Metabolic Conversions during Pupation of the Cecropia Silkworm

1. DEPOSITION AND UTILIZATION OF NUTRIENT RESERVES

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In biochemical studies of insect metamorphosis, much attention has been given to the deposition and utilization of nutrient reserves, and it has been inferred that insects may carry out net conversion of fat into carbohydrate (cf. Deuel & Morehouse, 1946; Buck, 1953; Chauvin, 1956). Since vertebrate animals are not at present known to possess any pathway for the net conversion of fatty acids into carbohydrates, the existence of such a pathway in a major invertebrate group would be of great interest. In micro-organisms and higher plants, the utilization of fat and metabolically related substances such as acetate for carbohydrate synthesis is well established (Kornberg & Krebs, 1957). The occurrence of such conversion in the nematode, *Ascaris*, has also been indicated though the mechanism has not been elucidated (Passey & Fairbairn, 1957). In insects, however, none of the evidence adduced to support net conversion of fat into carbohydrate is unequivocal.

Values of respiratory quotients below 0.7, suggesting oxidation of substrates to products other than carbon dioxide, have frequently been recorded for insects (see Buck, 1953). The measurement of respiratory quotients in insects, however, is complicated by several factors, e.g. the release of carbon dioxide in periodical bursts (Schneiderman & Williams, 1953) and the excretion of bicarbonate (Brown, 1938).

The earliest assertion of the conversion of fat into carbohydrate in an insect was based on the observation in silkworms (*Bombyx mori*) that, after the cessation of food intake, glycogen increased concomitantly with decrease in lipids (Couvreur, 1895). The alleged increase in glycogen during pupation of *Bombyx* is supported by Vaney & Maignon (1905), Bialasiewicz (1937) and Zaluska (1959), though not by others (Kotake & Sera, 1909; Yonezawa & Yamafuji, 1935). Increases of carbohydrate during the prepupal or pupal periods, i.e. non-feeding stages, have been reported also for other insect species: an unspecified blowfly (Frew, 1929), the blowfly, *Phormia regina* (Haub &

Hitchcock, 1941; Patton, Hitchcock & Haub, 1941), the Southern army-worm, *Prodenia eridania* (Babers, 1941), and the Japanese beetle, *Popillia japonica* (Ludwig & Rothstein, 1949). With the exception of Babers, all of these authors proposed in explanation synthesis at the expense of fat. Interpretation of changes in carbohydrate constituents, however, depends on completeness of the analyses. Several investigators have considered total reducing substances to represent free sugar, although it is known that most of the reducing substances of insects are generally not sugars. None considered the non-reducing sugar, trehalose, recently found to be abundant in many insects (Wyatt & Kalf, 1957). None except Zaluska (1959) considered the long-known carbohydrate, chitin, as a potential source of carbon for glycogen synthesis.

In this paper, we present results of quantitative analyses of cecropia silkworms from the mature larva to the early pupa, as well as some data on transfer of radioactive carbon, and show that sources other than fat can account for the glycogen that is formed during pupation. The next paper (Bade, 1962) describes tests for the operation of the glyoxylate cycle in these animals.

MATERIALS AND METHODS

Materials

Chemicals. All chemicals and solvents were reagent grade. Dye and enzymes for glucose-oxidase reagent ('Glucostat') were obtained from the Worthington Biochemical Corp., Freehold, N.J., U.S.A. Trehalase was prepared from *Galleria* larvae according to the method of Kalf & Rieder (1958). New England Nuclear Corp., Boston, Mass., U.S.A., supplied [^{14}C]glucose which was stated to have a specific activity of $3.6\mu\text{C}/\text{mg.}$, 97.5% of the activity being in [^{14}C]glucose. Dowex 50 X 12 (low porosity), 200–400 mesh, was obtained in H^+ form from Dow Chemical Co., Midland, Mich., U.S.A., and purified according to the method of Moore & Stein (1951). Analytical-grade anion-exchange resin AG 1 X 10, 200–400 mesh (prepared from Dowex 1), was obtained in the chloride form from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. It was converted into the formate form by percolation of 2N-ammonium formate until the effluent was free of chloride.

Biological material. Larvae of the American silkworm, *Hyalophora cecropia* (Lepidoptera, Saturniidae), were

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reared on wild cherry trees in the open air; the breeding stock was of mixed Massachusetts and Nebraska origin. As needed, late instar IV or V larvae were selected in the field and transferred to the laboratory, where they continued to feed on fresh cherry foliage until ready for pupation. Those not used during the prepupal period formed pupae in a normal manner.

The tracer experiments required larvae about to undergo their fourth moult. This stage was recognized by inspection of the yellow dorsal tubercles with a stereoscopic microscope. About 2 days before the moult, the tubercles become transparent as the epidermis separates from the cuticle. Then the new cuticle bulges progressively into them, spines appear and then darken in colour, and finally the moult takes place. Larvae were selected for injection when the new cuticle occupied about half of the tubercle, but spines were still wanting; they usually moulted 24–36 hr. later.

At an average room temperature of 27°, taking the time of the last emptying of the gut of larva V and the spinning of first few threads as day 0, spinning and coating of the outer cocoon occurred on day 1 and that of the inner cocoon on day 2. Shedding of larval skin occurred on day 8.

Quantitative analyses

Preparation of tissue fractions. In the procedure finally adopted, animals at selected stages of development were weighed, frozen on solid CO₂, and dissected while partially frozen in a room at 2°. Soft tissues (exclusive of silk gland in zero- and 2-day animals) were homogenized with about 30 ml. of ice-water in a motor-driven all-glass Potter-Elvehjem homogenizer. Homogenate (5.0 ml.) was deproteinized by adding 5 ml. of 0.6N-HClO₄. The process up to this point was carried out within 20 min.

Into a series of tared beakers were placed: 4.0 ml. of homogenate (for dry-weight determination), the scraped rinsed cuticle, the cocoon, the shed cuticle, and the silk gland, if any. These were frozen, freeze-dried, and then dried to 'constant weight' over P₂O₅ *in vacuo*. Constant weight is defined as a change of less than 2% of the net weight in two successive weighings separated by at least 1 day. The average of two such weighings was taken as the final weight. From the portion of homogenate treated with HClO₄, protein was spun down, washed twice with 0.3N-HClO₄, and discarded. Extract and washings were combined into a fraction designated as 'acid extract' which was stored at 4°. The tissue homogenate not used in these procedures was freeze-dried and samples from the same biological day were combined. The pooled powders were stored over anhydrous CaSO₄. The dried cuticles were similarly stored.

The material for a number of preliminary analyses was prepared by a somewhat less rigorous procedure. This differed from that described above in that (a) animals in the desired developmental stage were bled and haemolymph and carcasses stored frozen at -10° for periods up to several months before analysis, (b) dissection was done at ordinary temperature, just before analyses, (c) cocoons and samples of freeze-dried homogenate were weighed without being dried to constant weight. The results for lipids, chitin, and soluble carbohydrates differed little from those obtained in the final procedure. Glycogen analyses from this series of animals, however, showed considerable scatter

and low values relative to later results, especially for early prepupae. Results tended to be lower the longer the animals had been stored frozen. Further, it was shown that during standing for 30 min. in ice-water an homogenate could lose up to 10% of its glycogen content, presumably by glycolysis.

Glycogen and soluble carbohydrates. Acid extract (5.0 ml.) was mixed with 25 ml. of ethanol to precipitate glycogen and kept overnight at 38°. The flocculated glycogen was spun down and the clear supernatant decanted. The residue was warmed with 10 ml. of aq. 60% (v/v) ethanol, then chilled and again centrifuged. The combined supernatant and washings were designated 'ethanol extract' and analysed for sugars. The residue was allowed to drain and then dissolved in 50 ml. of water, to be analysed for glycogen.

Glycogen and sugars were determined by reaction with anthrone. Samples containing ethanol were evaporated to dryness under a stream of air; then all samples were made to 0.5 ml. with water and mixed with 3.0 ml. of reagent prepared according to Mokrasch (1954). Colour was developed by heating at 100° for 15 min. and read at 630 mμ in the Beckman spectrophotometer. Duplicates which did not agree within 0.012 extinction unit were rejected and the determination was repeated.

Ethanol-soluble anthrone-positive substances (sugars) were expressed as trehalose. That this sugar is the major component of this fraction was confirmed by the use of trehalase and glucose oxidase, as well as by radioautography of paper chromatograms prepared from silkworms which had received [¹⁴C]glucose. As some other compounds give some colour with the anthrone reagent (Ashwell, 1957), samples of the ethanol-soluble fraction were de-ionized by passing them through small columns of Dowex 1 (formate form) layered upon Dowex 50 (H⁺ form), evaporated, and subjected to paper chromatography (in butan-1-ol-acetic acid-water, 5:1:2, by vol.) and high-voltage paper electrophoresis in borate buffer (Crumpton, 1959). The papers were sprayed with sodium periodate followed by ammoniacal silver nitrate (Evans & Dethier, 1957) or benzidine (Wolfson & Miller, 1956). Substances were revealed which corresponded in mobility in both systems to glucose, galactose and several unknowns, as well as trehalose. Spots corresponding to *N*-acetylglucosamine, *N*-acetyl-galactosamine, glycerol, and sorbitol were also found; these would not interfere in the anthrone method (Koehler, 1952; Ashwell, 1957). No spots corresponding to ribose or fucose could be detected although the cysteine-H₂SO₄ test (Dische, 1955) indicated the presence of pentoses. No analysis was made for sugar phosphates.

Replication of carbohydrate determinations showed close agreement; also, prolonged storage of the acid extract at 4° did not measurably affect results. Analysis of a zero-day homogenate for glycogen by Pflüger's method (Good, Kramer & Somogyi, 1933) gave results identical with those from the acid-extract method. When the haemolymph was analysed separately for anthrone-positive substances, samples were deproteinized with HClO₄. In the acid extract from haemolymph, no glycogen could be found.

Chitin. The cuticle (ground, crushed, or whole) was digested for 2 hr. at 165° with 10–20 ml. of saturated aq. KOH to deacetylate the chitin (Campbell, 1929). The residual chitosan was filtered off on a tared, dried, Gooch crucible and washed with 500 ml. of water. After ethanol

and ether rinses, crucible and contents were dried at 110° and weighed. Duplicate determinations gave very close agreement. Completeness of conversion into chitosan was tested as described by Campbell (1929).

Analysis for tracheal chitin by digestion of 150 mg. of freeze-dried homogenate gave less than 1% of dry weight for zero-day larvae; in homogenate from 10-day animals the amount was too small to be measured by this method. However, newly laid-down, hence thin, tracheae may have been completely digested by the KOH treatment and lost (cf. Richards, 1951).

Lipids. (a) In preliminary analyses, samples (80–150 mg.) of freeze-dried homogenate from individual animals were refluxed with ethanol-ether (3:1, v/v) for 12 hr. The extracts were filtered, concentrated by evaporation, and extracted in a liquid-liquid extractor with light petroleum (b.p. 30–60°) for 18 hr. The light petroleum was evaporated and the residue weighed as lipid. Results were reproducible, and showed changes during the prepupal period similar to those found later with pooled samples. Because of inaccuracy in measuring tissue dry weights, however, further determinations were required. These were done by the following simpler procedure.

(b) The combined freeze-dried homogenates from animals of a given biological day were ground in a mortar. A sample (200 mg.) of the powder was then ground with 0.9 ml. of water in a Potter-Elvehjem homogenizer; this 'reconstituted homogenate' was extracted with chloroform-methanol (2:1, v/v) and the extract washed with aq. NaCl (0.73M) (Folch, Lees & Sloane-Stanley, 1957). When the Folch method was applied to samples of tissue that had been freeze-dried but not exhaustively desiccated, results were identical with those obtained by procedure (a). With material that had been dried over P₂O₅, however, results were erratic and far too low. Therefore, dry weights were determined from separate samples.

Samples of ground cuticle analysed by procedure (a) had lipid contents in the range 3–7% of the cuticle dry weight, i.e. on day 5 about 10 mg. of lipids/animal. This contribution to total body lipids was negligible.

Radioactive tracer studies

Injection and harvesting of animals. Instar IV larvae in an early stage of moulting were weighed, anaesthetized with CO₂, and radioactive solution was injected into a proleg (Bergold, 1943) with an Agla micrometer syringe (Burrroughs Wellcome and Co., Beckenham, Kent) and a 30-gauge steel needle. The solution contained 24 µC of [1-¹⁴C]-glucose/ml. of 'insect Ringer' solution (Ephrussi & Beadle, 1936), and 0.002 ml. was injected/0.1 g. of caterpillar. Animals that bled from the injection site were rejected.

Injected larvae moulted within 12–36 hr. and then fed and spun normally. They were harvested on days 0, 5, 7, and 10 from the beginning of spinning. During dissection, small samples of tissues (fat body, blood, gut, cuticle, tracheae, silk or silk glands, and moulting fluid and gut contents when available) were removed and spread on tared aluminium planchets to be assayed for total radioactivity.

Isolation of fractions. Glycogen was isolated from HClO₄ extract and purified by reprecipitation from water. It had specific rotation $[\alpha]_D^{25} + 184^\circ$ (c 0.5 in water). Reeves (1944) gave +185° [c 0.5 in aq. 3% (w/v) sodium chloride].

Hydrolysis of several glycogen samples in N-H₂SO₄ followed by paper chromatography in two solvent systems and radioautography showed only two radioactive spots which corresponded to glucose and a small amount of disaccharide, presumably maltose. Assay of two ether-dried samples by the cysteine-H₂SO₄ method of Dische (1955) gave 100% of the dry weight in terms of hexoses. (Several of these tests were very kindly carried out by Dr Joel Dain of the Thannhauser Laboratory, Tufts University.)

Ethanol-soluble neutral compounds in the supernatant remaining after precipitation of glycogen were isolated by neutralizing with KOH to precipitate KClO₄, then deionizing with a column of Dowex 50 (H⁺ form) overlaid with Dowex 1 (formate form). The water effluent (1 bed vol.) was evaporated to dryness *in vacuo* and the residue dissolved in 10 ml. of water. Samples were subjected to paper chromatography and electrophoresis. Radioautography of the chromatograms showed some degree of radioactivity for all spots made visible by the spray reagents. Polyols comprised 10–25% of the residual dry weight as shown by the method of Burton (1957). These, as well as amino sugar derivatives, are included in the 'neutral-soluble' fraction which was oxidized to CO₂ for counting, though sugar phosphates are absent from it; this fraction, therefore, is not quite identical with the sugar fraction defined on the basis of the anthrone reaction.

Whole cuticles were converted into chitosan by treatment with saturated KOH at 165°.

Total lipids were isolated from freeze-dried homogenate by a slight modification of procedure (a).

Counting procedures. All radioactive samples except lipids were plated on aluminium planchets and counted in a windowless gas-flow counter operated in the proportional range. The tissue samples taken during the dissections were spread on the planchets and allowed to dry.

The carbohydrate fractions were converted into CO₂ by wet combustion with K₂S₂O₈ (Abraham & Hassid, 1957) and counted as BaCO₃ which was plated by allowing it to settle undisturbed from suspension in acetone. Chitosan was dissolved in 1% (v/v) H₂SO₄ by heating above 60°. Specific activities for chitosan and neutral-soluble samples were calculated on the basis of the weight of BaCO₃ plated; for chitosan this corresponded to 88–120% of the amount calculated from the weight of the sample used. With glycogen, extra carbon was contributed by ethanol which had been used to precipitate the glycogen and was not completely removed; therefore, specific activities were based on the glycogen content of the solution as determined by anthrone analysis.

Counting rates were converted into rates at infinite thinness by the method of Hendler (1959). Standard curves constructed from radioactive samples of BaCO₃ and of tissue homogenate gave a satisfactory linear relationship between the correction factor defined by Hendler and sample thickness up to several times the saturation value. For cuticular and tracheal tissue samples, which could not be spread uniformly on the planchets, corrections were based on estimates of the actual area covered.

Lipid samples were dissolved in a toluene-phosphor mixture which contained 50 mg. of 1,4-bis-(5-phenyloxazolyl)benzene and 4 g. of 2,5-diphenyloxazole (both scintillation grade from Packard Instrument Co., LaGrange, Ill., U.S.A.)/l. of toluene. They were counted in a scintillation counter (Technical Measurements Corp., New Haven,

Table 1. *Dry weight and percentage composition of cecropia silkworms during pupation*

Except where otherwise indicated, weights and values for glycogen are from determinations on three individuals at each stage, done in 1959. Results for lipids are from pooled 1959 samples; those for chitin and sugars are from 3-8 replicate determinations done in 1958 and 1959.

Day*	Dry weight (g.)			Percentage of dry weight (mean $\pm$ s.e.m.)			
	Total including cocoon	Cuticle		Lipid	Glycogen	Sugars	Chitin
		Larval	Pupal				
0	3.00	0.44	—	18.1	11.1 $\pm$ 0.9	3.4 $\pm$ 0.2	5.2 $\pm$ 0.3
2	2.52	0.38	—	22.6	8.5 $\pm$ 1.05	2.0 $\pm$ 0.2	7.0 $\pm$ 0.4
5	2.26	0.31	—	20.8	9.1 $\pm$ 1.04	1.1 $\pm$ 0.1	4.6 $\pm$ 0.3
7	2.27†	0.32†	—	—	—	—	4.8†
8	2.40	0.043	0.084	21.4	10.3 $\pm$ 1.3	1.3 $\pm$ 0.1	0.85 $\pm$ 0.09
10	1.94	0.029	0.123	19.3	11.3 $\pm$ 0.4	1.6 $\pm$ 0.1	1.4 $\pm$ 0.1
15	2.31	0.038	0.170	19.1	11.9 $\pm$ 0.2	1.2 $\pm$ 0.2	2.2 $\pm$ 0.2
30	1.88	0.037	0.138	20.3	8.9 $\pm$ 0.9	1.1 $\pm$ 0.03	1.9 $\pm$ 0.7

* Time intervals from beginning of spinning.

† Two analyses only.

Conn., U.S.A.). Their yellow colour quenched a variable proportion of the counts; a correction for this was obtained by recounting after the addition of a colourless radioactive-lipid standard. Counts obtained in the scintillation counter were converted into their equivalent in the gas-flow counter by a factor obtained from counting a standard [¹⁴C]glucose sample by both techniques. Specific activities for lipids were calculated on the assumption of 76% C content.

RESULTS

Quantitative analyses

During three seasons, a total of 78 cecropia larvae, prepupae, and pupae were collected at timed stages, dissected, and used for quantitative analysis. Many of the results obtained during 1957 and 1958 were vitiated by (a) insufficient attention to obtaining reliable dry weights or (b) some degree of glycolysis during storage or dissection. Animals taken in 1959 were, on the average, larger and had a lipid content several per cent above those taken in preceding years; this difference may probably be ascribed to use, in that year, of some breeding stock from Nebraska. The quantitative results presented are mainly those obtained in 1959 which, although based on a relatively small number of animals, are believed to be the most reliable. The earlier results are in general agreement with them.

The average total dry weight of cecropia silkworms analysed in 1959 decreased in the course of pupation from an initial 2.995 g. at spinning to a final 1.884 g. after 30 days. Average total dry weights and cuticle weights are recorded in Table 1; females were generally heavier than males, but results from the two sexes are combined in the tabulations. Figure 1 shows the total dry weights expressed as percentages of zero-day dry weight and curves for dry-weight losses computed from the curve of respiration rate of Schneiderman &

Fig. 1. Mean dry weights of cecropia silkworms expressed as percentage of zero-day dry weight. Vertical limits indicate s.e.m. Curve A is a smooth curve through the experimental points. Upper and lower curves (R.Q. 0.707 and 1.00 respectively) indicate limits of dry weight losses calculated for oxidation of a mixture of fats and carbohydrates.

Williams (1953), assuming non-protein R.Q. values of 1.00 (for combustion of carbohydrate) and 0.707 (for combustion of fat) respectively. For this calculation, a fresh-weight curve for the first 30 days after spinning was constructed from the weights of 10-15 animals/day of sampling; an 'average' animal was assumed to have a fresh weight of 5.7 g., corresponding to 2 g. (35%) dry wt., 3 weeks after pupation. The observed decrease in dry weight is well within the range to be expected if both carbohydrate and fat are being oxidized.

Table 1 also shows the results of analyses for body constituents. Females tended to be lower in lipid and higher in glycogen content than males (cf. Zaluska, 1959). However, such differences were small compared with individual variations, and since the time course of the curves was nearly identical for both sexes, results are here combined. The analytical data from Table 1 are presented in

Fig. 2. Content of lipids and carbohydrates as percentage of estimated zero-day dry weight of cecropia silkworms; mean values are calculated as described in the text. Δ , Lipids; $\square$, total carbohydrates; $\blacktriangle$, glycogen; $\circ$, chitin; $\bullet$, sugars. S, Interval of spinning; P, pupal moult.

Fig. 2 as percentages of 'estimated zero-day dry weight'. This percentage is obtained by multiplying the percentage found on a given day by a factor read from Fig. 1, and is used to show the true quantitative changes of the body constituents. Changes in anthrone-positive compounds (chiefly trehalose) in perchloric acid-deproteinized cecropia haemolymph are shown in Fig. 3.

Noteworthy are the sharp drops in cuticle weight (Table 1) and in chitin (Fig. 2) just before the moult, at which time glycogen and sugars are rising.

Transfer of radioactive carbon

To test whether products released from the cuticle might be utilized in glycogen synthesis, larvae were injected with $[1-^{14}\text{C}]$ glucose late in instar IV. It was expected that the instar V cuticle would incorporate isotope in the ensuing moult, and, being presumably inert during the intermoult period, retain it. In the cellular tissues, on the other hand, radioactivity would be dissipated

Fig. 3. Levels of sugars (calculated as trehalose) in perchloric acid-deproteinized haemolymph of cecropia silkworms during pupation. Vertical limits indicate s.e.m. S, Interval of spinning; P, pupal moult.

during the active feeding and respiration of instar V. Thus, in the prepupal period, the animals would be labelled principally in their cuticular components, and transfer of carbon from these could then be followed.

Specific activities of samples of several tissues taken on different days (Table 2) show that at day 0 activity is concentrated in chitin-containing structures such as cuticle and tracheae (and, to a lesser extent, gut). Silk gland and silk had very few counts. The third zero-day animal, which had low counts in all tissues, is regarded as anomalous, and is omitted from all subsequent tabulations. Thus it seems that about 10% of the injected ^{14}C was retained at the beginning of spinning, and, of this, about 90% was present in the cuticle. Gradual uptake of radioactivity by the other tissues during the prepupal period is evident.

Table 3 shows specific activities of various purified fractions from the same series of animals. Specific activities of all fractions including lipids increase during pupation, and the increased radioactivity is most likely to originate in the cuticle. The non-chitin components of the cuticle (chiefly protein) were not isolated for counting. However, it is possible to estimate roughly their content of ^{14}C by difference, as shown in Table 4. It is apparent that there are even more counts in the non-chitosan constituents of the cuticle than in the chitosan. In fact, considering the error attaching to these calculations, the similar percentages of radioactivity and of weight contributed by chitosan suggest that the cuticle is approximately uniformly labelled.

DISCUSSION

Metabolism of nutrient reserves

Up to the pupal moult (day 8), the prepupa utilizes at a considerable rate both fat and carbohydrates (Figs. 2 and 3), and dry-weight losses

Table 2. *Radioactivity in tissues of cecropia silkworms injected with [1-¹⁴C]glucose in late instar IV*

Replicate values are from individual animals.

Specific activity (counts/min./mg. of dried tissue)

Day*	Haemolymph	Intestine	Fat body	Tracheae	Cuticle		Moulting fluid
					Larval	Pupal	
0	49	158	29	554	4 032	—	—
	211	178	30	316	2 098	—	—
	30	68	10	131	436	—	—
5	105	146	48	413	1 951	—	—
	103	119	46	479	1 950	—	53
	134	334	76	849	2 903	—	49
7	180	150	118	932	4 557	280	322
	152	216	62	2 122	4 379	93	75
10	459	404	492	956	15 230	1 592	—
	246	232	138	787	3 434	1 511	—
	211	224	135	773	6 549	1 469	—
	265	248	224	714	—	926	—

* Time intervals from beginning of spinning.

Table 3. *Radioactivity in chemical fractions from silkworms injected with [1-¹⁴C]glucose in late instar IV*Specific activity (mean counts/min./mg. of C $\pm$ s.e.m.)

Day*	Glycogen	Neutral-soluble fraction				Lipid
		Chitosan				
0	40 ± 20	73 ± 26	3 454 ± 1 331		29 ± 7	
5	45 ± 6	357 ± 157	5 508 ± 1 153		38 ± 2	
7	90 ± 30	540 ± 111	8 340 ± 1 836		37 ± 4	
10	1 047 ± 154	2 350 ± 642	Larval cuticle	Pupal cuticle	63 ± 4	
			15 790 ± 1 640			

* Time intervals from beginning of spinning.

Table 4. *Distribution of radioactivity in chitinous and other constituents of the cuticle*

Day	10 ⁻³ $\times$ total radioactivity (mean counts/min.)			Percentage of total contributed by chitosan	
	Whole cuticle*	Chitosan†	Other constituents, by difference	Counts	
				Weight	
0	1280	201	1079	16	28
5	889	242	647	27	28
7	1418	291	1129	21	33
10‡	469	74	395	16	21

* Specific activity (counts/min./mg. of tissue) $\times$ total wt. of cuticle.† Specific activity (counts/min./mg. of BaCO₃) $\times$ mg. of BaCO₃ formed by combusting total wt. of chitosan in cuticle.

‡ Values for (shed) larval and pupal cuticles are combined.

correspond to those calculated for catabolism of both (Fig. 1). After the pupal moult, utilization of both reserves is relatively slow, and there even appears to be some temporary net increase in total carbohydrate (Fig. 2).

During the first 2 days of the prepupal period, glycogen and sugars disappear rapidly (Figs. 2 and 3); much of this carbohydrate must be catabolized, as this is a time of great physical exertion in the spinning of cocoons, and some may be con-

verted into lipid, which appears to increase slightly. Between days 2 and 10, the quantity of lipid falls, but glycogen remains roughly constant, even rising slightly towards the end of this period; soluble sugars also rise between days 5 and 10. Chitin is extensively broken down during the prepupal period, the fall being especially abrupt between days 7 and 8. The apparent rise in glycogen before day 10, although not significant from the present data, presumably corresponds to the greater rise in

glycogen during the prepupal period of other insects. Our data may be explained by assuming (a) preferential utilization of fat and free sugars following spinning, and (b) resynthesis of glycogen from cuticular chitin at about the time of the moult.

That material is actually transferred from the cuticle to the internal nutrient reserves is established by the radioisotope experiments. Both glycogen and the neutral-soluble fraction show a rise in specific activity, gradual from days 0 to 7 and then rapid between days 7 and 10. The specific activity of the lipid also increases, though to a lesser extent. The results suggest uptake of material from a highly radioactive source. This source is presumably the cuticle, which in these experiments had far higher specific activity than any other body component and which has already been shown by the quantitative analyses to lose material gradually from days 0 to 5 and then suddenly between days 5 and 8 (Table 1). More than sufficient labelled carbon is lost from the cuticle, even if chitin alone is considered, to provide the counts taken up into glycogen, soluble fraction, and lipids (Fig. 4).

Just after pupation, between days 8 and 10, there appears to be a slight rise in total carbohydrate (Fig. 2). This may be spurious, reflecting incompleteness of the analyses. Very thin chitinous structures which are abundantly present immediately after the pupal moult may have dissolved in the isolation procedure. Carbohydrate fractions not measured by our quantitative methods would include amino sugars and glycoproteins. Amino sugars were detected qualitatively on our chromato-

grams and have been found in insect moulting fluid by Passonneau & Williams (1953) and Zielinska & Laskowska (1958). If the rise in total carbohydrate is real it may be the result of gluconeogenesis from resorbed cuticular protein. This protein contains abundant radioactivity which was incorporated with remarkable efficiency from glucose (Table 4). The likelihood of such a conversion was discussed by Needham (1929).

It is unnecessary therefore to invoke conversion of fat into carbohydrate, or any other metabolic pathway unusual in animal tissues, to explain the changing of nutrient reserves seen in the pupating cecropia silkworm. Any increase in glycogen or similar body constituents may be traced to mobilization of components of the larval cuticle. The insect, which will not feed again in its life cycle, thus handles the cuticle as an additional source of reserve nutrients.

The present findings are in substantial agreement with the studies of such changes in the Oriental silkworm, *Bombyx mori*, by Vaney & Maignon (1905), Bialasiewicz (1937) and Zaluska (1959). In a smaller insect (such as *Bombyx*) the integument might well comprise a greater proportion of the total body mass and any changes resulting from release of material from the cuticle would then be exaggerated relative to the picture in cecropia. The metabolic conversions in cecropia may thus be representative of those occurring in other insects.

Breakdown and deposition of the cuticle

Most of the breakdown of the larval cuticle occurs during the last day before the pupal moult. This is consistent with previous observations on insect moulting (Passonneau & Williams, 1953; Kühn & Piepho, 1938).

The moulting fluid, through which the products of cuticular degradation must pass, never acquires very high radioactivity (Table 2). This implies that products released from the larval cuticle must be immediately transferred to the new cuticle or resorbed into the haemolymph. There is never much moulting fluid present at any given time, and, as some 65% of the larval chitin is resorbed during the last day before the moult, the transfer rate must be very rapid.

The steady increase in specific activity of the chitosan fraction during the prepupal period (Table 3) may result from the ordered building up and breaking down of layers of the cuticle. The exocuticle is presumably laid down just before a moult, as shown by Kühn & Piepho for *Ephesia*; as [^{14}C]glucose was injected into the cecropia larvae at this stage, the exocuticle is highly labelled, and the endocuticle, deposited after the moult, is less so. Breakdown before the next

Fig. 4. Mean total counts during pupation in purified chemical fractions of cecropia silkworms which had received an injection of [^{14}C]glucose in late instar IV. □, Glycogen; ■, neutral-soluble fraction; ▨, lipids; ▩, chitosan (cuticle); ▤, chitosan (shed cuticle). Values are the product of the specific activity of each constituent times the quantity of the constituent as determined by analysis. Vertical limits indicate actual range of determinations. Days denote interval from beginning of spinning.

moult occurs in the reverse sequence, so that the less radioactive inner layers are progressively dissolved and, finally, the highly labelled exocuticle is shed.

The specific activity of pupal-cuticle chitosan on day 10 is as high as that of the larval cuticle during prepupal days 0 to 5. Since no other prepupal carbohydrate fraction has such high specific activity, this suggests direct utilization of material from the larval cuticle for synthesis of that of the pupa—a possibility already considered by Lafon (1943) for the coleopteran, *Tenebrio molitor*, and by Zaluska (1959) for *Bombyx*. For such a mechanism, since at least half of the pupal chitin is added after the larval cuticle has been shed (Fig. 2), products from the larval cuticle would have to be temporarily stored in the animal at the time of the moult, and then be preferentially utilized in synthesis of the pupal cuticle. Yet, none of the fractions examined (Table 3) has high enough specific activity to fill this role. One or more neutral-soluble components may acquire high activity during the last hours before the moult, after our day 7 samples were taken. More probably, the (radioactive) carbon destined to be utilized for the pupal cuticle may, after its removal from the larval cuticle, be sequestered in a fraction for which no analysis was made in the present study, namely glycoproteins. During the moulting periods, glycoproteins show great increases in the blood of a roach (Lipke, Grainger & Siakotos, 1960) and a crab, *Carcinus maenas* (Frentz, 1958). The presence of glycoproteins could also explain the fact that Wyatt & Kalf (1957), with Pflüger's method, detected apparent glycogen in larval blood of the cecropia silkworm whereas none was found after perchloric acid extraction in the present study.

SUMMARY

1. Cecropia silkworms taken at intervals during the prepupal period were analysed quantitatively to secure evidence on metabolic conversions.

2. The loss of total dry weight falls within the limits calculated from the rate of respiration, assuming a non-protein respiratory quotient corresponding to catabolism of mixed carbohydrate and fat.

3. Amounts of lipids decrease steadily during the prepupal period. Those of glycogen and free sugars decrease during spinning, then show an apparent increase about the time of the pupal moult. The amount of chitin, as well as total weight of cuticle, decreases sharply just before the moult. It is thus possible to account for the synthesis of glycogen and sugars by conversion of substances from the cuticle without invoking conversion of lipids.

4. Larvae were injected with [^{14}C]glucose just before their fourth moult. By the end of instar V, the retained label was almost exclusively in the cuticle. Then, during the pupal moult, isotopic carbon was transferred to glycogen, sugars, and lipids.

5. Chitin in the newly synthesized pupal cuticle has a high specific activity similar to that of the larval cuticle. Specific re-use of materials from the latter is suggested.

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REFERENCES

- Abraham, S. & Hassid, W. Z. (1957). In *Methods in Enzymology*, vol. 4, p. 550. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Ashwell, G. (1957). In *Methods in Enzymology*, vol. 3, p. 84. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Babers, F. H. (1941). *J. agric. Res.* **62**, 509.
- Bade, M. L. (1962). *Biochem. J.* **83**, 478.
- Bergold, G. (1943). *Biol. Zbl.* **63**, 1.
- Bialasiewicz, K. (1937). *Acta Biol. exp., Varsovie*, **11**, 20.
- Brown, A. W. A. (1938). *Biochem. J.* **32**, 903.
- Buck, J. B. (1953). In *Insect Physiology*, pp. 213, 214. Ed. by Roeder, K. D. New York: J. Wiley and Sons.
- Burton, R. M. (1957). In *Methods in Enzymology*, vol. 3, p. 246. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Campbell, F. L. (1929). *Ann. ent. Soc. Amer.* **22**, 401.
- Chauvin, R. (1956). *Physiologie de l'Insecte*, p. 113. The Hague: W. Junk.
- Couvreux, M. E. (1895). *C.R. Soc. Biol., Paris*, **47**, 796.
- Crompton, M. J. (1959). *Biochem. J.* **72**, 479.
- Deuel, H. J. & Morehouse, M. G. (1946). *Advanc. Carbohydr. Chem.* **2**, 119.
- Dische, Z. (1955). *Meth. biochem. Anal.* **2**, 322.
- Ephrussi, B. & Beadle, G. W. (1936). *Amer. Nat.* **70**, 218. Cited by Buck, J. B. (1953). In *Insect Physiology*, p. 161. Ed. by Roeder, K. D. New York: J. Wiley and Sons.
- Evans, D. & Dethier, V. G. (1957). *J. Insect Physiol.* **1**, 3.
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
- Frentz, R. (1958). *C.R. Acad. Sci., Paris*, **247**, 2204.
- Frew, J. G. H. (1929). *J. exp. Biol.* **6**, 205.
- Good, C. A., Kramer, H. & Somogyi, M. (1933). *J. biol. Chem.* **100**, 485.
- Haub, J. G. & Hitchcock, F. A. (1941). *Ann. ent. Soc. Amer.* **34**, 32.
- Hendler, R. W. (1959). *Science*, **130**, 772, 1701; **131**, 1886.
- Kalf, G. H. & Rieder, S. V. (1958). *J. biol. Chem.* **230**, 691.
- Koehler, L. H. (1952). *Analyt. Chem.* **24**, 1576.
- Kornberg, H. L. & Krebs, H. A. (1957). *Nature, Lond.*, **179**, 988.
- Kotake, Y. & Sera, Y. (1909). *Hoppe-Seyl. Z.* **62**, 115.
- Kühn, A. & Piepho, H. (1938). *Biol. Zbl.* **58**, 13.
- Lafon, M. (1943). *Arch. Zool. exp. gén.* **83**, 58.

- Lipke, H., Grainger, M. & Siakotos, A. N. (1960). *Proc. 11th int. Congr. Entomol., Vienna* (in the Press).
- Ludwig, D. & Rothstein, F. (1949). *Physiol. Zool.* **22**, 308.
- Mokrasch, L. C. (1954). *J. biol. Chem.* **208**, 55.
- Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
- Needham, D. M. (1929). *Biol. Rev.* **4**, 307.
- Passey, R. F. & Fairbairn, D. (1957). *Canad. J. Biochem. Physiol.* **35**, 511.
- Passonneau, J. V. & Williams, C. M. (1953). *J. exp. Biol.* **30**, 545.
- Patton, M. B., Hitchcock, F. A. & Haub, J. G. (1941). *Ann. ent. Soc. Amer.* **34**, 26.
- Reeves, R. E. (1944). *J. biol. Chem.* **154**, 49.
- Richards, A. G. (1951). *The Integument of Arthropods*, p. 184. Minneapolis: University of Minnesota Press.
- Schneiderman, H. A. & Williams, C. M. (1953). *Biol. Bull., Woods Hole*, **105**, 320.
- Vaney, C. & Maignon, F. (1905). *Rapp. Lab. Soie, Lyon*, **12**, 13; *C.R. Acad. Sci., Paris*, **140**, 1192.
- Wolfson, M. L. & Miller, J. B. (1956). *Analyt. Chem.* **28**, 1037.
- Wyatt, G. R. & Kalf, G. F. (1957). *J. gen. Physiol.* **40**, 833.
- Yonezawa, Y. & Yamafuji, K. (1935). *Bull. sci. Fak. terk. Kjušu Univ.* **6**, 125.
- Zaluska, H. (1959). *Acta Biol. exp., Varsovie*, **19**, 339.
- Zielinska, Z. M. & Laskowska, T. (1958). *Acta Biol. exp., Varsovie*, **18**, 209.

Biochem. J. (1962) **83**, 478

Metabolic Conversions during Pupation of the Cecropia Silkworm

2. TESTS FOR THE OPERATION OF THE GLYOXYLATE CYCLE

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It has been widely maintained that insects may carry out net conversion of fat into carbohydrates. Although quantitative analyses of cecropia silkworms taken during the non-feeding prepupal stage led to an alternative explanation of the source of glycogen synthesized at the end of this time (Bade & Wyatt, 1962), the possibility that some of this glycogen might be derived from fat could not be ruled out. Tissues of these insects have therefore been examined for activity of the glyoxylate cycle, an enzymic pathway by which fat is utilized for carbohydrate synthesis in micro-organisms and plants (Kornberg & Collins, 1957; Kornberg, Phizackerley & Sadler, 1960; Canvin & Beevers, 1961).

MATERIALS AND METHODS

Chemicals. All chemicals and solvents were of reagent grade, except that the diethyl ether used for chromatography was anaesthetic grade. CoA was obtained from the Pabst Brewing Company, Milwaukee, Wis., U.S.A., and oxaloacetic acid, glutathione (reduced), glyoxylic acid (sodium salt hydrate) and ATP (disodium salt) from Sigma Chemical Co., St Louis, Mo., U.S.A. They were stored in a desiccator at -10° . The glyoxylate gave a 2,4-dinitrophenylhydrazine (prepared in 65% yield by Dr Anne

Sayigh) which had m.p. $194-195^{\circ}$ and gave only two spots on chromatography in a basic solvent system. Crystalline bovine serum albumin was a product of Pentex Inc., Kankakee, Ill., U.S.A. New England Nuclear Corp., Boston, Mass., U.S.A., supplied sodium $[2-^{14}\text{D}]$ acetate which was stated to have a specific activity of 2.00 mc/m-mole. Ion-exchange resins were as described by Bade & Wyatt (1962).

Biological material. Specimens of *Hyalophora cecropia* were reared as described by Bade & Wyatt (1962). Tissues utilized for enzyme assays were obtained from prepupae 5 or 6 days after the beginning of spinning. For comparison, assays were also carried out with tissue homogenates from feeding larvae in instar V, and from early pupae (30 days after spinning).

Determination of protein. Protein was assayed by the biuret method (Layne, 1957). Triplicate samples of each homogenate were prepared by precipitating the proteins in 0.1 ml. with 75% (v/v) ethanol, washing with ether and drying at 110° . For the assay, they were dissolved in 1 ml. of hot N-NaOH.

Preparation of tissues and assay conditions. Preliminary experiments on incorporation of $[2-^{14}\text{C}]$ acetate into total ethanol-soluble non-volatile components of fat-body homogenate established that a good rate of incorporation could be obtained in presence of histidine-HCl buffer (0.05M) with little difference over the pH range 6.5-7.5. Exogenous phosphate was not required. Keeping the enzyme preparation at 0° for 40 min. reduced incorporation only slightly, freezing and thawing somewhat more so. Addition of CoA and ATP was necessary for maximal incorporation.

Fat body and midgut from one individual were used as enzyme sources for each assay. Tissues were dissected out

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