Development-Specific Incorporation of [^{14}C]5-Aminolevulinate and [^{3}H]Leucine into Cytochrome *c* and Biliprotein in the Butterfly, *Pieris brassicae.* Correlation with the Ecdysteroid Titer in the Pupa

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Incorporation of [^{14}C]5-aminolevulinate and [^{3}H]leucine into cytochrome *c*, biliprotein and total soluble protein was followed from the last larval instar to the adult stage in *Pieris brassicae*. The titer of ecdysteroids during the pupal stage was determined with a radioimmunoassay to correlate synthesis of heme products and of protein(s) with adult differentiation. Incorporation of both precursors showed a developmental profile with high synthetic activities in feeding larvae and in pupae after the release of ecdysteroids. Variation of the hormone titer during pupal life differed significantly in males and females. Labeling of cytochrome *c* by both ^{14}C and ^{3}H was as expected from the variation of its concentration reported in a preceding paper; highest incorporation was around adult emergence. The results demonstrate that i) the accumulation of cytochrome *c* in the developing adult insect is primarily due to *de novo* synthesis of both heme and apocytochrome *c*, performed under coordinate control, and ii) the concentration of 5-aminolevulinate is not rate-limiting in the formation of cytochrome *c*. Biliverdin IXγ, the major tetrapyrrolic product in this insect, seems to be directly derived from (free) heme and relatively short-lived as deduced from a time-course study. Formation of the bilin, *i.e.* destruction of heme, increased concomitantly to the initiation of adult differentiation by ecdysteroids in the pupa but later decreased at adult emergence. Synthesis of cytochrome *c* takes place as a late event during terminal development. Thus, the pathways leading to the two major heme products seem to be differently regulated during development.

Introduction

In pterygote insects the development of adult forms is characterized by the growth and differentiation of the massive flight muscles [1, 2]. In this tissue up to one third of the volume is made up by a special type of mitochondria known as sarcosomes; for this reason there is a dramatic accumulation of mitochondrial cytochromes during the terminal differentiation of the flight muscles as discussed in detail in a preceding paper [3].

In the Large White Butterfly, *Pieris brassicae*, we found a 43- to 50-fold increase (depending on sex) in cytochrome *c* concentration taking place during a few days before and after adult emergence [3]. Apart from cytochromes this insect produces a biliprotein [4] the concentration of which also varies with development [3]. According to radiotracer experiments, performed with newly emerged butterflies, the prosthetic groups and the corresponding apoproteins of cytochrome *c* and biliprotein are

synthesized *de novo* at that time; the bilin, presumably derived from heme, turned out to represent a major product of the heme pathway in the butterflies [3].

Since heme synthesis was expected to display a distinct developmental pattern, we injected [^{14}C]5-aminolevulinate (ALA) as a specific precursor to tetrapyrrolic compounds [*cf*. 3], in combination with [^{3}H]leucine as a protein label, and followed their incorporation into cytochrome *c* and biliprotein from the last instar of the larva to the fully developed butterfly. As adult differentiation is triggered by ecdysteroid hormones in the pupa we also measured the hormone titers for correlation with changes in the synthetic activities of the heme pathway.

These studies were undertaken to gain insight into the developmental regulation of the heme pathway by ecdysteroids and on the metabolic significance of the formation of bilins in an insect that does not produce hemoglobin as a possible source of these pigments. A detailed developmental study on the formation of heme products using radio-

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labeled precursors has not yet been performed in insects. Some preliminary results have been presented [5].

Materials and Methods

Insects

The culture of our laboratory stock of *Pieris brassicae* L. (Lepidoptera) has been described previously [3]. The developmental homogeneity, achieved by selection of individual insects at the preceding ecdysis, was ± 2 h in last instar larvae, $\pm 5-7$ h in pupae, and ± 1.5 h in adult insects. "Newly emerged" butterflies were those at the age of 5 ± 1.5 h when the cuticle was sufficiently hard.

Assay for ecdysteroids

The hemolymph titers of ecdysteroid hormones were determined in pupae staged to ± 2.5 h at ecdysis; males and females were studied separately. For each day of pupal development $3-9$ samples from individual insects were collected and appropriately diluted with water containing 0.1% phenylthiourea to block phenoloxidases. Ecdysteroids were assayed with a radioimmunoassay using a rabbit antiserum against ecdysterone coupled to bovine serum albumin as described in [6]. The antiserum was kindly provided by Dr. W. Behrens. Hormone concentrations were expressed as equivalents of ecdysterone; ecdysone was about half as active in this assay.

Injection of isotopes

Each insect received 3 μl of a mixture containing 2.38×10^6 dpm L-[4,5-^{3}H]leucine (spec. act. 1.70 TBq/mmol) and 2.83×10^5 dpm 5-amino[4-^{14}C]levulinic acid (ALA) hydrochloride (spec. act. 2.15 GBq/mmol); both were purchased from Amersham-Buchler (Braunschweig). The insects were injected as described [3] and kept at 21 °C. After 2 h (in developmental studies) they were weighed in random groups of 6 (adults; girdle stage insects), 7 (pupae), and $10-12$ (feeding larvae) respectively and killed by immersion into liquid nitrogen.

The time-course study $(15-180$ min) of incorporation was performed with 1-day $(\pm 2$ h) old butterflies (sexes mixed). Five insects were used for one extract.

Chromatographic procedures and quantification of cytochrome c

The procedures were as detailed in a preceding paper [3]. They involved chromatography on TEAE- and CM-cellulose and quantitative determination of purified cytochrome *c* by a sensitive pH-difference spectroscopic method. The biliprotein was obtained in the flow-through of the CM-cellulose column and precipitated with 7% PCA (perchloric acid).

Determination of radioactivity

Radioactivity of the ^{3}H/^{14}C-labeled samples was measured with a Packard Tri-carb scintillation spectrometer with optimized window adjustments; counting efficiencies of 39% for ^{3}H and of 78% for ^{14}C were obtained with all samples. These were the $200\,000 \times g$ supernatant of the extract with 0.2 M potassium phosphate buffer pH 7.2 (total soluble radioactivity), the redissolved TCA (trichloroacetic acid) precipitate of the same extract (total soluble protein), the supernatant of the PCA precipitate of the flow-through of the CM-cellulose column (non-incorporated precursors), the resuspended PCA precipitate of the same flow-through (biliprotein as the main protein), and the solution of the purified cytochrome *c* after pH-difference spectroscopy. Routinely, samples were counted in duplicate from $4-6$ different extracts. Other details were the same as described in [3].

Results

Ecdysteroid concentrations

The variation of the ecdysteroid titer in the hemolymph during pupal life is presented in Fig. 1. The hormone concentration rose by day 2, reached peak values at days $4-5$ and was low again 2 days later. The most remarkable observation was that ecdysteroid concentrations were higher in males than in females. This was highly significant $(P \ll 0.01)$ at days $4-6$; it seems to be in part due to a difference in time at which the hormonal output takes place in the two sexes.

Time-course of incorporation

As a prerequisite for the developmental studies the time-course of utilization of labeled leucine and ALA, injected into 1-day old butterflies, was followed over a period of 3 h. The results are shown in Fig. 2. In cytochrome *c* (Fig. 2A, B) radioactivity
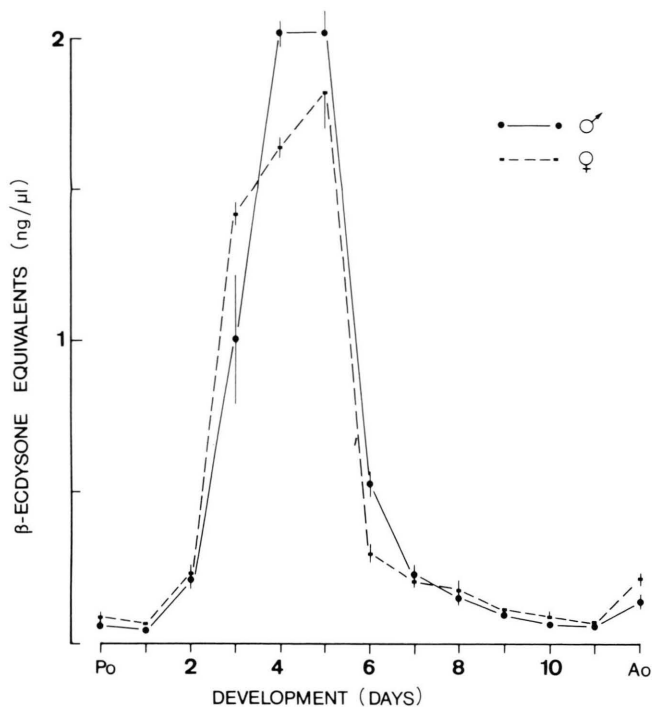
Fig. 1. Variation of hemolymph titer of ecdysteroids during the pupal stage of male and female *P. brassicae*. P_0 – newly formed pupa; A_0 – newly emerged adult. Means ($\pm$ S.E.M.) from 3–9 individual insects.
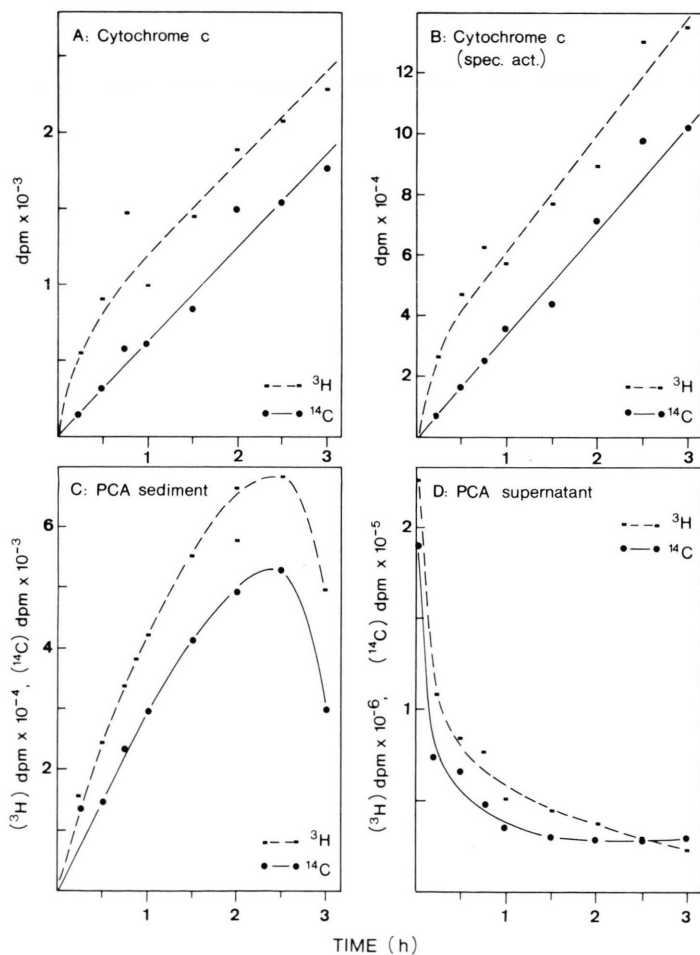

Fig. 2. Time-course of incorporation of a mixture of $[^{14}C]$5-aminolevulinate and $[^3H]$-leucine into cytochrome *c* (A, B) and biliprotein (C) by 1-day old butterflies of *P. brassicae*. Non-incorporated precursors are shown in D. Data are means from 2–4 differnt extracts and expressed in dpm per insect (A, C, D) or dpm per mg of cytochrome *c* (B).

from both precursors increased linearly with time over the whole period. With leucine, however, the initial rate of incorporation (for approx. 30 min) was higher than during the following time.

The PCA sediment (Fig. 2C) contained the biliprotein as the major constituent. Radioactivity from both precursors increased linearly up to 2 h in this fraction but then, after a maximum at about 2.5 h, it declined again in contrast to cytochrome *c*. There was no lag phase in the labeling of either protein.

The disappearance of the injected radioactive precursors was measured in the PCA supernatant (Fig. 2D). Radioactivity from both leucine and ALA rapidly dropped down after injection; during the first 15 min more than 60% of the initial dose was lost in this fraction demonstrating high turnover in both substances.

On the basis of these results a labeling period of 2 h was found to be optimal for studies on the synthesis of cytochrome *c* and biliprotein during development.

Developmental studies

The utilization and incorporation into cytochrome *c* and biliprotein of [^{3}H]leucine and [^{14}C]ALA was studied from day 2 of the last larval instar to the fully developed butterfly 4 days after emergence. The variation of the ecdysteroid titer is presented in each figure for correlation of the synthetic activities with development during pupal life.

Total soluble radioactivity. As shown in Fig. 3 the radioactivity of the extracts varied with the developmental stage. During the larval feeding period and adult development only about 60% of the
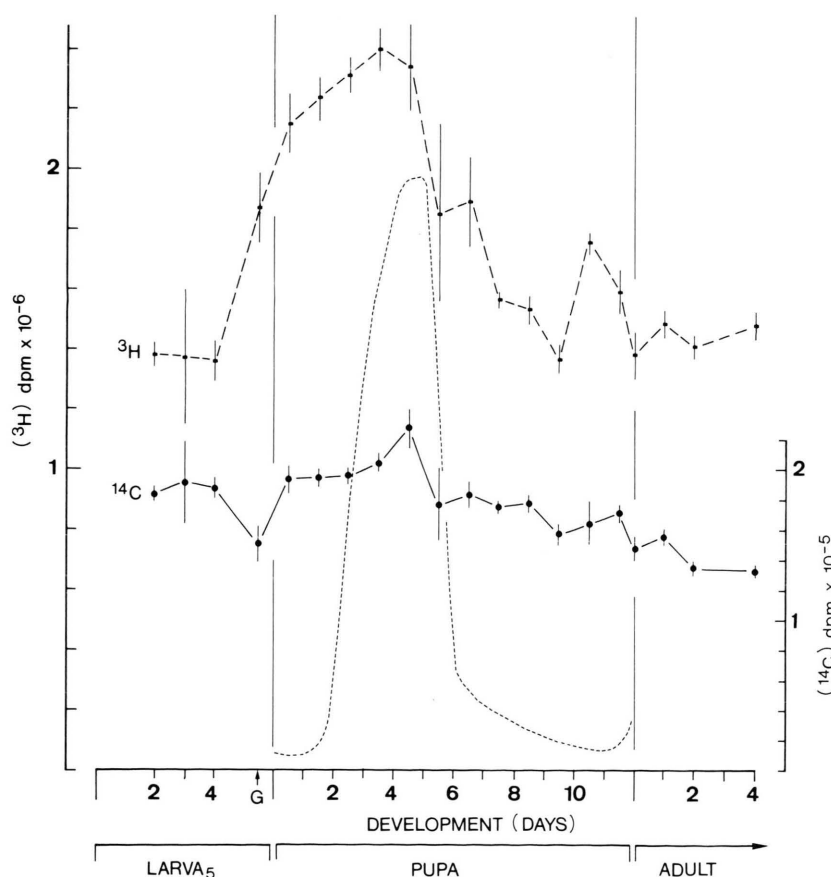

Fig. 3. Soluble radioactivity in extracts from *P. brassicae*. Last instar larvae (Larva$_5$), pupae and adults were injected a mixture of [^{14}C]5-aminolevulinate and [^{3}H]leucine and killed 2 h later (21 °C). Each point represents the mean (± S.E.M.) from 4 different extracts. The variation of ecdysteroid titer (mean curve from Fig. 1) is included for correlation with development. G — spinning of the girdle.

injected ³H-label remained soluble; a major part of leucine was apparently incorporated into insoluble protein. At the time prior to the release of ecdysteroid hormones in the pupa 100% of the injected ³H remained soluble; other data show (see Fig. 8) that it was not utilized in protein synthesis at that time. Soluble radioactivity from ALA did not display such a clear developmental profile. A maximum of 80% of the injected ¹⁴C was found in the supernatant; this proportion decreased to 47% in the adult insect though the major product of ALA was a soluble compound (see Figs. 6, 8).

Total soluble protein. Labeling of the total protein from the extracts varied markedly with development (Fig. 4). The most prominent feature was a rapid increase in protein radioactivity from both [³H]leucine and [¹⁴C]ALA following the peak of ecdysteroid titer. Highest incorporation of ¹⁴C was observed 2 days prior to adult emergence, that of ³H was in 1-day old butterflies. Incorporation of both precursors decreased again rapidly in young butterflies. There was also a transient increase in labeling by ALA in the young pupa prior to the hormone

peak; this was not seen with leucine incorporation which displayed an U-shaped variation during development.

Cytochrome c. Incorporation of leucine and ALA into cytochrome *c* varied similarly to each other over the developmental period studied (Fig. 5). There was a very clear U-shaped developmental variation of incorporated radioactivity from both precursors with a low minimum, especially with ¹⁴C, in the young pupa. Shortly after the ecdysteroids had reached peak titers label in cytochrome *c* began to increase markedly; increase in ¹⁴C started 2 days later than in ³H. The maximum of incorporation of both precursors was around adult emergence; a rapid decrease followed during the first days of adult life. High labeling was also found in young last instar larvae.

Biliprotein. As shown in Fig. 6 there was a continuous increase in radioactivity in the biliprotein fraction by both [³H]leucine and [¹⁴C]ALA following the release of ecdysteroids in the pupa. Labeling dropped down by adult emergence in ¹⁴C and 2 days later in ³H. The high incorporation of ³H in feeding last instar larvae was most probably due to
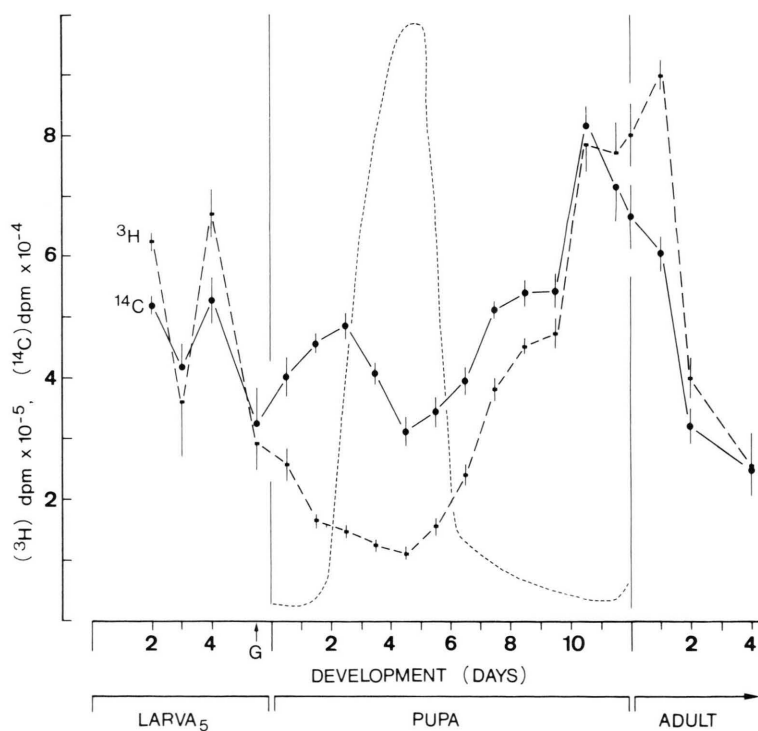

Fig. 4. Protein-bound radioactivity in extracts from *P. brassicae.* For further explanation see legend of Fig. 3.
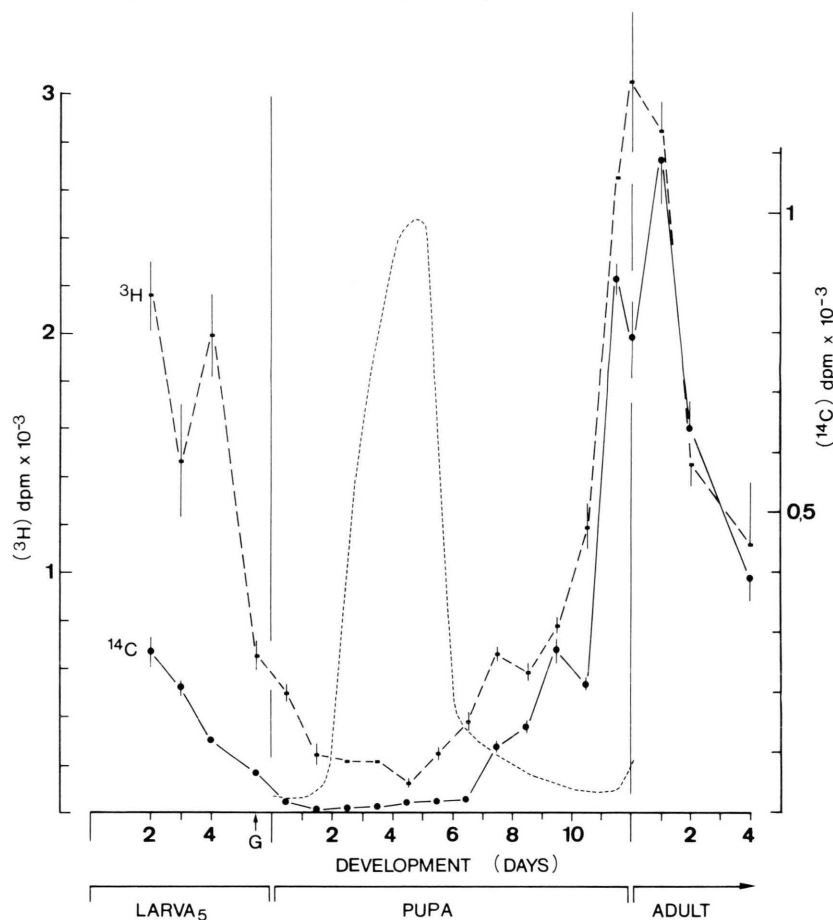
Fig. 5. Radioactivity incorporated into cytochrome c from *P. brassicae*. For further explanation see legend of Fig. 3.

contamination of the biliprotein by other labeled proteins that were also obtained in the flow-through fraction. It should be noted that labeling by [^{14}C]ALA in the young pupa remained at the late larval level and did not decrease as in cytochrome c (cf. Fig. 5).

Non-utilized precursors. As a prerequisite to estimate the proportion of [^{3}H]leucine and [^{14}C]ALA that had not been incorporated into soluble proteins the chromatographic behaviour of the precursors (mixed with a cold extract) on the tandem-operated TEAE- and CM-cellulose columns was followed. As illustrated in Fig. 7 only 67% of labeled ALA but 95% of leucine had been eluted from the TEAE-column by the time when this was detached from the CM-cellulose. This was performed when all cytochrome c and biliprotein had reached the second column (inset in Fig. 7). No radioactivity from the free precursors was bound to the cation exchanger and hence all label that had been eluted

from the first column was recovered in the PCA supernatant of the flow-through of the CM-cellulose column. Assuming no incorporation of precursors has taken place then 2.26×10^6 dpm from [^{3}H]leucine and 1.9×10^5 dpm from [^{14}C]ALA can be obtained in the PCA supernatant.

According to the data presented in Fig. 8 utilization of both precursors was strongly dependent on the developmental stage. The low recoveries during the feeding period of the larvae and during adult development, which followed the release of ecdysteroids, corresponded to periods of high protein synthesis as demonstrated in Figs. 3–6. Just prior to the hormone peak essentially all injected ^{3}H-label was recovered again in the PCA supernatant, *i.e.* was not incorporated into protein. The corresponding values for [^{14}C]ALA never exceeded 70% indicating that transformation of the heme precursor took place at nearly any developmental stage.
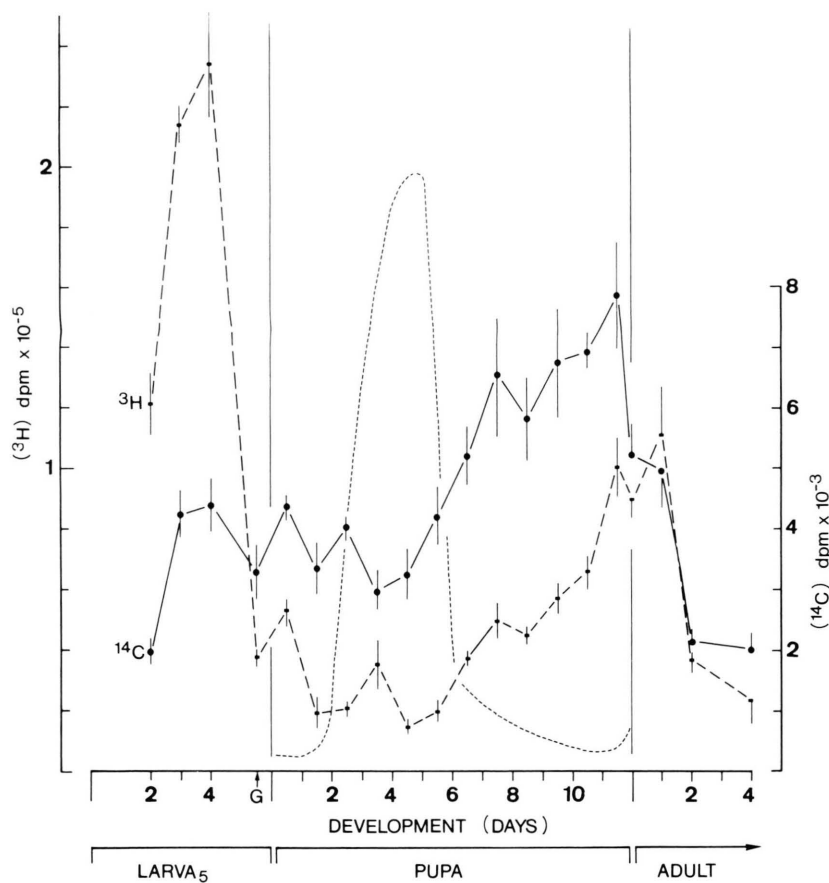
Fig. 6. Radioactivity incorporated into the biliprotein fraction from *P. brassicae*. For further explanation see legend of Fig. 3.
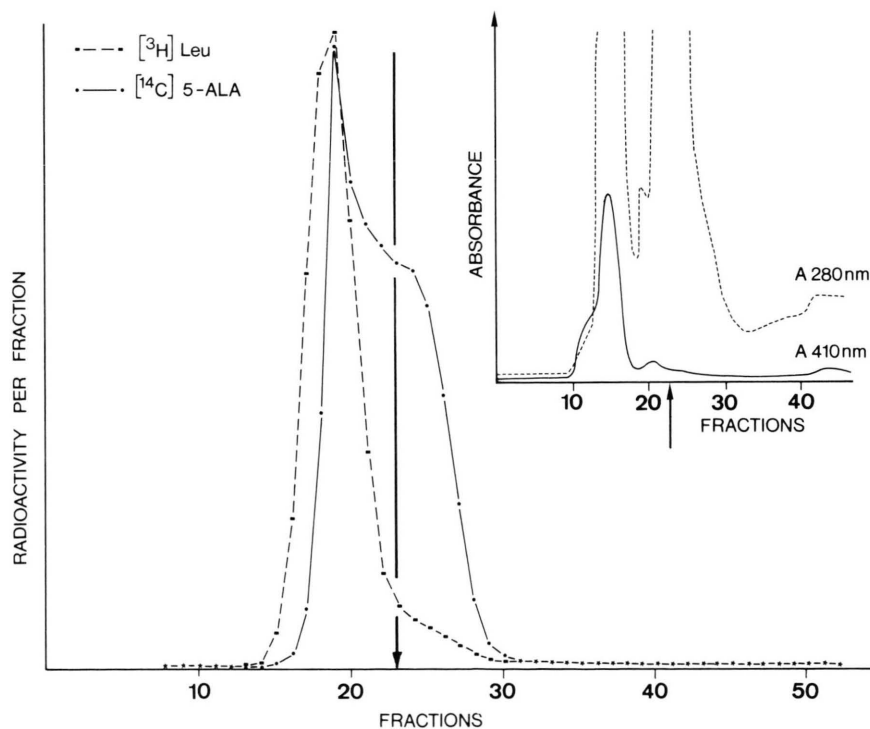

Fig. 7. Chromatographic behaviour of [^{14}C]5-aminolevulinate and [^{3}H]leucine, mixed with a cold extract of adult *P. brassicae*, on TEAE-cellulose. The arrow indicates detachment of the column of TEAE-cellulose from the CM-cellulose column; at that time 67% of labeled 5-aminolevulinate and 95% of leucine were eluted from the TEAE-cellulose and finally recovered in the PCA supernatant (see Fig. 8). The inset shows the corresponding records of absorbance at 280 nm respectively 410 nm (cytochrome *c*: fractions 10–15; biliprotein: fractions 13–19).
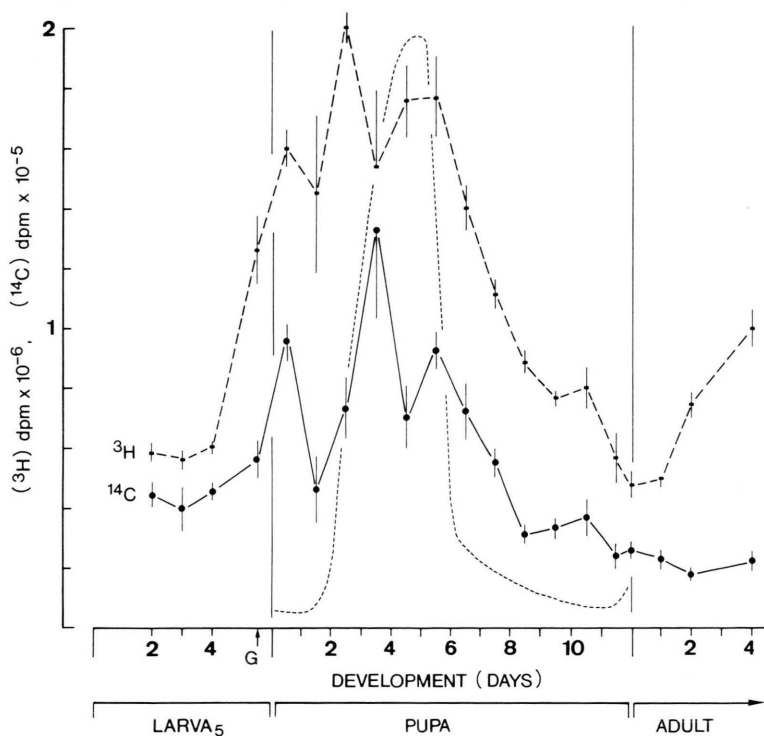
Fig. 8. Non-incorporated [^{14}C]5-amino-levulinate and [^{3}H]leucine in *P. brassicae* (see Fig. 7). For further explanation see legend of Fig. 3.

Discussion

The major results of the present study can be summarized as follows: i) there is a small but significant sexual difference in the variation of the hemolymph titer of ecdysteroids in the pupa, ii) the bilin appears as a straight forward product of the heme pathway, iii) the availability of ALA is not rate-limiting in cytochrome synthesis, and iv) formation of cytochrome c and biliprotein are regulated independently from each other.

Pupal ecdysteroid titers in *P. brassicae* have been reported by several authors [7−9] but the sexes have never been examined separately as in other lepidopteran species, too. It is obvious from our results that in female pupae the hormone concentration rises (and later decreases) some hours earlier and reaches lower peak values than in male pupae. Provided adult development *per se* takes the same time in both sexes, the first statement is supported by our observation that the pupal stage in males takes about 10−20 h longer than in females (not the reverse as claimed in [9]). Apart from this novel feature the variation and the peak values of the hormone titer is in good agreement with the data by

Claret *et al.* [8]; we, too, did not find a preceding smaller peak of ecdysteroids as reported by others [7].

In vertebrates bilins are predominantly formed by degradation of hemoglobin [10]. Radioactivity from a heme precursor thus appears in these pigments at a late time corresponding to the life span of erythrocytes [11]. In our study on an insect the bilin became labeled by ALA with no lag phase similar to cytochrome c; this indicates that the insect bilin is immediately produced from newly synthesized heme and not *via* a hemoprotein. Apparently, there is a rapidly turning-over pool of free heme that is not used for other synthetic activities. It is not yet known in which tissue or even body part the bilin respectively biliprotein is synthesized. This is in contrast to cytochrome c ~ 90% of which is built up in the flight muscles of the thorax which is devoid of bilin. So it seems safe to assume that different pools of heme are engaged in the synthesis of the two chromoproteins. Porphyrins are not immediate precursors to bilins since it is now firmly established that formation of bilins must proceed *via* heme for mechanistic reasons [12, 13].

In biosynthetic work it is essential to know the true specific radioactivity of the immediate pre-

cursor [14]. Though we have determined the concentration of ALA during *Pieris* development [15] nothing is known about tissue location and intracellular compartimentation. Therefore, the correct specific radioactivities of the ALA pools for cytochrome and bilin synthesis, respectively, and hence biosynthetic rates cannot be calculated. Assuming all larval ALA is for bilin synthesis there would be a dilution of the injected [^{14}C]ALA by one third; correspondingly, the rate of bilin formation would be underestimated. Similar considerations hold for leucine; here the injected amount represented less than 1% of that present in the hemolymph of at last instar larva for example [16].

In most tissues heme synthesis is rate-limited by the activity of the first enzyme, ALA-synthase, and thus by the concentration of ALA [17]. In the present study we injected about the 5-fold amount of this precursor as is present in the total pharate insect [15]. Nevertheless, incorporation of ALA into cytochrome *c* was essentially as expected from the variation of cytochrome *c* levels determined previously [3] and no stimulation of cytochrome synthesis by the injection of the precursor was observed [*cf.* 18] as claimed by others [19]. It is thus evident that formation of cytochrome *c* is limited by other steps such as synthesis of the apoprotein or its receptor-mediated transfer across the outer mitochondrial membrane concomitant with binding to heme [20]. A similar conclusion was reached from studies on cytochrome synthesis in fat body mitochondria of a roach [21]. The essentially parallel incorporation of both ^{14}C and ^{3}H into cytochrome *c* during sarcosome development is in favour of a tight coupling between the formation of heme and synthesis of the polypeptide chain. Previous studies with cycloheximide support this [3].

In case of the biliprotein formation of the pigment complex seemed not to be limited by the apoprotein in young butterflies [3] though incorporation of leucine and ALA into the biliprotein ran parallel, as shown in this study. It is concluded that the two components of the biliprotein are produced simultaneously but not in such a tightly coupled manner as in cytochrome *c*.

The results of the present study support the view that cytochromes and the biliprotein represent independent and even alternate products of the heme pathway in *Pieris*. Degradation of heme (or of porphyrin *via* heme) to a bilin is a widespread and conserved process that could represent an old means to regulate the cellular concentration of free heme. In vertebrates pools of rapidly turning-over heme are known which could give rise to the "early peak" of bilirubin formation [22]. A similar situation may lead to the rapid labeling of the insect bilin which itself seems to be relatively short-lived at least in young butterflies.

Cytochrome synthesis in flight muscle cells is a late event in a series of differentiation processes triggered by ecdysteroids. On the other hand, stimulation of heme destruction, as deduced from the increased incorporation of ALA into the biliprotein, starts at the time of apolysis and is therefore regarded as an early metabolic response to the hormones. A similar pattern has been established for the activity of ALA-dehydratase, the second enzyme of the heme pathway [15]. Thus, both heme synthesis and turnover to bilin seems to be stimulated by ecdysteroids in the pupa. Detailed work at the levels of enzyme regulation and transcription of specific mRNA species is required to substantiate this view.

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