HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ECDYSONE METABOLITES APPLIED TO THE CABBAGE BUTTERFLY, *PIERIS BRASSICAE* L.

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ABSTRACT

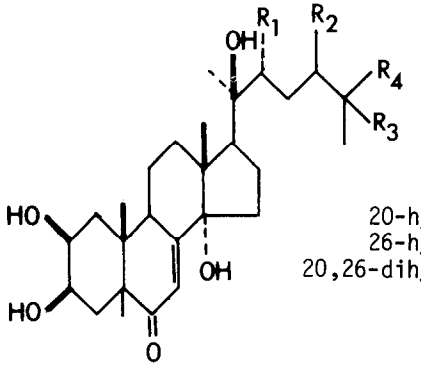
HPLC allowed separation of twelve major labeled compounds after injection of ^{3}H-ecdysone into *Pieris* pharate pupae. These compounds were identified as six pairs of metabolites (3α and 3β epimers), comprising ecdysone, 20-hydroxyecdysone, 26-hydroxyecdysone, 20,26-dihydroxy-ecdysone and the polar metabolites P and 20-hydroxy-P. These last two products could not be enzymatically split by any hydrolase tested and are weak acids arising respectively from 26-hydroxyecdysone and 20,26-dihydroxyecdysone. They might be 26-oic compounds.

Epimerization appears as a fundamental inactivation process in *Pieris* and could well be a general characteristic of *closed systems* (eggs and pupae). No significant amounts of hydrolyzable conjugates were detected in our biological system (pharate pupae and pupae).

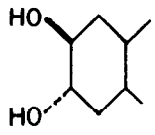
INTRODUCTION

Ecdysteroid metabolism in insects and crustaceans is quite well documented (1,2). Much work has been devoted to isolating several compounds which occur naturally in these animals [ecdysone (3), 20-hydroxyecdysone (4), 26-hydroxyecdysone (5), 20,26-dihydroxyecdysone (6), makisterone A (7) and 2-deoxyecdysone (8), as well as the 3α epimers of several ecdysteroids (9)]. Parallel tracer experiments have defined the relationships between these compounds, and have also provided evidence for the formation of various as yet unidentified polar metabolites (1). These metabo-

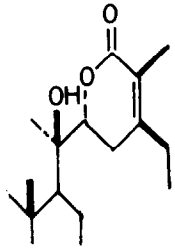
STRUCTURAL FORMULAE OF ECDYSONE AND RELATED COMPOUNDS



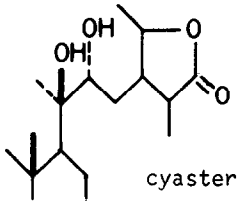
	R_1	R_2	R_3	R_4
ecdysone	H	H	OH	CH_3
20-hydroxyecdysone	OH	H	OH	CH_3
26-hydroxyecdysone	H	H	OH	CH_2OH
20,26-dihydroxyecdysone	OH	H	OH	CH_2OH
makisterone	OH	CH_3	OH	CH_3
inokosterone	OH	H	H	CH_2OH



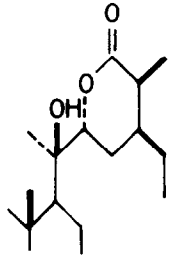
series of
3α epimers



ajugalactone



cyasterone



capitasterone

TRIVIAL AND IUPAC EQUIVALENT NAMES OF THE MAJOR ZOOECDYSTEROIDS

ecdysone = 2β,3β,14α,22R,25-Pentahydroxy-5β-cholest-7-en-6-one
20-hydroxyecdysone = 2β,3β,14α,20R,22R,25-Hexahydroxy-5β-cholest-7-en-6-one
26-hydroxyecdysone = 2β,3β,14α,22R,25,26-Hexahydroxy-5β-cholest-7-en-6-one
20,26-dihydroxyecdysone = 2β,3β,14α,20R,22R,25,26-Heptahydroxy-5β-cholest-7-en-6-one
3-epiecdysone = 2β,3α,14α,22R,25-Pentahydroxy-5β-cholest-7-en-6-one
3-epi-20-hydroxyecdysone = 2β,3α,14α,20R,22R,25-Hexahydroxy-5β-cholest-7-en-6-one

lites were in many cases enzymatically hydrolyzed and appeared to be conjugates (sulfo-, gluco- or phosphoconjugates), whereas others seemed resistent to hydrolysis. The metabolic pathways described for insects consist of general basic reactions e.g. hydroxylation in position C-20 and of reactions described in only a few species (10,11) like dehydrogenation or epimerization in position C-3. In the case of *Lepidoptera*, ecdysteroid metabolism has been studied in several species, namely *Antheraea polyphemus* (12), *Bombyx mori* (13,14), *Hyalophora cecropia* (15), *Prodenia eridania* (16), *Manduca sexta* (17) and *Choristoneura fumiferana* (18). These investigations were performed at various developmental stages (eggs, embryos, larvae or pupae) and provided evidence for the scheme summarized in figure 1.

Several points, however, need further examination, especially the question of polar metabolites. The general development of high-perfor-

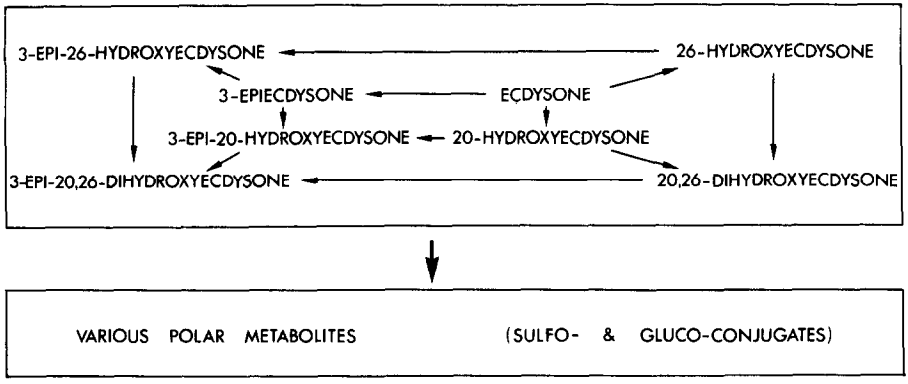


Fig. 1. Summary of present data concerning ecdysone metabolic pathways in *Lepidoptera*, based on references quoted in the text.

mance liquid chromatography (HPLC) has enabled us to examine the fate of ecdysone and 20-hydroxyecdysone in our research animal, *Pieris brassicae* (*Lepidoptera*). The aim of the present work is essentially qualitative, i.e. concerned with the chemical nature of the various metabolites.

MATERIALS AND METHODS

Animals. *Pieris* larvae were reared as previously described (19), i.e. at constant temperature (21 ± 1°C) under long-day photoperiod (16L : 8D). Under these conditions, the last larval instar lasts five days and the pupal stage, twelve days. The present experiments used pharate pupae (12 hours before pupal ecdysis).

Chemicals. (^{3}H) ecdysone and 20-hydroxyecdysone of high specific activity (50-60 Ci/mmol) were gifts from Dr. Hoffmann (Strasbourg, France). They were injected as water solutions (0.1 to 0.2 µCi per animal, i.e. about 20-40 pmol.). Amounts injected were always far below the physiological levels previously found in this species (20).

Reference ecdysteroids (non-labeled forms) were ecdysone and 20-hydroxyecdysone (Simes, Milan, Italy), 26-hydroxyecdysone (a gift of Dr. Kaplanis, to Dr. Delbecque) and 20,26-dihydroxyecdysone (a gift of Dr. Kaplanis, to Dr. Hoffmann). Reference ecdysone and 20-hydroxyecdysone 3α epimers were prepared enzymatically according to Nigg *et al.* (11).

Sample preparations for HPLC. Labeled compounds were injected into pharate pupae and the animals sacrificed 24 hours thereafter (as young pupae). They were extracted twice with large volumes of methanol and the crude extracts were processed according to figure 2. The purification procedure included differential precipitation, silicic acid column chromatography and in some cases thin layer chromatography (TLC) on silica plates with a fluorescent indicator (type 60 F 254 from Merck, Darmstadt, Germany). All solvents were from Mallinckrodt or Merck.

HPLC analyses. They were performed either with the whole spectrum of metabolites or with specific fractions previously isolated by TLC. For isocratic analyses, we used a Waters 6000 A module pump and a universal septumless U6K injector. Gradient analyses were conducted using a DuPont system (Module 848 pump and preprogramed gradient former) with a 50 µl sample loop Rheodyne injector (21, 22). Absorbance was monitored at 254 nm (fixed wavelength detector from Waters).

Two types of columns were used (both were 25 cm long and 4.6 mm i.d.) :

- reversed-phase columns (Zorbax C8 from DuPont) ; elution was carried out with a linear acetonitrile gradient in pH 8.5 buffer (20 mM TRIS, adjusted with perchloric acid to the appropriate pH) or water. The use of a constant pressure delivery system did not allow complete reproductibility of ecdysteroid standard retention times, which varied, especially with column ageing . Consequently, some variability was observed in the present experiments.

- straight phase columns (silica) were used for further purification of TLC or reversed-phase HPLC (RP-HPLC) fractions. Two columns (Partisil 10 from Whatman and Zorbax-Sil from DuPont) were used with two different solvents systems-methylene chloride/*iso*-propanol/water (125:25:2) and methylene chloride/methanol (9:1). The reasons for this choice will be given under Results.

Hydrolysis procedures. In order to define the nature of the polar metabolites found in *Pieris*, hydrolysis experiments were performed according to procedures already described (23), using either purified enzymes (from Sigma) or a crude *Helix pomatia* juice (Industrie Biologique Française, Genevilliers).

RESULTS

Metabolite pattern. Ecdysone and 20-hydroxyecdysone were injected into pharate pupae, i.e. during the descending phase of the large hemolymph 20-hydroxyecdysone peak (20). The metabolizing mechanisms are very efficient at this time and give rise to many compounds which can be separated by RP-HPLC on the C8 column (figures 3A and 3B). Some of the observed peaks comigrate with authentic standards of ecdysone, 20-hydroxyecdysone, 26-hydroxyecdysone and 20,26-dihydroxyecdysone. The major part of the radioactivity is contained in polar metabolites which are eluted early. Figures 3A and 3B clearly show that the different metabolites are eluted as compound pairs (i.e. 3 pairs in figure 3A and 6 in figure 3B), and this result suggests the existence of isomeric forms for each compound. In order to assess this idea, certain metabolites were further analyzed on silica columns. Using a Zorbax-Sil column and a water-containing eluent, the ecdysone/20-hydroxyecdysone band (from a TLC separation) gave single sharp peaks comigrating with authentic hormones (figure 4A). Due to the high efficiency of this chromatographic system (11,000 theoretical plates), we concluded that no isomers (epimers) were present in *Pieris* (22), but this conclusion made the results of figures 3A and 3B impossible to understand. However, when the same mixtures were analyzed with a water-free solvent, two peaks were easily distinguishable although some

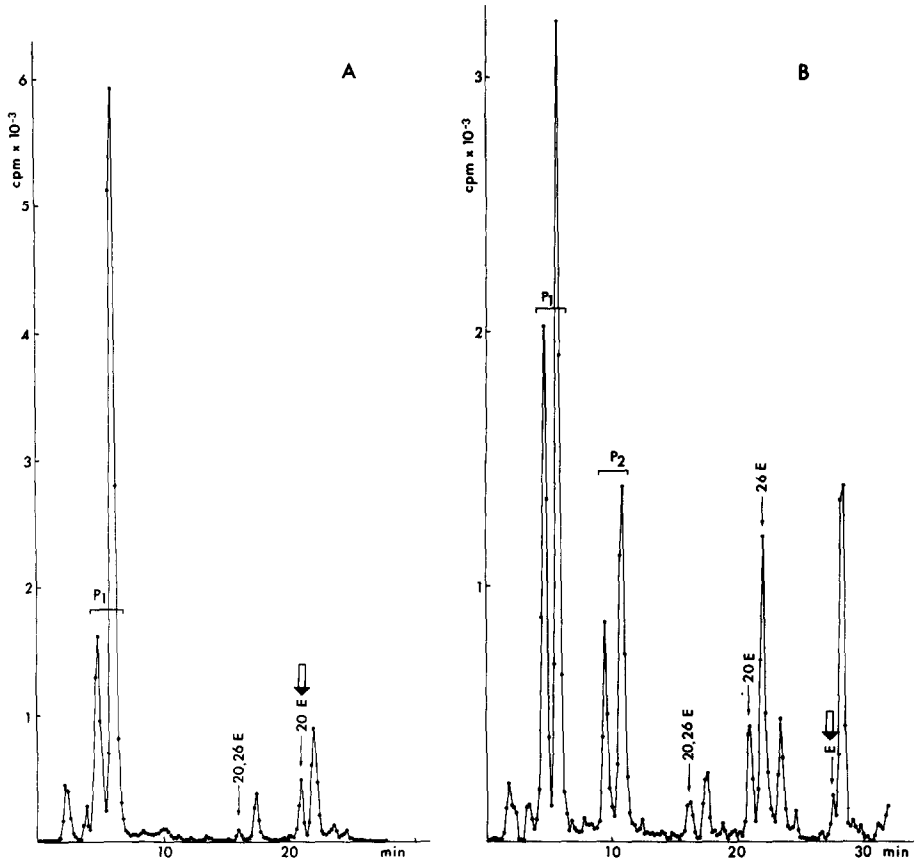


Fig. 3. Pattern of metabolites formed in *Pieris* pharate pupae after ^{3}H-20-hydroxyecdysone (A) or ^{3}H-ecdysone (B) injection. Operating conditions : column Zorbax C8, pressure 2,500 psi. Primary solvent 8 % acetonitrile in 50 mM Tris/perchlorate buffer pH 8.5. Secondary solvent 40 % acetonitrile in the same buffer. Gradient conditions : 0 to 100 % secondary solvent in 50 min., linear gradient. Fractions : 0.2 min. Some of the labeled compounds eluted with ecdysteroid standards (2026 E : 20, 26-dihydroxyecdysone ; 20E : 20-hydroxyecdysone ; 26E : 26-hydroxyecdysone ; E : ecdysone). Arrows (⇨) indicate the compounds injected. P_1 and P_2 refer to polar metabolite pairs.

peak tailing impeded baseline resolution (figure 4B).

This finding was consistent with earlier observations in which similar chromatographic systems were used to separate the 3β and 3α epimers of ecdysone and 20-hydroxyecdysone (11,26). It was therefore con-

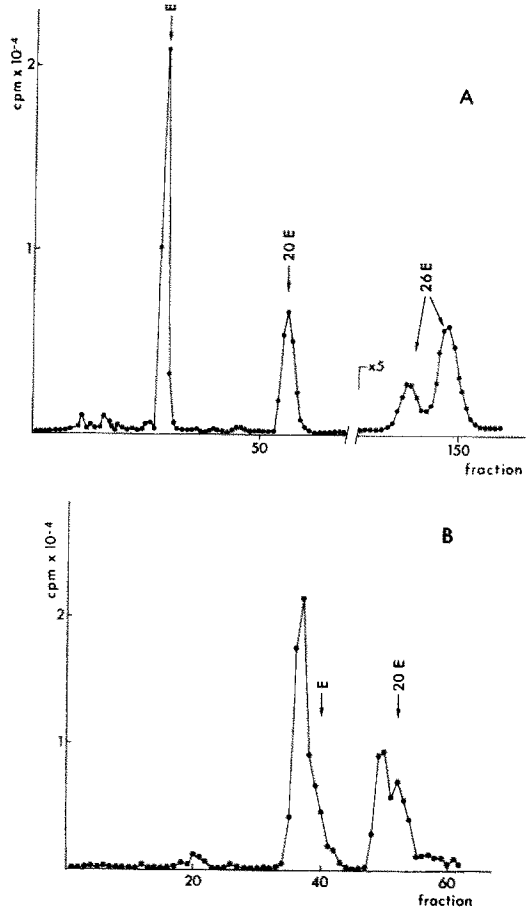


Fig. 4. HPLC analysis on a Zorbax-Sil column of the "non-polar metabolite band" from a TLC separation (animals injected with ecdysone).
Operating conditions :
A. solvent Methylene chloride/*iso*-propanol/water (125:25:2), flow rate 1 ml.min^{-1}, fractions 1 ml.
B. solvent Methylene chloride/methanol (9:1), flow rate 1.5 ml. min^{-1}, fractions 0.75 ml.
Reference compounds as in figure 3. Note in figure 4A that 26-hydroxyecdysone (labeled *or* standard) gives two peaks, probably corresponding to the 25S and 25R isomers respectively (24).

cluded that the compound pairs observed in figures 3A and 3B might be due to the simultaneous presence of the 3α and 3β forms of six different metabolites, i.e. the four mentioned above plus the two polar metabolites P_1 and P_2. Moreover, only one pair of polar metabolites (P_1) was found after 20-hydroxyecdysone injection, but two pairs (P_1 and P_2) were observed after ecdysone injection. As *in vivo* ecdysone is efficiently converted into 20-hydroxyecdysone, this result is consistent with the hypothesis that P_1 and P_2 differ by the presence or absence of a 20-hydroxyl group. Their chemical nature will be considered below.

Evidence for the presence of 3α and 3β epimers. We prepared labeled 3α epimers of ecdysone and 20-hydroxyecdysone by incubating the 3β forms with midgut homogenates and a NADPH generating system, according to a previously described procedure (11). The reactions proceeded with a very good yield and the two epimers were separated by RP-HPLC (figure 5). The 3α compounds were identified upon chromatographic properties and

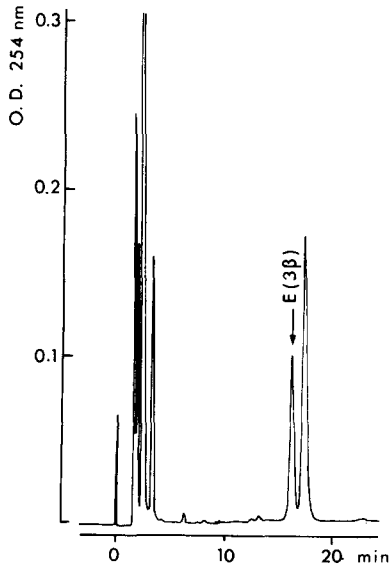


Fig. 5. Separation of the 3β and 3α ecdysone epimers from an incubation of *Pieris* midgut homogenates *in vitro*. The mixture was purified according to figure 2 and a TLC band containing both isomers was injected on the Zorbax C8 column. Operating conditions : pressure 2,500 psi, primary solvent, 18 % acetonitrile in 50 mM buffer, secondary solvent, 40 % acetonitrile in buffer. Linear gradient : 0 to 30 % secondary in 30 min. The 3α epimer was identified from the absence of acetonide formation.

reaction with acetone in the presence of paratoluenesulfonic acid.

The purified 3α epimers of ecdysone and 20-hydroxyecdysone were injected into pharate pupae. The pattern of 3α-ecdysone metabolites is shown in figure 6. Six major single peaks were observed, and no compound pairs were found. Similarly, 3α-20-hydroxyecdysone metabolites gave three single peaks (not shown here). The experiments reported in figures 3B, 5 and 6 strongly support our hypothesis on the formation of epimers. Additionally, figure 6 clearly shows that the epimerization reaction is irreversible, at least *in vivo*.

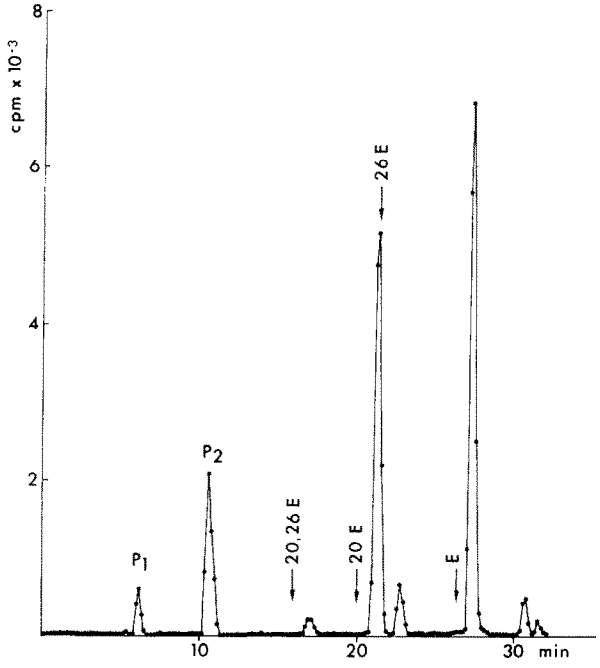


Fig. 6. Pattern of metabolites obtained after injection of labeled 3α-ecdysone. Same conditions as in figure 3 (compare this result with figure 3B). The procedure for sample preparation in this case includes incubation with *Helix* juice (otherwise the pattern of metabolites would be more complex). The reasons for this treatment are given in the discussion.

Alternative experiments were performed with *in vitro* cultures of *Pieris* imaginal discs, which were able to transform the hormones (24). Ecdysone was converted into 26-hydroxyecdysone and a polar metabolite, and 20-hydroxyecdysone mainly formed a polar metabolite- more polar than

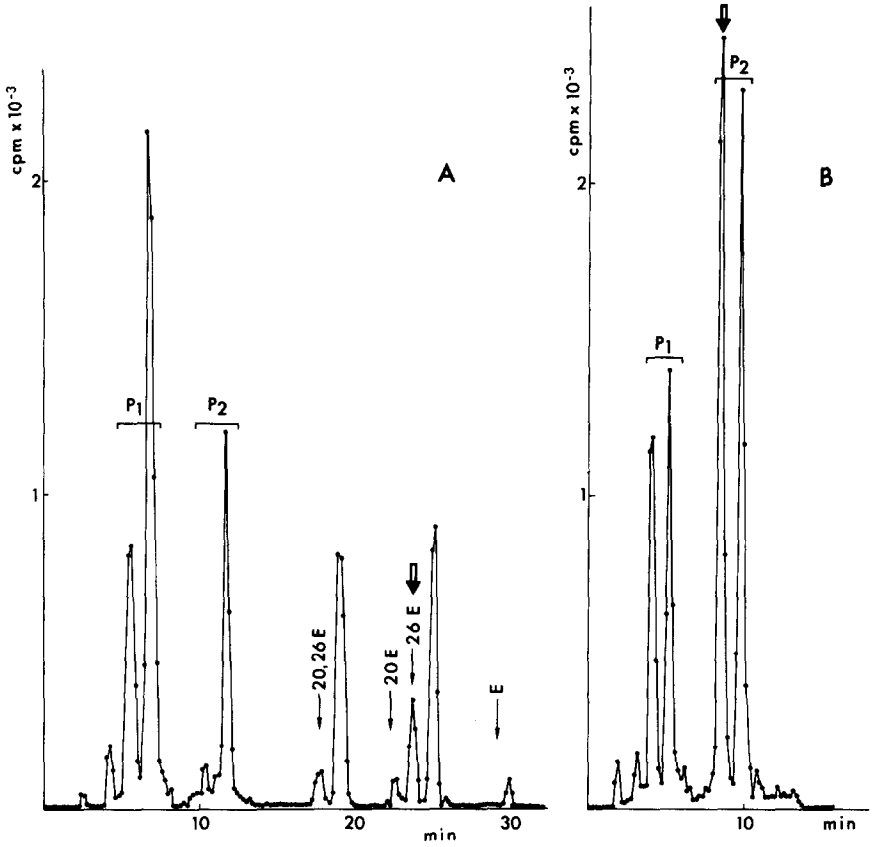


Fig. 7. *In vivo* conversion by pharate pupae of *Pieris* of 26-hydroxyecdysone and of a polar metabolite formed *in vitro* by imaginal wing discs incubation with labeled ecdysone. Operating conditions as in figure 3. A : injection of 26-hydroxyecdysone ; B : injection of P_2 (arrows correspond to injected compounds).

the preceding one. Wing discs were unable to achieve 20-hydroxylation or 3-epimerization. The polar metabolites which they formed from ecdysone and 20-hydroxyecdysone were respectively identified as P_2 and P_1 (3β forms only), thus providing additional evidence that P_1 is probably 20-hydroxy-P_2. This *in vitro* conversion of hormones by discs was used for the preparation of labeled 26-hydroxyecdysone and P_2. These two metabolites were subsequently injected into pharate pupae (figure 7). The exa-

mination of figures 7A and 7B suggests several comments : first, all the metabolites appear as pairs, which means that epimerization can occur irrespective of the hydroxylation pattern. Second, figure 7 shows the conversion *in vivo* of 26-hydroxyecdysone into 20,26-dihydroxyecdysone, and of P_2 into P_1 : thus, 20-hydroxylation is also a general reaction. Third, figure 7A gives fundamental information concerning the origin of the polar metabolites P_1 and P_2, showing not only the expected conversion of 26-hydroxyecdysone into 20,26-dihydroxyecdysone and into the corresponding 3α epimers, but also its conversion into P_1 and P_2. This result will be of importance for our discussion about the chemical nature of these polar metabolites.

The polar metabolites P_1 and P_2. These metabolites were at first thought to be conjugates, and various hydrolysis experiments were performed either with purified enzymes or crude snail juice as previously described (23). All these experiments were unsuccessful with P_1 and P_2, as had also been the case for the I_C class of polar metabolites described in *Locusta* (23).

The analogy with *Pieris* polar metabolites led us to compare them with I_C in more detail. Accordingly, *Locusta* adults were injected with (^{3}H) ecdysone and their feces extracted as described in figure 2. TLC analysis showed that I_C and (P_1 + P_2) had similar R_f (figure 8). HPLC analysis of I_C on the C8 column showed that it was a mixture of P_1 and P_2 without the 3α epimers. Besides I_C, the *Locusta* extract contained a more polar class of metabolite, probably the one termed I_A (23). Hydrolysis experiments were unsuccessful with I_C, but positive with I_A, in agreement with previous data (23). *Helix pomatia* juice is a complex and very active mixture of enzymes that is able to split all the conjugates

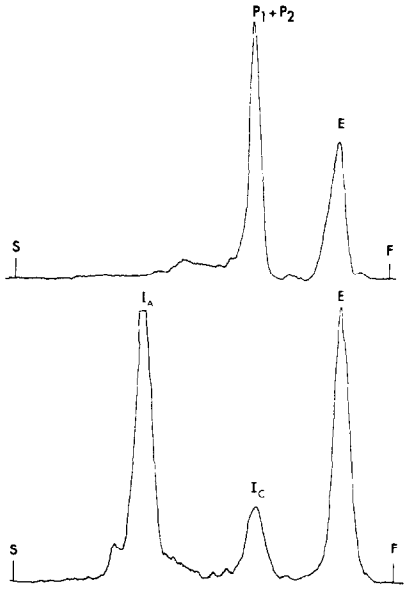


Fig. 8. TLC of ecdysone metabolites in *Pieris* pharate pupae (A) and *Locusta* adult feces (B). Solvent : ethyl acetate/ethanol/water (20:80:10). See text for further details.
S : start ; F : front.

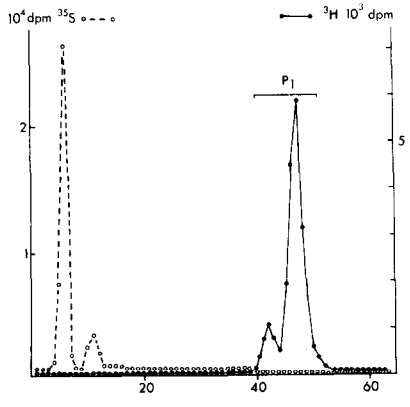


Fig. 9. HPLC of the polar metabolite band of *Pieris* after simultaneous injection of (^{3}H) 20-hydroxyecdysone and ^{35}S sodium sulfate into pharate pupae. The TLC band containing both isotopes was further examined on the C8 column.
Operating conditions : pressure, 2,000 psi ; primary solvent, 1 % acetonitrile in pH 9 buffer ; secondary solvent, 40 % acetonitrile in the same buffer. Linear gradient : 0 to 100 % secondary solvent in 50 min. 0.3 min. fractions were collected.

so far described for insects (sulfates, glucosides, glucuronides and phosphate esters). Our hydrolytic conditions were efficient, as I_A was totally hydrolyzed. This means that P_1 and P_2 are probably not conjuga-

tes. Additional evidence against the sulfoconjugate nature of P_1 and P_2 was provided by double-labeling experiments with (^3H) ecdysone and (^{35}S) sodium sulfate. Here, the TLC band containing P_1 and P_2 also contained some ^{35}S, but subsequent HPLC clearly resolved the ^3H and ^{35}S peaks (figure 9). This is also good evidence that TLC comigration cannot be regarded as a sufficient criterion for the identification of polar metabolites.

Having rejected the conjugate nature of *Pieris* polar metabolites on experimental evidence, we attempted to characterize them by other means. Earlier electrophoresis experiments with I_C (23) indicated that these compounds are weak acids. This was confirmed by our HPLC studies, in which reduction of the pH of the buffer used in the mobile phase increased P_1 and P_2 retention (while ecdysone and 20-hydroxyecdysone retention remained constant) (22). From the data thus obtained, the pK of this acid function can be evaluated at 4-5 (i.e. analogous to that of a carboxylic acid). The presence of a carboxylic acid function in the polar metabolites was checked by treating them with diazomethane, according to the usual procedures (25). A large decrease in the polarity of labeled compounds was observed, as one might expect from the formation of methyl esters (figure 10). The resulting derivatives did not appear very stable, and some hydrolysis occured during RP-HPLC, even when pure water replaced pH 8.5 buffer in the mobile phase. The polarity of the P_1 derivatives was close to that of 20-hydroxyecdysone (figure 10B) when analyzed by RP-HPLC. This would be consistent with the presence of five hydroxyl groups in the molecule ; in the RP mode, the number of -OH groups is a very important factor and migrations of 20-hydroxyecdysone, 26-hydroxyecdysone and inokosterone are very close together.

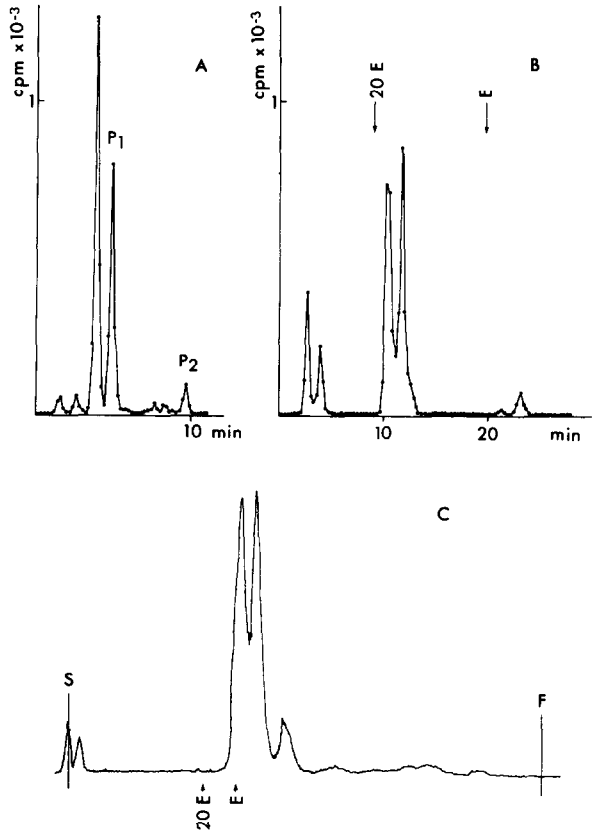


Fig. 10. Effect of treatment of *Pieris* polar metabolites on their chromatographic behavior.
A : polar metabolites before treatment, analyzed as in figure 3.
B : polar metabolites after diazomethane treatment ; same chromatographic conditions as in figure 5, except that water was used instead of buffer.
C : radiochromatogram of TLC separation of polar metabolites after diazomethane treatment ; solvent chloroform/methanol (80:20), two successive developments ; S : start ; F : front.

DISCUSSION

Advantages of the use of HPLC for metabolic studies. As previously reported (21,22), HPLC is an excellent means of analyzing ecdysteroids and their metabolites. Reversed-phase columns in the gradient mode allow baseline resolution of most compounds, except for 3-epi-20-hydroxyecdysone and 26-hydroxyecdysone (figure 3B). These two compounds are however well resolved using a straight phase system (figure 4A).

Silica columns can apparently be used in two ways : when the methylene chloride/*iso*-propanol/water solvent is used, compounds that differ at the side-chain level can be separated ; for instance, ecdysone can be separated from 22-isoecdysone (22), or the 26-hydroxyecdysone 25R isomer from the 25S isomer. This solvent, which *contains water*, seems unable to resolve 3α from 3β epimers when a 6 µm column is used. Paradoxically, the 10 µm column can give some resolution of these two epimers, although the plate number is smaller. Previous analyses performed with this solvent system led us to the erroneous conclusion that no epimers were present in *Pieris* (22), a confusion enhanced by the comigration of 26-hydroxyecdysone and 3α-20-hydroxyecdysone on the C8 column. In fact, when silica columns were used with water-free solvents, better epimer separation was obtained, although excessive peak tailing (especially with the 6 µm column) made baseline resolution impossible. In such cases, peak tailing was smaller with the 10 µm column, i.e. with larger particles. A similar result was also reported by Nigg *et al.* (11). The 3α and 3β epimers were very clearly distinguished with methylene chloride/methanol mixtures, either 9:1 (the present study) or 93:7 [as in a work on *Sarcophaga peregrina* (26)], but the separation was in fact better with conventional TLC (chloroform/methanol 80:20 ; two successive

developments). Best results were obtained with reversed phase columns (see figure 5), provided that the operating conditions were optimized : for instance, it appeared that acetonitrile could be used but not methanol, since with the latter, epimer separation proved impossible on the Zorbax C8 column. However, the use of C8 columns from other manufacturers might lead to different results. A separation process giving similar results to ours was recently described using two sequentially linked µBondapak-C18 columns and 40 % methanol as eluent (27). In any case, all the metabolites found in *Pieris* were unambiguously separated by analysis both with TLC *and* HPLC (with normal silica *and* reversed-phase columns). The TLC step also appears necessary when several classes of polar metabolites are present, as is the case in *Locusta* adults ; this is because all these classes give peaks which elute in the same region, when RP-HPLC and an alkaline buffer are used.

Nature of metabolites in *Pieris* pharate pupae. The metabolites found in *Pieris* form six pairs of 3β and 3α isomers : ecdysone, 20-hydroxyecdysone, 26-hydroxyecdysone, 20,26-dihydroxyecdysone, P_1 and P_2. The present situation is very similar to that described in another lepidopteran species, *Manduca sexta*, in which the 3β and 3α epimers of ecdysone, 20-hydroxyecdysone and 20,26-dihydroxyecdysone were isolated in their pure form from pharate adults. Although 26-hydroxylation is active, 26-hydroxy-compounds do not accumulate but are more or less rapidly transformed into the polar metabolites P_1 and P_2. This is particularly evident when *Pieris* imaginal wing discs are incubated *in vitro* with (^{3}H) 20-hydroxyecdysone (24) ; in this case, a polar metabolite (P_1) accumulates, although almost no 20,26-dihydroxyecdysone can be detected. Are 26-hydroxy-derivatives inactivation products or do they act as true hormones within

target cells ? In our view, such non-accumulating substances might be good candidates for active hormonal factors, but additional experiments are necessary to answer this important question.

Unambiguous identification of P_1 and P_2 requires more defined chemical experiments. The present data show that they are probably not conjugates and that they have a carboxylic function. Non-hydrolyzable polar metabolites were previously reported in several species, including *Locusta* (23), *Calliphora* (28), *Gryllus* (29) and *Tenebrio* (30). The presence of a carboxylic function and the metabolic origin of these compounds (they arise from 26-hydroxy compounds) suggest to us that they might themselves be 26-carboxy compounds. The 26-hydroxyl group is the sole primary alcoholic function of the precursor molecules, and can be readily oxidized into a carboxylic acid function by chemical means [see for instance the previously reported conversion of inokosterone into the 26-oic derivative (31)]. It should also be noted that several phytoecdysteroids contain a lactone ring that involves a carboxylic function at C-26 (e.g. cyasterone, capitasterone and ajugalactone). Finally, oxidation in this position is also known to take place during cholic acid biosynthesis. The postulated reaction would therefore not occur in *Pieris* only.

Absence of conjugates in *Pieris*. Conjugation is a general detoxication process for compounds poorly soluble in water. For instance, vertebrate steroids are detoxified and excreted by this means, but ecdysteroids are much more water-soluble and large amounts of free forms have been reported in the feces of several species (32). On the other hand, insects have general detoxifying mechanisms similar to those described in vertebrates (33), so one may wonder why some insect species conjugate ecdysteroids and others do not. The complexity of the problem is well illus-

trated by *Locusta* adults, which simultaneously excrete free hormones, acid steroids (I_C) and conjugates (I_A) after (^3H) ecdysone injection. It should be noted that conjugates have never been isolated in pure form, and that evidence for their presence results from labeling experiments. We are therefore entitled to ask, firstly, whether conjugates are natural compounds or whether their formation is a consequence of ecdysone injection, especially when no endogenous hormone is present. Thus, *Locusta* larvae essentially excrete I_C (in the presence of endogenous hormone) whereas adult males mainly excrete conjugates (see (23) ; Lafont and Sommé, unpublished data). Secondly, the question arises of whether conjugate formation is due to injection of an excess of hormone. This hypothesis was investigated by injecting up to 50 µg of 20-hydroxyecdysone into *Pieris* pharate pupae but the results did not support it ; no conjugates appeared, and only a small proportion of the injected hormone was converted into P_1 within 24 hours. A third question is the possibility that conjugate formation might be due to injection of a hormone analog instead of ecdysone ; as a foreign substance, this analog would thus be detoxified. It is true that hydrolyzable conjugates were formed by *Pieris* pharate pupae when 3-epiecdysone was injected, but they only accounted for about 10 % of the total radioactivity and their absence from animals injected with ecdysone (in which case there is epimerization) might be due to a compartmental organization of metabolism . Similarly, conjugates were formed by *Manduca* after 22,25-dideoxyecdysone injections (34). In actual fact, *in vitro* incubation of fat body with hormones emerges as the most obvious process leading to conjugate formation. Unfortunately, however, the conjugates thus formed were not identical with the polar metabolites formed *in vivo* (17). Consequently, as long as conjuga-

tes cannot be definitely characterized and demonstrated to occur without hormone injection, this important question will remain open. In any case, they are not present in *Pieris* extracts during the entire pupal-adult development, and hormone inactivation proceeds by other means.

Importance of epimerization reactions. Epimerization is considered as an inactivating mechanism, as 3α-compounds are far less active than 3β-compounds in the bioassays (11). Until now, the presence of 3α-compounds has been reported in a few species only : in *Manduca sexta*, they were found in whole pupae and adult meconium (9) ; in *Sarcophaga peregrina* pharate pupae, 3-epi-20-hydroxyecdysone was identified as a metabolite of 20-hydroxyecdysone (26) ; and in *Galleria mellonella* eggs, 3α-compounds were thought to be present in relatively large amounts (35). In the present study, epimerization was found to take place in pharate pupae and during early pupal development. All the cases reported have a common characteristic : they correspond to *closed systems* - eggs or pupae. By contrast, no epimerization took place in *Locusta* or in *Sarcophaga* larvae (26) - open systems.

In *Pieris* pharate pupae, epimerization is a very active process, but becomes less active in young pupae, when endogenous hormone levels are low ; it is possible that the activity of the epimerizing system varies according to endogenous ecdysone levels. The reaction is irreversible and constitutes a major inactivation process.

Conclusions. The entire catabolic pathway might appear rather complex at first sight. In fact, it proceeds through a limited number of reactions : epimerization at C-3, hydroxylation at C-20 and C-26, and possibly oxidation at C-26. These reactions are responsible for the metabolite pattern observed (figure 11), and are probably catalyzed by

poorly specific enzymes which accept several different substrates. Experiments are under way to define the variations in the activity of these systems in relation to hemolymph hormone levels.

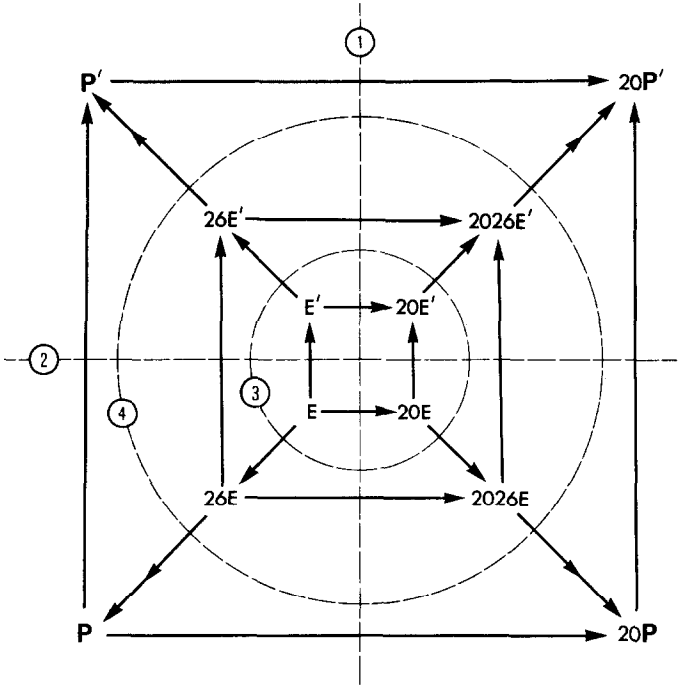


Fig. 11. Summarized view of the metabolic relationship between the compounds identified in *Pieris*. Broken lines refer to enzyme systems (1 : ecdysone 20-monooxygenase ; 2 : ecdysone-3-epimerase ; 3 : ecdysone-26-hydroxylase ; 4 : to be determined). E : ecdysone ; 20E : 20-hydroxyecdysone ; ... The prime (e.g. E') refers to 3α-compounds. P = P_1 and 20P = P_2 (see figure 3).

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