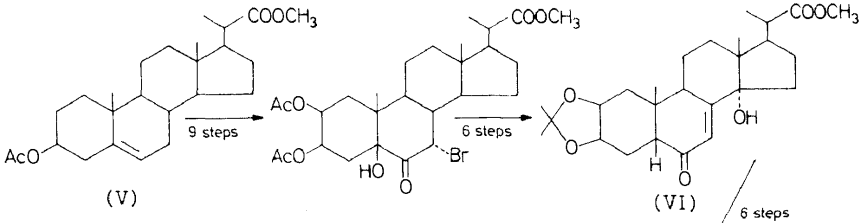
Thus, one hydroxyl group was assigned to C–14; a second one was located in the side chain, *viz.* at C–25, as was evident from the n.m.r. spectrum: there was no hydrogen beneath the two terminal methyl groups. Position 3 could be expected to bear an oxygen function, in this case hydroxyl, and since there 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 discussed[19]. The full structure, including stereochemistry, was finally elucidated by the x-ray work of Huber and Hoppe[20]. 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 × 0·35 × 0·15 mm (weighing about 30 μg), 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 hydrogens[22]. The structure thus determined can be described as 2β,3β,14α,22R,25-pentahydroxy-Δ^7-5β-cholesten-6-one (IV): This structural formula, published a year ago, has in the meantime been confirmed by two independent syntheses in the

laboratories of Schering/Hoffmann-La Roche[23] and in the Syntex labora-



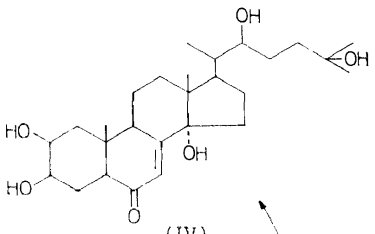
(IV)

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

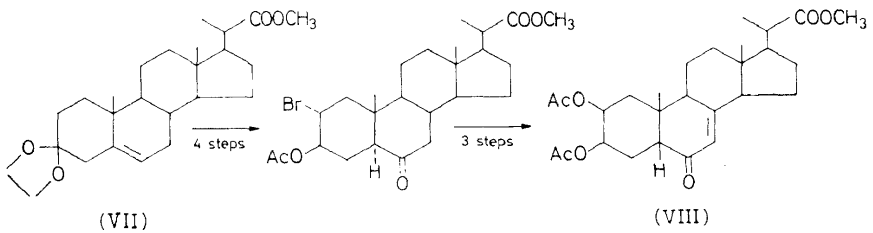


(V) (VI)

Synthesis carried out at Syntex Laboratories



(IV)
Ecdysone



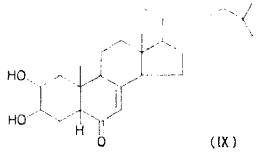
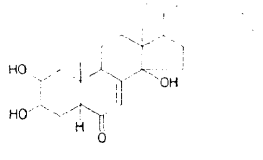
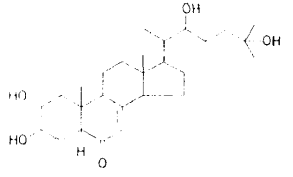
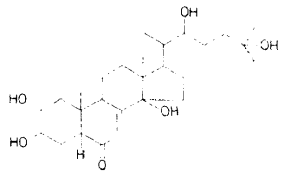
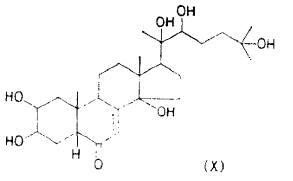
(VII) (VIII)

Synthesis carried out at Schering / Hoffmann-La Roche Laboratories

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*
 (IX)	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
 (X)	0·04	1·25	Plus one OH-group

bioassay. *Table* 1[26] lists the compounds that are active. The last compound listed is the natural 20-hydroxyecdysone (X) isolated by Hocks and Wiechert[14], presumably identical with ecdysterone[13], crustecdysone, and β-ecdysone[16]. It should be mentioned that the products of the 5α-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 laboratory[17]) 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 radioactive[27].

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 needs[28]. 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 shown[29] 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 form[30]. Recent experiments have shown that ecdysone is indeed degraded *in vivo* as well as *in vitro*. Further work on this subject is in progress.

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.*

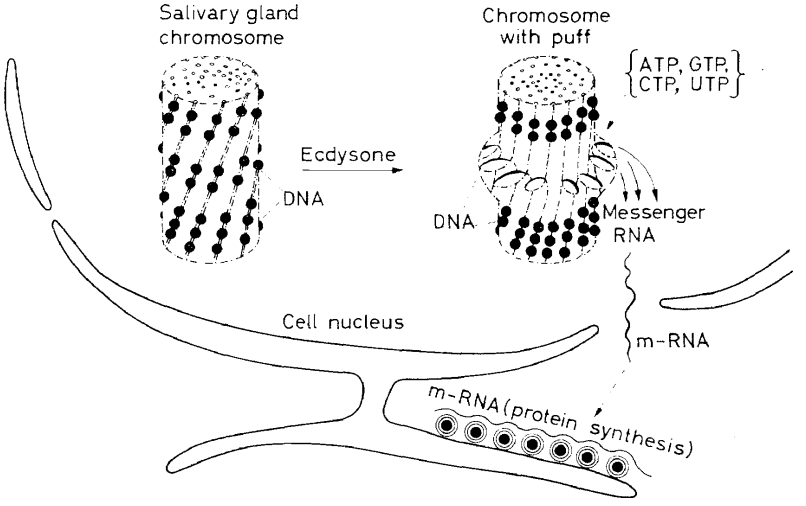


Figure 2. Mechanism of action of ecdysone

The site of action is the cell nucleus. The earliest effect observed *in vivo* is the induction of puffs in giant chromosomes[31]; they become visible 15 min after ecdysone injection into the whole animal. The dose needed to give a positive response is extremely small, 2×10^{-6} μg 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 cytoplasm[32]. Increase of RNA formation can indeed be measured after ecdysone injection into *Calliphora* larvae[33]; this RNA has all the characteristics of messenger RNA[34]. Even isolated nuclei respond to ecdysone with enhanced RNA synthesis[35]. 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 formed[38]. 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 controversial[41]. Substances with biological activity are apparently widespread in nature, they have been obtained from micro-organisms, plants, invertebrate, and vertebrate animals[6] and even from newspapers[42]. We found[43] that excreta from the mealworm, *Tenebrio molitor*, yield active extracts, and my former coworker Schmialek[44] 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* moths[45]. 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 Law[46] 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 1959[51]. 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 moth[54–56]. 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 silkmoth

Pioneering chemical work on the sex attractants has been done by Butenandt and coworkers. They used the commercial silkmoth, *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 attractant[59]. 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 structure[60] 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:

Cleavage products: $H_3C{\cdot}(CH_2)_2{\cdot}COOH$, $HOOC{\cdot}COOH$, $HOOC{\cdot}(CH_2)_8{\cdot}CH_2{\cdot}O{\sim}R$

Bombykol (XI): $H_3C{\cdot}(CH_2)_2{\cdot}\overset{H}{C}:\overset{H}{C}{\cdot}C:C{\cdot}(CH_2)_8{\cdot}CH_2OH$

The stereochemistry is Δ^{10}-*trans*-Δ^{12}-*cis*. The formula was confirmed by synthesis, which also made available the other three stereoisomers[61]. 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 electroantennogramms. As mentioned above, the attractant is perceived by the male through the chemoreceptors of the antennae. It is possible to insert microelectrodes 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 cm^3 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

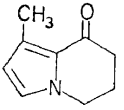
Bombykol was the first sex pheromone of the lepidoptera to be isolated and identified. The choice of *Bombyx* was for practical reasons—*Bombyx* is a highly domesticated animal well suited for laboratory work. In other laboratories, species of economic importance have been predominantly studied, since 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-1[65]; 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 assay[67]. 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 incorrect[70], and the chemical nature of the cockroach pheromone is still open[71].

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 identified[72] as 7-methyl-2,3-dihydropyrrolizidin-1-one (XII), cetyl acetate (XIII) and Δ^{11} *cis*-vaccenyl acetate (XIV).



(XII)

$CH_3 \cdot (CH_2)_4 \cdot CH_2OCOCH_3$

(XIII)

$CH_3 \cdot (CH_2)_5 \cdot \overset{H}{C}=\overset{H}{C} \cdot (CH_2)_9 \cdot CH_2OCOCH_3$

(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 Tam[73] 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 secretion[74]. 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 investigation[75] 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 pheromones[76]. 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 terpenoids[77].

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 assay[78]. 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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