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On the hormonal control of insect metamorphosis. A historical review

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Already in 1917, Stefan Kopec found that the brain exerted a hormonal control of moulting (Kopec, 1917, 1922) The first report appeared in a Journal of the Academy in Cracow, a rather hidden place; but the second paper in the "Biological Bulletin, Woods Hole" found also little resonance. The reason may have been that at that time, it was hard to believe that the brain produced a hormone. Kopec himself then turned to other problems of insect physiology.

In 1934, Wigglesworth (1934, 1936) started his investigations on the control of moulting and metamorphosis in the blood-sucking bug *Rhodnius prolixus*. After a blood meal, the processes leading to moulting begin. This can be prevented by decapitation, a corroboration of the postulate of Kopec that the brain produces a hormone involved in the control of moulting. A series of very ingenious parabiosis experiments gave additional evidence. Wigglesworth also pointed out that the corpora allata are important for the control of insect development.

In the lepidoptera, ligation experiments showed the same result. Kühn and Piepho (1936; Piepho, 1938) ligated the head part of caterpillars and showed that this prevented moulting. Reimplantation of the brain into the abdomen led to moulting (Plagge, 1938). But it is not the "brain hormone" that acts on the epidermis. The brain hormone activates another gland that is situated in the prothorax and is therefore called prothoracic gland. It was Fukuda in Japan who gave clear-cut evidence (experiments with the silkworm, *Bombyx mori*). His papers appeared in 1940 and 1944, but were hardly available in the USA and in Europe during the war.

For further research on the moulting and metamorphosis hormone, work by G. Fraenkel (1934, 1935) was important. Fraenkel was a German emigrant working in London. He showed that ligation of mature larvae of the blowfly, *Calliphora vicina*, had the result that only the head part formed a puparium; the hind part survived as permanent larva. Injections of blood from larvae that were about to form a puparium led the hind part to pupariate as well

These experiments formed the basis of the *Calliphora* bioassay for the moulting hormone. It was Erich Becker, a co-worker of Kühn, who took up the problem and worked out the bioassay. He also prepared extracts from *Calliphora* pupae that showed activity in the bioassay, and devised some steps of purification, e.g. extraction with butanol. Becker's last paper (1941) closed with the words: "Das Problem ist nun soweit gefördert, daß es aus der Hand des chemisch arbeitenden Biologen in die Hand des Biochemikers übergehen sollte. Herr Prof. Butenandt wird zu gegebener Zeit darüber berichten".

Only two months later, Erich Becker – a very gifted scientist – lost his life in the war. Butenandt then asked me if I would be interested to work on this problem, and I agreed.

The first thing to do was to repeat Becker's work on the *Calliphora* bioassay. This assay needed some refinement, the main point was to exactly measure the amount of fluid injected. This could be done with a microinjection syringe developed by G. Bergold for his work on an insect virus (Bergold, 1941). Starting material for preparing the extracts were still blowfly pupae, but it was very tedious to collect kilograms of these pupae for extraction. Thus, progress was slow.

There was another reason for the slow progress. In the summer 1944, the Institute of Butenandt was forced to leave Berlin because of the air raids in the war. Our new domicile became the University of Tübingen where we could work as guests in several laboratories which where not in use. It took a lot of work and time to establish all the facilities that were needed to continue the research on the metamorphosis hormone

It was now close to the end of the war. Only a few months later, on April 20, French troops occupied Tübingen. Again I had to

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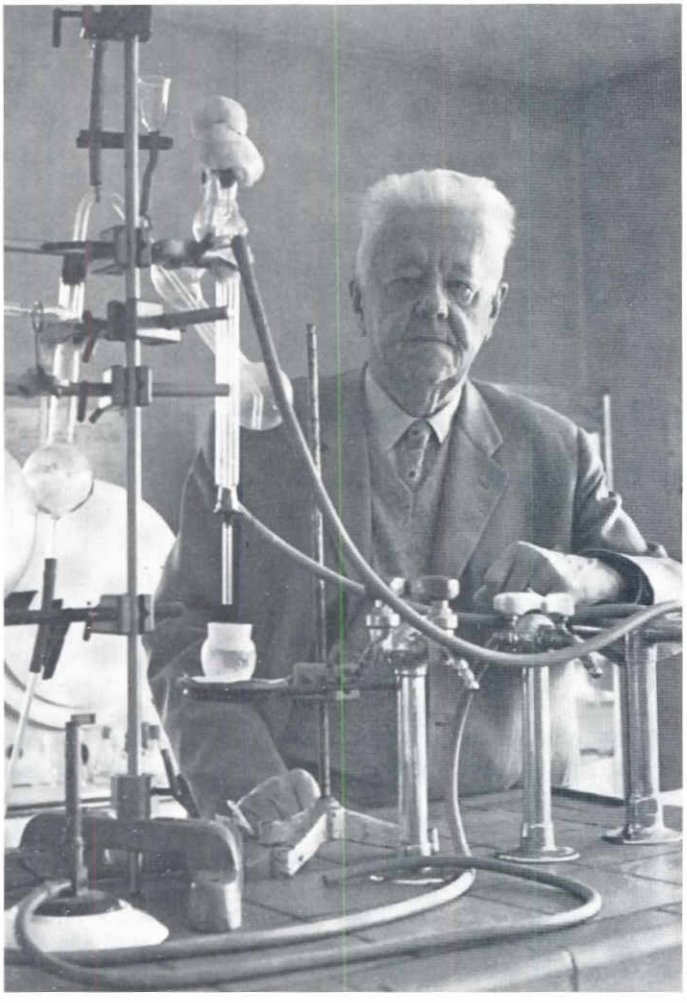


Fig. 1. Alfred Kühn, a pioneer of insect developmental biology. *Taken from 5. Biolog Jahresheft, Yerband Deutscher Biologen, Iserlohn 1972.*

move my laboratory because it was in the Clinics of Surgery, and this building was occupied by the French troops. For the next six months, we could not continue experimental work, or rather, we did but only to a very limited extent. But at the end of 1945, things turned to the better. Butenandt became Professor of Physiological Chemistry at the University of Tübingen and Director of the Institute of Physiological Chemistry, and the Kaiser-Wilhelm-Institute was housed there as guest. Thus, I had a laboratory again and could gradually resume my work in the metamorphosis hormone, or, as we used to say, the "Verpuppungshormon".

A next important step forward was the discovery that pupae of the silk worm *Bombyx mori* could be used as source for the metamorphosis hormone. These pupae were available in our laboratory because they were used for the work on the insect sex attractant, now known as Bombykol (Butenandt *et al.*, 1961).

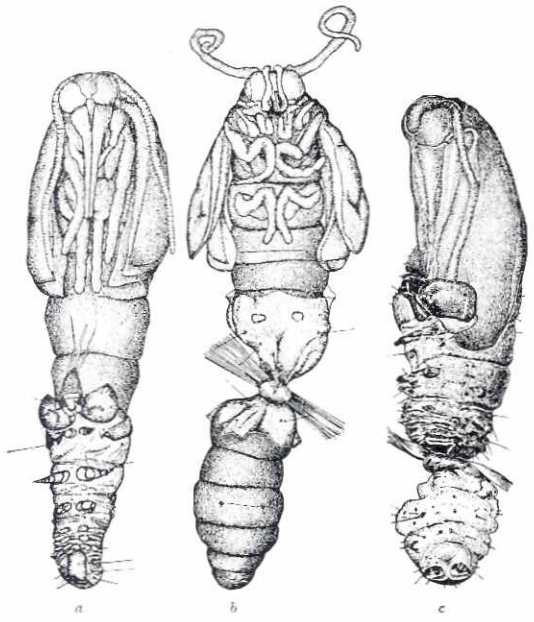


Fig. 2. Ligation experiments with larvae and pupae of *Ephestia kuhniella*. **(a)** *Nonoperated pupa, the old cuticle is partly shed;* **(b)** *a prepupa after ligation: both parts have formed a pupa. The old cuticle is removed.* **(c)** *A larva ligated earlier. The front part has formed a pupa, the hind part is a permanent larva.*

Silkworms were reared in quantities, but only the females had the scent glands, and the males could be used for my purpose (with the exception of a few that were needed for the bioassay of the sex attractant).

Guided by the *Calliphora* bioassay and using various methods of separation, e.g. chromatography on alumina or counter-

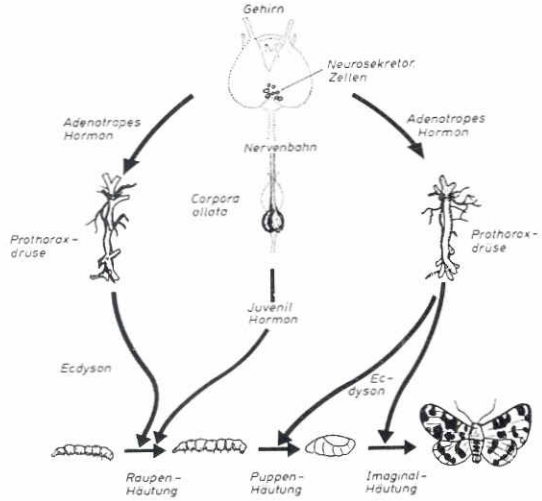


Fig. 3. Scheme of the interaction of hormones during insect development. *Neurosecretory cells in the brain produce an adenotropic hormone that activates the prothoracic glands to produce the moulting hormone, ecdysone R larval moult results if the corpora allata produce the juvenile hormone. If the corpora allata remain silent, a pupal molt and later an imaginal moult occur. Taken from 5. Biolog Jahresheft, Yerband Deutscher Biologen, Iserlohn 1972.*

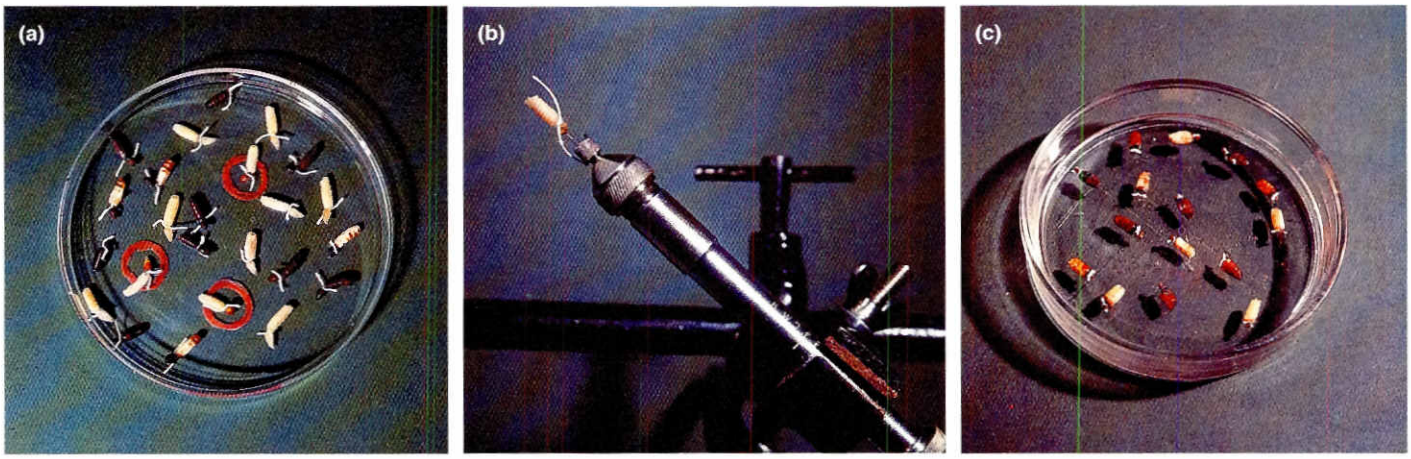


Fig. 4. The *Calliphora* bioassay. (a) *Shows a number of larvae 24 h after ligation. Those encircled have formed a puparium in the head part; they are used for the assay.* **(b)** *Shows the injection of the solution to be assayed.* **(c)** *Shows the result after 24 h: many larvae have formed a puparium. If their number is 50%, then the dosis corresponded to 1 Calliphora unit.*

current distribution, we arrived at good purification factors. But it became clear to me that we needed much more starting material to get a breakthrough and isolate the hormone in pure form.

I knew that in the early fifties, silk worm rearing was still done commercially in Germany. Before and during the war, it was propagated because the silk was needed for the production of parachutes. Now it was used for silk stockings which were very rare.

Fig. 5. The author (left) and his assistant, Ingeborg Brachmann, preparing a crude extract from silkworm pupae using a press.

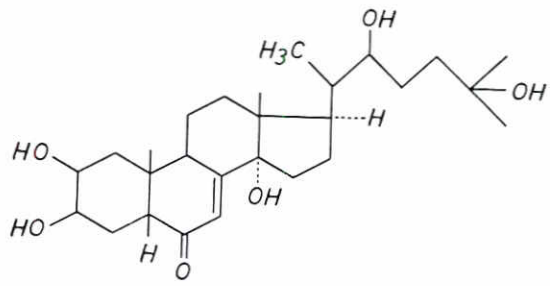
I then went to Butenandt and persuaded him that we should buy the German harvest of silk cocoons, transfer them to Tübingen and use them for our scientific projects, mainly the metamorphosis hormone and the sex attractant. The necessary money came from a grant of the Deutsche Forschungsgemeinschaft. The organization of this operation was done in collaboration with an Institute in Celle that was responsible for the German silk production

Our task was not simple. When we received the cocoons in Tübingen, they had to be cut open and the pupae removed. Then they had to be sorted out to separate the females and the males (the pupae show a morphological difference on the last abdominal segments that allowed the determination of the sex). The females were allowed to eclose, then their heads were cut off to get material for the analysis of their eye pigments, and the scent glands at the tip oitres of methanol. It was impossible to handle these quantities in the laboratory; we needed help from the chemical industry. The Grenzach factory promised to do the necessary operations, but they required my presence to survey the extractions and manipulations. Thus, in the fall of 1953, I worked for three weeks as chemist in a factory; it was an interesting time.

To cut a long story short, the operation resulted in a concentrate of 5 kg from 500 kg of pupae. With this material, I returned to Tübingen and started the further purifications. Our guide was, as mentioned, the *Calliphora* bioassay. Gradually, we arrived at preparations with very high activities.

In March 1954, I discovered crystals in a small vessel set aside. At first I did not believe that they could be important, we had isolated crystalline material from our extracts on more than one occasion (Butenandt *et al.*, 1951a,b). But in the bioassay the material was active, even at low concentrations Indeed we had isolated the moulting and metamorphosis hormone, now known as ecdysone. The yield was only 25 mg from the 500 kg of pupae, but this meant a purification factor of about $1:10^7$.

Ecdysone was the first insect hormone to be isolated. The paper appeared in June 1954 in "Zeitschrift fur Naturforschung"



Ecdyson

Fig. 6. Structural formula of ecdysone. *Taken from 5. Biolog Jahresheft, Yerband Deutscher Biologen, Iserlohn 1972.*

(Butenandt and Karlson, 1954). I had already started a correspondence with Carrol Williams at Harvard University, and now I asked him if he would be willing to test ecdysone in his bioassay on diapausing pupae of Cecropia. Indeed, ecdysone showed the expected activity for the metamorphosis hormone. This was important since many insect physiologists did not believe in the *Calliphora* bioassay They thought the puparium formation was due to some quinones responsible for tanning of the cuticle (Dennell, 1949).

In the following years, many colleagues asked for samples of ecdysone for their experiments. I was for years the only person who had ecdysone. In most cases, I gave the necessary quantity, often in form of highly purified extracts – crystalline ecdysone was too precious. Moreover, we needed all we had for chemical experiments towards the elucidation of the structure.

This proved very difficult. In the mid-fifties, many powerful physical methods were not yet available, e.g. nuclear magnetic resonance and mass spectroscopy. We had just acquired an infrared spectrometer and the experts interpreted the spectrum of ecdysone as evidence for a peptide bond. Later, it turned out that ecdysone did not contain nitrogen. A regrettable error was our incorrect determination of the molecular mass. This was due to the fact that crystalline ecdysone contained half a mole of crystal water. Thus, our measurement led to the empirical formula of $C_{18}H_{30}O_4$; only later, we found that the correct formula is $C_{27}H_{44}O_6$ (Karlson *et al.,* 1963).Chemical degradation experiments as well as the analysis of the crystals showed that ecdysone is a steroid. The full elucidation of the structure was achieved in 1965, again by a combination of chemical experiments (Karlson *et al.,* 1965) and X-ray analysis of the crystals (Huber and Hoppe, 1965).

So we were back to steroids. Butenandt had started his scientific career with the isolation of the sex hormones estrone, androsterone and progesterone, and the elucidation of their structures, they all were steroids. Then he deliberately left this field to work on other problems – the mechanism of gene action in the moth *Ephestia*, and as already mentioned the sex attractant of *Bombyx*. I did my Ph.D. Thesis on a problem of steroid stereochemistry, thus I also started out with steroids. And now, ten years later, I found myself back in the field of steroids! But let us return to Developmental Biology. Ecdysone allowed us to make an important discovery: that hormones act by the activation of genes. The idea was born in a discussion which I had with Ulrich Clever in the summer of 1959 at the Max-Planck-Institute for Biology in Tübingen. Clever had approached me asking for ecdysone to use it in tissue culture. He was studying the puffing pattern in the giant chromosomes in the midge *Chironomus tentans*. Puffs are local enlargements of certain bands on the chromosomes, they are visible signs of gene transcription. During the transition of mature larvae to pupae the puffing pattern changed considerably, some puffs regressed, others appeared anew.

In the discussion I said to Clever that this might be due to endogenous ecdysone. We decided to test this hypothesis, and in October I went again to Tübingen, this time with my micro syringe and a solution of ecdysone. We did the injections, and soon after my return to Munich I got a letter from Clever telling me that ecdysone indeed produced puffing (Clever and Karlson, 1960). The response was very quick, visible already 30 minutes after injection, and the dose necessary was rather low, one hundredth of a *Calliphora* unit – that corresponds to 10^{-10} gram.

This result was exciting For the biochemist, it meant the elucidation of the mode of action of a hormone. It later turned out that all steroid hormones act in this way. For the biologist, it meant that a hormone can be a "timing device" to control gene activity (Karlson, 1963).

A historical review should not come too close to the present time. I will therefore end here, leaving the developments of the last 30 years to other contributors of this issue.

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