De novo Synthesis and Levels of Cytochrome *c* and a Biliprotein during Pupal-Adult Development of a Butterfly, *Pieris brassicae*

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Using a sensitive pH-difference spectroscopic method in combination with a three-column procedure of ion-exchange chromatography (overall yield 94%) the levels of cytochrome *c* in the large white butterfly, *Pieris brassicae*, were determined from the last larval instar to the adult insect. In the larva cytochrome concentration reached a maximum at mid instar and subsequently decreased to very low values in the pupa. During adult development the level of cytochrome *c* increased 50-fold in males and 43-fold in females; this sexual difference was expressed only after adult emergence. Biliverdin IXγ which occurs as a specific biliprotein complex was accumulated during the last larval instar and also in young butterflies. *De novo* synthesis of heme *c*, biliverdin IXγ and the corresponding apoproteins was demonstrated in newly emerged butterflies by injections of radiolabeled 5-aminolevulinate, lysine, leucine, and succinate, respectively. Cycloheximide inhibited labeling of both apoproteins and of heme *c* to 90% but that of the bilin to only 25%. This suggests that in cytochrome *c* but not in the biliprotein formation of the holoprotein depended on a coordinated synthesis of both constituents. Incorporation of 5-aminolevulinate into the biliprotein exceeded that into cytochrome *c* sevenfold indicating that biliverdin IXγ is the major product of the heme pathway in *P. brassicae*. The results are discussed in relation to the formation of mitochondria and flight muscles during postembryonic development of insects.

Introduction

Heme plays a manyfold role in all cells by its function as the prosthetic group of a number of enzymes as well as a regulator of protein synthesis now established in various cell types (for review see [1]). The formation of heme and hemoproteins has been extensively studied in vertebrates including man to elucidate cellular and endocrine mechanisms involved in the regulation of the heme pathway.

Among the hemoproteins of wide interest are the mitochondrial cytochromes as components of the respiratory chain. In vertebrates biosynthetic studies on cytochromes are hampered by the comparatively low rates of synthesis and by excess formation of other heme-containing proteins such as hemoglobins. Insects, in general, offer at least two advantages in this field: they have no need for hemoglobins but, on the other hand, contain high concentrations of mitochondrial cytochromes which are built up during the relative short period of flight muscle development.

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The insect flight muscle displays the highest metabolic activity of any known tissue. This activity is strictly aerobic and based on the high number of mitochondria known as sarcosomes for their exceptional size and specific arrangement between the muscle fibers [2]. Flight muscles are absent at the larval stage; they develop during transformation to the mature insect [3]. The final differentiation of the muscle fibers is characterized by the accumulation of large amounts of sarcosomes with a high concentration of cytochromes. This offers ideal conditions for studies on the biosynthesis of these hemoproteins and on control mechanisms involved. No detailed work has been published in this field.

Our interest concentrates on cytochrome *c* which is the only soluble component of the respiratory chain and relatively easy to purify. Furthermore, it represents the principal cytochrome of our experimental insect, the large white butterfly *Pieris brassicae* [4]. We choose this species for several reasons: development is synchronous and comparatively rapid, endocrinology has well been studied [5–8] and comparative work on diapausing and non-diapausing pupae is possible. In addition, *P. brassicae*, as many other lepidopteran species [9],

produces remarkable amounts of a bile pigment identified as biliverdin IXγ [10]. So, we can also examine the metabolic significance of heme destruction in insects devoid of hemoglobins which represent the principal source for bilins in vertebrates.

The present paper describes a micro method for the determination of cytochrome *c* in column eluates and basic results on the variation of the levels of cytochrome *c* and the biliprotein during larval-pupal-adult transformation of *P. brassicae*. In addition, *de novo* synthesis of these two major heme products in the butterflies has been demonstrated. This work is continued in an accompanying paper [11] to correlate the synthetic activities with the hemolymph titer of ecdysteroid hormones. Some preliminary results have been presented previously [12, 13]. Our further aims are to study formation of heme products at the levels of translation, transcription and gene expression during postembryonic development of insects.

Experimental Section

Materials

All chemicals were purchased from Merck (Darmstadt) in the purest grade available unless stated otherwise. TEAE-cellulose No. 134 (0.63–0.75 mequiv/g) was obtained from Schleicher-Schüll (Dassel), CM-cellulose Whatman CM 32 (1 mequiv/g) was purchased from Bender-Hobein (Karlsruhe). Chloramphenicol was from Serva (Heidelberg), cycloheximide and horse heart cytochrome *c* (type VI) were from Sigma (Munich). Prosil-28 was a product of PCR Research Chemicals (Gainesville, USA) obtained from Ventron (Karlsruhe). Precoated thin-layers of silica gel type Polygram Sil-G were a product of Macherey-Nagel (Düren). The chromatographic columns (1.5 and 0.9 cm i.d.) were from Pharmacia (Freiburg); the micro columns (0.6 cm i.d.) were those from Omnifit with adjustable plungers and polyester filter supports.

5-Amino[4-^{14}C]levulinic acid (ALA) hydrochloride (spec. act. 2.15 GBq/mmol), L-[U-^{14}C]lysine hydrochloride (spec. act. 13.1 GBq/mmol), [2,3-^{14}C]-succinic acid (spec. act. 0.81 GBq/mmol) and L-[4,5-^{3}H]leucine (spec. act. 1.70 TBq/mmol) were all obtained from Amersham-Buchler (Braunschweig). Aqualuma was purchased from Baker Chemicals (Groß-Gerau).

All glassware that had to come into contact with cytochrome *c* was siliconized using Prosil-28 or dimethyldichlorosilane to avoid ionic adsorption of this protein.

Insects

The insects, *Pieris brassicae* L. (Lepidoptera), were taken from an established laboratory colony. The larvae were fed an artificial diet based on the formulation of David and Gardiner [14]; some essential modifications had to be introduced, however, which will be reported elsewhere. The larvae were exposed to a light-dark cycle of 16:8 h to prevent pupal diapause [15]. All experimental insects were kept at 21 ($\pm$ 0.5) °C; they had entered their developmental stage within a 4 h period typically.

Isolation of cytochrome c

The procedure for quantitative extraction and chromatographic purification on analytical scale is based on the method of Kadenbach and Urban [16, 17] with some essential modifications to be described in detail.

Extraction. Approximately 2–4 g of frozen larvae or pupae (6–30 insects depending on size) or 0.5–0.9 g of butterflies (3 insects) were homogenized in 5–7 ml of prechilled 0.2 M potassium phosphate buffer, pH 7.2, using an Ultra-Turrax TP 18 for two cycles of 1 min each. A few crystals of phenylthiourea had been added to the buffer to block phenoloxidase activity. The homogenate was sonicated for 6 × 15 s at maximal intensity using a Sonifier B12 (Branson) equipped with the normal tip. The whole procedure was performed in an ice bath and intermitting periods were allowed for cooling. The final homogenate was centrifuged at 200000 × *g* (rotor Ti 70, 1) for 45 min in a Beckman ultracentrifuge at 0 °C and the supernatant immediately used for chromatography or stored at – 20 °C for later processing.

Chromatography. Purification of cytochrome *c* was achieved by ion exchange on TEAE-cellulose and CM-cellulose performed at 6 °C. The celluloses were always fully precycled according to standard procedures. Usually, 4 ml of the extract were diluted with 2 ml of water and applied to a column

of TEAE-cellulose (1.5 × 25 cm) equilibrated with 10 mM Tris/HCl, pH 7.5 at 6 °C. The sample was rapidly introduced into the cellulose by air pressure and chromatographed with the same Tris buffer. Large amounts of red ommochrome pigments were adsorbed at the top of the TEAE-cellulose column. The eluate was led through two photometer units (Uvicord II, LKB) for monitoring transmission at 280 nm and 410 nm, respectively, and then diluted 1:1 *via* a 3-way fitting with 0.02 M phosphate buffer, pH 6.5, containing 0.1 mM $K_3[Fe(CN)_6]$ to oxidize cytochrome *c* before reaching the column of CM-cellulose (0.9 × 27 cm) which was equilibrated with 0.02 M phosphate buffer, pH 6.5. The flow speed was 20 ml/h in the TEAE-cellulose and 40 ml/h in the CM-cellulose. When all cytochrome *c* and biliprotein had reached the second column (as monitored at 410 nm) this was detached from the TEAE-column and re-equilibrated with 0.02 M phosphate buffer. Cytochrome *c* remained bound at the top of the CM-cellulose column as a red band, whereas the blue biliprotein was obtained in the flow-through. Complete oxidation of cytochrome *c* was assured by addition of 1 ml of a 1 mM solution of $K_3[Fe(CN)_6]$ in equilibration buffer. Elution of the CM-cellulose column was performed with a linear gradient of phosphate buffer concentration (0.02–0.3 M; pH 6.5). Again, transmission of the eluate was monitored at 280 nm and 410 nm and 60 fractions (1.5 ml each) were collected. Flow velocity was 30 ml/h.

Concentration of cytochrome c

Fractions containing cytochrome *c* were pooled, diluted 1:5 with water and pumped onto a micro column of CM-cellulose (0.6 × 2 cm) equilibrated as before. The cellulose was successively washed with 1 ml of 1 mM $K_3[Fe(CN)_6]$ in 0.02 M phosphate buffer pH 6.5, 5 ml of the buffer alone and 5 ml of water. Then, cytochrome *c* was slowly eluted with 0.02 N NaOH as a sharp red band into a graded 5 ml tube. At least 2.5 ml were collected and used for pH-difference spectra.

In radioisotopic studies possible contamination of cytochrome *c* by other labeled proteins was avoided by differential elution after replacement of phosphate by cacodylate, which does not bind to cytochrome *c* [18], as follows: after oxidation of the cytochrome the micro column was successively washed with 5 ml of 0.02 M sodium cacodylate buffer pH 6.5, 15 ml of 0.08 M cacodylate buffer pH 7.5, and 5 ml of water prior to elution of cytochrome *c*.

Radioisotopic studies

The tracer solutions were injected into the thorax with a 50 µl Hamilton microsyringe in combination with a PB 600-1 repeating dispenser. Each insect received 2–4 µl of the tracer solution depending on the labeled compound. Insects which leaked were discarded. Cycloheximide and chloramphenicol were dissolved in water and 1 µl, containing the desired amount, was injected prior to the labeled compound. Controls were injected the same volume of water and kept under identical conditions. The insects were killed by immersion into liquid nitrogen. Extraction and chromatographic purification of radioactive samples were performed as in the routine method; some modifications were necessary in the concentrating step as detailed there.

Chromatographic experiments were run to confirm that the proteins under study were obtained free from any non-incorporated radioactivity.

After quantification of cytochrome *c* by pH-difference spectroscopy two aliquots of the solution were counted with 10 ml Aqualuma in a Packard Tri-carb scintillation spectrometer. The proteins in the flow-through of the CM-cellulose column were precipitated with 7% perchloric acid (PCA). The co-precipitating $KClO_4$ facilitated sedimentation and resuspension of the proteins. After standing overnight at 5 °C the precipitate was collected by centrifugation, washed twice with 7% PCA and then dispersed in 3 ml of water with an Ultra-Turrax. Two aliquots of each the precipitate suspension, with the biliprotein as the main constituent, and the first supernatant, containing the precursor, were counted for radioactivity as described above. An aliquot of the extract (200 000 × *g* supernatant) was also counted for total soluble radioactivity. Counting efficiencies of 85% were obtained with ^{14}C and of 45% with ^{3}H.

The distribution of radioactivity on thin-layer chromatograms was recorded with a windowless gas flow counter (scanner system BF 210-23; Berthold and Frieseke).

Absolute and pH-difference spectroscopy of cytochrome c

UV/VIS spectra were recorded with a Zeiss DMR 21 photometer using quartz cuvettes with a light path of 1 cm or 2 cm. Absolute spectra of cytochrome *c* were recorded in phosphate buffer (0.1–0.2 M; pH 6.5–7.2); for quantitative determinations the extinction coefficients for horse cytochrome *c* [19] were used. The purity of cytochrome *c* was calculated from the quotient $A_{409\,nm}/A_{280\,nm}$ of the oxidized protein; a value of 4.75 has been obtained for highly purified cytochrome *c* from *P. brassicae* [4].

For quantification of micro amounts of cytochrome *c* a sensitive method was developed which is based on difference spectra of the Soret band at acidic and basic pH values. The procedure is as follows: 1 ml of the concentrated cytochrome solution in 0.02 N NaOH (eluate of the micro column of CM-cellulose) was pipetted into each of two photometric cuvettes; 10 µl of 5 N HCl were added to one cuvette and 10 µl of 0.02 N NaOH to the other one. The difference spectrum of the acidic against the basic solution was recorded in the range of 450–360 nm. For the extinction difference between the maximum at 409 nm and the minimum at 392 nm a $\Delta\varepsilon$-value of 160 $mM^{-1} \times cm^{-1}$ was established with horse cytochrome *c*. A molecular weight of 12 500 was used for calculations on a weight basis.

Extraction and determination of bilin

Biliverdin IXγ was extracted from freeze-dried insects and finally dissolved in methanol containing HCl as described [20]. Pigment concentration was determined from the absorption at 696 nm by using the coefficient of 30.8 $mM^{-1} \times cm^{-1}$ of the α-isomer [21].

Cleavage of in vivo labeled cytochrome c and biliprotein

The heme group of cytochrome *c* was easily obtained by a reaction [22] designed to cleave the tryptophanyl peptide bond of the apoprotein whereby heme is quantitatively removed as a side reaction. In the biliprotein (purity 77–82%) the chromophore was quantitatively removed with acetone at a final concentration of 66% (with 50% about 15% of the apoprotein remained in the supernatant; see Table II and [23]) or, alternatively, with 5% HCl in methanol as described above. The precipitated apoprotein was once washed with acetone respectively methanol and redissolved in 0.25 ml of 0.25 N NaOH containing 2% SDS. Aliquots of the bilin and apoprotein solutions were counted for radioactivity.

Results

Evaluation of the methods applied to cytochrome c

A tandem-combination of columns of TEAE-cellulose and CM-cellulose was successfully applied for the isolation of small amounts of cytochrome *c* from insects. However, when the homogenate (which contained pieces of cuticle and a lot of scales) was directly loaded onto the TEAE-cellulose, as performed with different extracts [16, 17], solvent flow was blocked and the yields of cytochrome *c* were very low. Therefore, the homogenate was subjected to high speed centrifugation which also removed much of the proteins. With purified horse cytochrome *c* reproducible recoveries of 94% ($n = 4$) were obtained after passage over the combined TEAE- and CM-cellulose columns. The losses were largely due to ionic adsorption to the TEAE-cellulose as shown by high salt elution. In a third chromatographic step performed after gradient elution of the CM-cellulose column cytochrome *c* was again concentrated on a micro column of CM-cellulose for quantitative determination. For this step mean recoveries of $100 \pm 2\%$ ($n = 5$) were obtained with amounts of cytochrome *c* ranging from 9 to 300 µg. The purity of the gradient-eluted cytochrome *c* from *Pieris* depended on the developmental stage of the insects. With extracts from butterflies typical purities were about 90–95% when calculated from the quotient $A_{409\,nm}/A_{280\,nm}$ of the oxidized protein. In the radioisotopic studies essentially pure cytochrome *c* was achieved by increasing the cation concentration in cacodylate buffer as described in the Experimental Section.

The pH-difference spectroscopic method applied in this study is about eightfold more sensitive than the classical redox difference spectroscopy using the wavelengths range above 500 nm. The pH-method allows quantification of cytochrome *c* down to 1 µg or less depending on the sensitivity of the spectrophotometer. Furthermore, the chromatographic

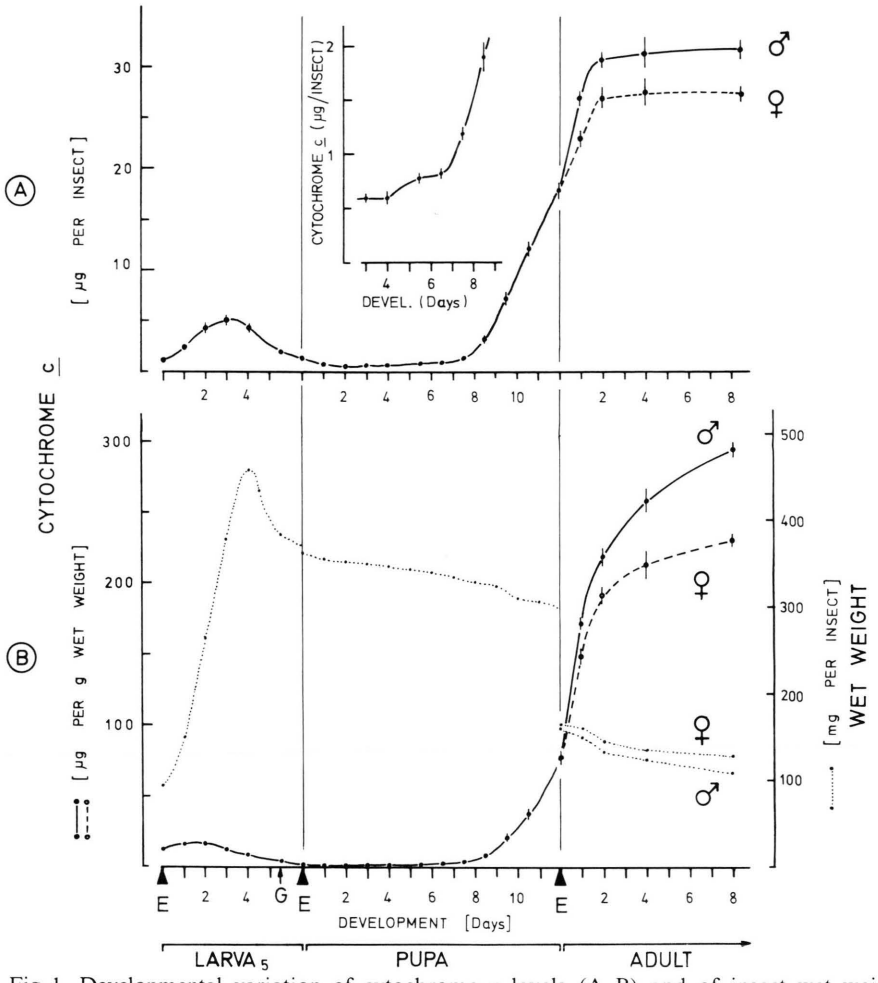

Fig. 1. Developmental variation of cytochrome *c* levels (A, B) and of insect wet weight (B) in *Pieris brassicae*. The inset in A displays days 3–9 of pupal life with an enlarged ordinate to show minor but statistically significant variations of the cytochrome level. Mean values (± S.E.M., not shown if too small for drawing) from 4–10 extracts. E – ecdysis; G – spinning of the girdle. Peaking ecdysteroid titers during pupal stage are at days 4–5 (see reference [11]).

procedure placed before eliminates other compounds (*e.g.* chromoproteins) that might also exhibit pH-sensitive spectra and hence could interfere with this method.

The small basic cytochrome *c* was the first protein to migrate along the TEAE-cellulose column followed by the biliprotein. On reaching the CM-cellulose cytochrome *c* was bound at the top of the column whereas the biliprotein was obtained in the flow-through of this column (*cf.* [23]).

Cytochrome c levels during development

The variation of cytochrome *c* concentration from the beginning of the last larval instar to the developed adult insect is shown in Fig. 1. In the larva a maximum of 5 µg per insect was reached on day 3, *i.e.* one day before the end of the feeding period and the maximum of larval weight. Thereafter, cytochrome *c* levels rapidly decreased below 1 µg per insect. At days 5 and 6 of pupal life a small but significant ($P < 0.01$; student's *t*-test) increase was recorded (inset in Fig. 1 A); this was followed by a steep rise which continued up to day 2 of the adult insect.

Male butterflies contained more cytochrome *c* than females. This feature developed only after adult emergence since significantly different cytochrome concentrations between the sexes were not

found during the pharate phase and in newly emerged butterflies. In the course of adult development cytochrome *c* accumulated 50-fold in males and 43-fold in females. About 90% of the total cytochrome was concentrated in the thorax (details not shown).

Bilin concentration during development

In *P. brassicae* biliverdin IXγ forms an equimolar non-covalent complex with a specific protein [23]. Extraction and quantification of the pigment therefore provided a measure for the concentration of the biliprotein.

In contrast to cytochrome *c* the bilin was mainly accumulated during the last larval instar as shown in Fig. 2. After pupation the pigment level remained fairly unchanged until a small but significant ($P \ll 0.0001$) increase at the end of the pupal stage. Adult emergence coincided with a rapid decrease in the bilin. This loss was due to excretion of the pigment with the meconium by the newly emerged butterflies as demonstrated by chromatography. A further accumulation of biliverdin took place in young butterflies with a maximum at day 2.

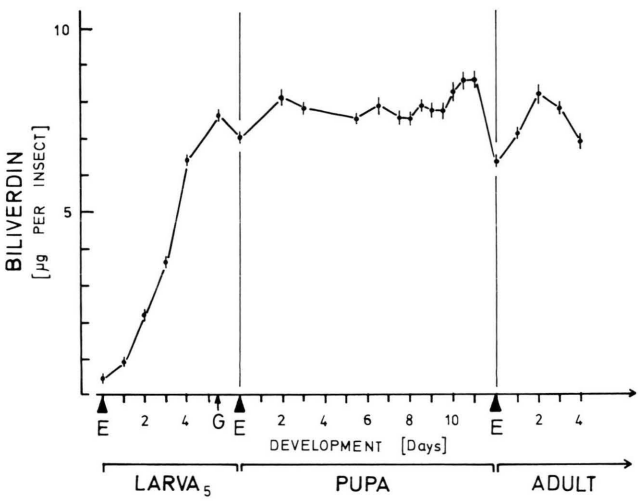

Fig. 2. Developmental variation of biliverdin IXγ levels in *Pieris brassicae*. Mean values (± S.E.M.) from 5–15 extracts. E – ecdysis; G – spinning of the girdle.

Incorporation of labeled precursors into cytochrome c and biliprotein

Newly emerged butterflies were injected [^{14}C]5-aminolevulinate, [^{14}C]lysine, [^{14}C]succinate, and [^{3}H]leucine, respectively, and the incorporation of radioactivity into cytochrome *c* and biliprotein was determined. Cytochrome *c* was obtained in pure form from the micro column of CM-cellulose. The biliprotein was determined in the flow-through of the long CM-cellulose column. In this eluate radioactivity from ALA paralleled the specific absorption of the biliprotein indicating that no other [^{14}C]labeled proteins were present. Similar results were obtained with respect to ^{3}H.

The results of the incorporation studies are summarized in Table I. Radioactivity from ALA was recovered to 0.47% in cytochrome *c* and to 3.4%

Table I. Incorporation of radiolabeled precursors into cytochrome *c* and biliprotein in adult *Pieris brassicae*. The butterflies were injected 4 h (± 2 h) after emergence.

Precursor (dose and period)	Number of exp.	Incorporation (mean values and ranges in % of injected radioactivity) into		Quotient B/C
		Cytochrome *c* (C)	Biliprotein (B)	
[^{14}C]5-aminolevulinate (2.2×10^5 dpm/120 min)	7	0.47 (0.22–0.88)	3.4 (1.4–6.8)	7.1 (6.6–7.9)
[^{14}C]L-lysine (4.4×10^5 dpm/90 min)	3	0.26 (0.17–0.31)	6.2 (4.4–7.3)	25.2 (23.5–26.1)
[^{3}H]L-leucine (2.2×10^6 dpm/120 min)	4	0.13 (0.11–0.17)	3.7 (3.0–4.2)	29.3 (24.2–34.2)
[^{14}C]succinate (5.9×10^5 dpm/120 min)	1	0.03	1.6	54.7

in the biliprotein. The corresponding values for lysine were 0.26% and 6.2% respectively. Incorporation of leucine was about half of that of lysine. Succinate labeled cytochrome *c* to 0.03% and the biliprotein to 1.6% only.

The specificity of the labeled compounds as precursors to the tetrapyrrolic prosthetic groups and their apoproteins respectively was mainly examined in the biliprotein since this was most labeled and the chromophore was much easier to remove than in cytochrome *c*. As summarized in Table II radioactivity from ALA was almost exclusively confined to the bile pigment, as determined with different methods, and to the heme group of cytochrome *c*. Lysine and leucine on the other hand were incorporated into the apoprotein of the biliprotein with high specificity. Radio TLC of the bilin dimethylesters revealed that there was essentially no radioactivity from either lysine or leucine in the pigment; by the same method it was also confirmed that label from ALA was associated with the bilin. Radioactivity from succinate was about equally distributed between the chromophore fraction and the protein moiety of the biliprotein.

Effects of translational inhibitors

Cycloheximide and chloramphenicol were examined for their effects on incorporation of labeled ALA and lysine into cytochrome *c* and biliprotein. Cycloheximide inhibited incorporation of lysine into the two proteins at fairly identical dose-dependent rates (Fig. 3). Free lysine, as measured in the PCA supernatant of the column flow-through, increased concomitantly up to 470% (inset in Fig. 3B), corresponding to approx. 70% of the injected dose, due to a general inhibition of cytoplasmatic protein synthesis. About 10% or less of incorporation were resistant to cycloheximide which had been administered 80 min prior to the labeled precursor. Incorporation of ALA into cytochrome *c* was about equally inhibited by cycloheximide as that of lysine (Fig. 3A). However, labeling of the biliprotein by the heme precursor was much less affected: maximal inhibition was only about 25% with 20 µg of cycloheximide per insect. Radioactivity in the PCA supernatant was only slightly increased (up to 130%) in this experiment; it indicated that in contrast to lysine most of ALA had been further used up in the presence of the inhibitor. When ALA and cycloheximide were injected simultaneously maximal inhibition was only about 65% in cytochrome *c* (60 min incorporation). This suggested that the agent required some time to express its full activity.

Chloramphenicol, an inhibitor of mitochondrial protein synthesis, exerted no significant effect on the incorporation of lysine and ALA, respectively, into either protein (details not shown); inhibitor doses of up to 20 µg per insects were injected 60 min prior to the labeled compounds.

Table II. Distribution of radioactivity between heme *c* respectively bilin and their apoproteins after *in vivo* labeling by various precursors in adult *Pieris brassicae*. The butterflies were injected 4 h (±2 h) after emergence.

Precursor	Incorporated dpm per sample	Recovery after cleavage [%]	Distribution of radioactivity (% of total recovered)		Cleavage method for biliprotein (see Exp. Sect.)
			Heme *c* resp. bilin	apoprotein	
[^{14}C]5-aminolevulinate	cytochrome *c*				
	6 990	104.7	95.3	4.7	–
	biliprotein				
	151 022	123.0	98.7	1.3	66% acetone
	476 044	124.9	98.2	1.8	66% acetone
	64 274	101.4	94.5	6.9	5% HCl/MeOH
	55 576	99.9	93.8	6.2	5% HCl/MeOH
[^{14}C]L-lysine	10 030	107.3	19.3	80.7	50% acetone
	27 942	108.4	13.5	86.5	50% acetone
[^{3}H]L-leucine	31 750	100.0	4.5	95.5	66% acetone
	21 744	100.2	3.3	96.7	66% acetone
[^{14}C]succinate	12 163	98.2	42.7	57.3	66% acetone

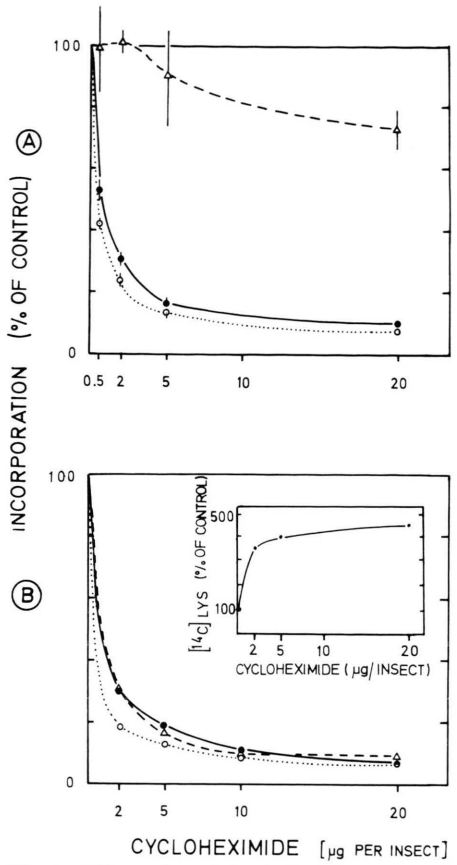

Fig. 3. Effect of cycloheximide on the incorporation of [^{14}C]5-aminolevulinate (A) and [^{14}C]L-lysine (B) into cytochrome *c* and biliprotein in adult *Pieris brassicae*. The age of the butterflies was 4 h (± 2 h) after emergence. Cycloheximide was injected 80 min prior to the radioactive compounds (doses and periods as specified in Table I). A – mean values (± S.E.M.) from 3–4 experiments, B – mean values from 2 experiments. The symbols refer to: ●—● cytochrome *c* (per insect); ○....○ cytochrome *c* (spec. act.); △----△ biliprotein (per insect). The inset in B shows free [^{14}C]lysine as a function of the dose of cycloheximide.

Discussion

This paper provides the first description of cytochrome *c* levels during larval-pupal-adult transformation of a butterfly and the demonstration of *de novo* synthesis of both heme *c* and the apoprotein in emerged adults. Corresponding studies were also performed on a biliprotein; biosynthetic work on such an insect protein has not been reported before.

A dramatic increase of cytochrome concentration takes place during the late pharate adult stage in *P. brassicae*. Similar results have been obtained with other insects such as the moths *Platysamia cecropia* [24] and *Samia cynthia* [25], the flies *Phormia regina* [26] and *Lucilia cuprina* [27], the honey bee [28, 29], the colorado beetle [30] and the locust, *Locusta migratoria* which has been very well studied [31]. The increase in cytochrome *c* reflects the formation of mitochondria which is a quantitative important aspect of insect flight muscle differentiation [2, 3]. As demonstrated in some species [28, 30] also the other respiratory cytochromes increase similarly in the thoraces during flight muscle development. Corresponding results were also obtained with *P. brassicae* [4].

The postemergence maturation of the respiratory system seems to be rather short in *P. brassicae*, taking only about two days. This agrees with other results which show that normal flying activity and associated mating behaviour is observed not before 2 to 3 days after emergence in this insect [32, 33]. Furthermore, male *Pieris* butterflies are known to be more active in flight than females; this obviously is related to the higher concentration of cytochrome *c* in mature males (the other cytochromes have not been measured in this respect).

The variation of cytochrome *c* levels during the last larval instar of *P. brassicae* parallels both the respiratory activity of intact larvae and the amounts of mitochondrial protein [34, 35]. The decrease in all three parameters, as observed at the end of the feeding period, may be due to degeneration of typical larval mitochondria and their possible replacement by a population with different properties [36, 37]. This would account for the developmental changes in respiratory and ATP-synthesizing capacities which have been reported for isolated mitochondria from *Pieris* larvae [34, 35].

The low cytochrome *c* level in the pupa is characteristic for this developmental stage. It has long been known from several moths species that most of this cytochrome disappears at pupation and will remain at nearly undetectable levels in diapausing pupae until resynthesis occurs with the termination of diapause [38]. The resulting typical U-shaped respiratory activity has also been established in *P. brassicae* pupae [35]; its increase is in good accord with the accumulation of cytochrome *c* in pharate adults as reported in the present paper.

Histological studies in *Pieris* revealed [4] that the release of ecdysteroids at mid pupal instar is soon

followed by the begin of flight muscle development. The accumulation of respiratory cytochromes, however, begins 3 days later as part of the terminal differentiation. It seems therefore that cytochrome synthesis in the muscle cells is not directly stimulated by ecdysteroids but is part of a sequence of events triggered by these hormones. The small early increase in cytochrome *c* at begin of adult differentiation may nevertheless be a direct hormonal effect taking place in a different cell type to match the increased demand for energy supply.

Apart from the mitochondrial cytochromes *Pieris* synthesizes a biliprotein as a major product of the heme pathway. The chromophore, biliverdin IXγ [10], is mainly accumulated during the last larval instar as already shown earlier [20]. Further increases in the bilin concentration are observed at the late pharate adult stage, when heme synthesis is expected to be high, and in young butterflies which have lost a considerable amount of bilin by excretion with the meconium at emergence.

In emerged butterflies most radioactivity from ALA, which specifically labeled both the bilin and heme *c*, was recovered in the biliprotein fraction though cytochrome synthesis was maximal at that time (see [11]). This demonstrates that the bilin represents a major product of the heme pathway in *Pieris*. Both the bilin and the apoprotein are synthesized *de novo* in young butterflies; corresponding results were also obtained for cytochrome *c* in *Pieris*. *De novo* formation of the polypeptide chain of cytochrome *c* during the time of its dramatic accumulation has also been demonstrated in other insects [25, 27, 29, 39].

Label from succinate was about equally distributed between the bilin and the apoprotein. This is explained by the precursor role of succinate for the heme precursor ALA *via* succinyl-CoA on one hand [1] and its transformation into amino acids *via* Krebs cycle intermediates on the other hand. Lysine was about twice effective as leucine in labeling both cytochrome *c* and the biliprotein. This is in accord with the relative abundance of these amino acids in the *Pieris* cytochrome [40] and the biliprotein [23].

It is now firmly established that the polypeptide chain of cytochrome *c* is synthesized on cytoplasmatic ribosomes [41, 42]. In agreement with this, incorporation of labeled lysine into cytochrome *c* in *Lucilia* was reduced to 2% by cycloheximide, an inhibitor of cytoplasmatic protein synthesis, but unaffected by chloramphenicol which specifically blocks mitochondrial translation [27]; see also [43]. Similar results have been obtained with *Pieris* where maximal inhibition was about 90–95% in both cytochrome *c* and biliprotein. Cycloheximide may be rapidly detoxified or eliminated by insects [44–46] with the consequence that protein synthesis may recover within the experimental period; this must be kept in mind when effects of the inhibitor are compared.

In *Pieris* cycloheximide inhibited the incorporation of both ALA and lysine into cytochrome *c* to the same extent. That, however, the heme pathway itself was only slightly affected by this agent followed from the much less reduced labeling of bilin. It seems therefore that formation of holocytochrome *c* is limited by the availability of its protein moiety whereas in the biliprotein a pool of apoprotein must exist which is sufficiently large to bind the bilin synthesized despite the presence of cycloheximide. A further conclusion is that the enzymes involved in synthesis (after ALA formation) and degradation of heme are long-lived relative to the period of exposure to the inhibitor (200 min). Detailed studies are now in progress to elucidate the control mechanisms involved in the synthesis of heme and cytochrome *c* during insect development.

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