Aspects of the Development of Flight-Muscle Sarcosomes in the Sheep Blowfly, *Lucilia cuprina*, in Relation to Changes in the Distribution of Protein and some Respiratory Enzymes during Metamorphosis

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1. Changes in the amounts and distribution of protein and respiratory enzymes have been estimated during the life cycle of the fly *Lucilia cuprina*. 2. The fully fed larva contains about 7 mg. of protein, the pupa and newly emerged fly about 4 mg., and the mature adult about 3 mg. 3. There are two periods of incorporation of protein into particles at the expense of the soluble protein; the first, immediately after pupation, may store protein (0.5 mg./insect) for use in adult development; the second, over the period of emergence, was due mainly to the development of the thoracic mitochondria of the adult (0.7 mg./insect). 4. In the thorax, cytochrome *c* oxidase and the dehydrogenases for glycerophosphate, isocitrate (NAD-dependent), succinate and malate appeared initially in small particles (less than 1 μ in diameter). 5. In adult development these enzymes were redistributed so that in the mature fly most of the activity was present in larger particles (1–10 μ in diameter). 6. During this redistribution the specific activity (μ l. of oxygen/hr./mg. of protein) of glycerophosphate dehydrogenase in the small particles was 690 at 1½ days before emergence, 955 at emergence and 980 at 7 days after emergence; the corresponding values for the large particles were 164, 760 and 1220. 7. In the mature fly the highest specific activities (μ l. of oxygen/hr./mg. of protein) estimated were: glycerophosphate dehydrogenase 1380, isocitrate dehydrogenase (NAD-dependent and requiring ADP and Mg^{2+}) 408, succinate dehydrogenase 122, malate dehydrogenase 190, and cytochrome *c* oxidase 1360. 8. The results are considered in relation to the development of the flight-muscle sarcosomes.

The metamorphosis of holometabolous insects like the blowfly is known to involve transformations, not only in morphology (Wigglesworth, 1965, p. 64) and in the activity of individual enzymes (Wigglesworth, 1965, p. 573), but also in the nature of the subcellular structures. Striking examples of the last change are the development of the flight-muscle sarcosomes (Brosemer, Vogell & Bücher, 1963; Lennie & Birt, 1966) and in the appearance and disappearance during pupal life of large proteinaceous particles, which are characterized by having an active tyrosine-dependent ATP-pyrophosphate exchange enzyme (Lennie & Birt, 1965) and which are possibly 'albuminoid spheres' (Kilby, 1963; Wigglesworth, 1965, p. 402).

The aim of the present paper is to describe the distribution of protein and some respiratory enzymes in subcellular fractions isolated at different stages in the metamorphosis of *Lucilia*. This genus has been used for a study of the changes in the

protein content/insect by Evans (1932), who concluded that there was no appreciable variation in the total protein during the pupal life of *L. sericata*; however, soluble protein declined after pupation and rose before emergence, as did that of *L. cuprina* (Howells & Birt, 1964).

Respiratory changes during metamorphosis have been studied in a variety of insects; in holometabolous forms many respiratory enzymes pass through a stage of minimum activity in mid-pupal life (Wigglesworth, 1965, p. 573) whereas in hemimetabolous species the rate of synthesis of such enzymes decreased during the period of moulting (Bücher, 1965). However, detailed information about the subcellular localization of the respiratory enzymes concerned is available only for the hemimetabolous locust (Brosemer *et al.* 1963); some information about changes in the structure and cytochrome content of particles isolated from the holometabolous bee has also been published

(Herold & Borei, 1963; Herold, 1965). Bücher (1965) concluded that the mitochondria of the locust are capable of both 'independent growth' (i.e. synchronous increase of enzymic and non-enzymic protein) and 'multiplication' (by fission).

The development of the flight-muscle sarcosomes of this insect begins in the nymph and continues into adult life. However, the development of thoracic sarcosomes in the fly, which begins much more abruptly during pupal life (probably after the pupal-adult moult), is more comparable with that of the bee. In the late pupal stage of this insect Herold (1965) detected a variety of possible mitochondrial precursors different in appearance and cytochrome complement to the mature mitochondrion. The precise role of these various particles is not clear. Similarly, it has been reported previously that particles of differing size and specific enzymic activity can be separated from the thoraces of developing flies. Some of these particles may be the precursors of the flight-muscle mitochondria, which develop with a considerable increase in enzymic activity and mitochondrial mass (Lennie & Birt, 1966); corresponding increases of mitochondrial mass have been reported in the blowfly *Phormia* (Watanabe & Williams, 1953).

The investigations reported in the present paper permit a description of the storage and redistribution of protein during the metamorphosis of the fly and a fuller discussion of the development of the flight-muscle sarcosomes.

EXPERIMENTAL

Chemicals

Cytochrome *c* (type III), disodium DL- α -glycerophosphate (grade X), trisodium DL-isocitrate, L-malic acid (type 1), ATP, ADP, NAD⁺ and NADP⁺ were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.; succinic acid, L-ascorbic acid, DCPIP* and INT were from British Drug Houses Ltd., Poole, Dorset; PMS was from Koch-Light Laboratories Ltd., Colnbrook, Bucks.; crystallized bovine serum albumin was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex. All other reagents were of A.R. grade or of the highest purity commercially available. Phosphate buffers were made from the potassium salts.

Insects

Flies were maintained at 30° throughout the entire life cycle; larvae were fed on ox liver and the adults on sucrose and water. Batches of tissue for each experiment were chosen from individuals that pupated within a period of 4 hr. All insects were chilled to 0° and washed in 0.25 M-sucrose at 0° before further treatment.

* Abbreviations: DCPIP, 2,6-dichlorophenol-indophenol. INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride. PMS, *N*-methylphenazonium methosulphate.

Tissue homogenates

Insects from selected stages of their life cycle were disrupted by homogenizing three to five insects/ml. of ice-cold 0.25 M-sucrose for 20 sec. in a glass vessel with a Teflon plunger (with a clearance of 0.01 in.) rotating at about 1000 rev./min. Thoraces were dissected away from heads and abdomens, freed from legs and wings, cut in half longitudinally and then gently homogenized by hand in the glass-Teflon homogenizer for 1 min. Pharate adults were removed from the pupal case before dissection. The suspensions were filtered, under gentle suction, through a pad of glass wool resting on a sintered-glass disk (porosity 1). Insects were cooled to 0° before all manipulations and all operations conducted at 0°–5°.

Fractionation of the homogenates

Three methods have been used; all manipulations were conducted 0–3°. Method 1 was used to isolate particles from homogenates of whole insects, and methods 2 and 3 were used for thoracic homogenates.

Method 1. The method was that described by Smith (1961), who defines the fractions in terms of the 'effective diameter' of the particles, which are assumed to be spheres of constant density. The following fractions were separated from the homogenates of the whole insect: particles of diameter less than 0.2 μ (the 'soluble' fraction), 0.2–1.0 μ , 1.0–3.0 μ and greater than 3 μ .

Method 2. This method involved centrifuging the filtered homogenates for 3 min. at 50 *g* to remove material greater than 10 μ in diameter. Then 2.0 ml. of the supernatant suspension was carefully layered on to 0.3 M-sucrose solution (14 ml.) in a 34 ml. centrifuge tube, as shown in Fig. 1 (tube *a*). The radial distance from the axis of rotation to the centre of the upper layer (r_1) was 9.6 cm. This system was centrifuged at 3000 rev./min. for 10 min. (1080 $g_{av.}$) in a Spinco SW 25.1 rotor (all subsequent centrifugings were carried out in this rotor). By using the constants and the equation quoted by Smith (1961), a distance r_2 (11.9 cm.) was calculated so that removal of fluid to this level would leave particles greater than 1 μ in diameter in the tube. The upper layer was mixed

Fig. 1. Diagrammatic representation of the system for separating particles from homogenates by methods 2 (tube *a*) and 3 (tube *b*). r_1 , r_2 , r_3 and r_4 are the radial distances (9.6, 11.9, 7.7 and 9.9 cm.) from the axis of rotation during the centrifuging. ▨, Homogenates; □, 0.3 M-sucrose; ▩, sucrose gradient (from sp.gr. 1.24 at bottom to sp.gr. 1.18 at top).

and centrifuged at 10000 rev./min. (12000 $g_{av.}$) for 10 min. to remove particles greater than 0.2μ in diameter; these were gently resuspended in 0.25M-sucrose. The particles in the lower layer were sedimented and resuspended in the same way. Thus three fractions were obtained, a 'soluble' fraction (containing cytoplasmic solutes and particles less than 0.2μ in diameter) and the fractions containing the small ($0.2-1\mu$ in diameter) and the large ($1-10\mu$ in diameter) particles.

Method 3. The procedure was the same as method 2, except that the 0.3M-sucrose was layered on to a continuous gradient of sucrose solution (8ml.) with sp.gr. range 1.24-1.18, the system before centrifuging as shown in Fig. 1 (tube b), where r_3 is 7.7 cm. The tubes were centrifuged at 3000 rev./min. for 11.6 min. An upper layer containing all the particles less than 1μ in diameter was removed (r_4 9.9 cm.) and centrifuged as in method 2. The lower layer (a combination of 0.3M-sucrose resting on the sucrose gradient) was centrifuged for 15 min. at 15000 rev./min. (23000 $g_{av.}$) to separate the mitochondria (sp.gr. 1.20) from fragments of muscle fibres (sp.gr. 1.21-1.24), nuclei and uric acid granules (sp.gr. greater than 1.24). The portion of the gradient containing the mitochondria was removed, freed from contamination by fragments of muscle fibres, nuclei and uric acid particles checked by phase-contrast microscopy and the suspension diluted until the sucrose concentration was about 0.3M. The particles were sedimented and resuspended in 0.25M-sucrose. This procedure permitted the isolation of a more highly purified fraction of the large particles.

Determination of activity of respiratory enzymes

Cytochrome c oxidase. This enzyme was assayed by the method of Smith (1955) by using a Zeiss PMQII spectrophotometer; ascorbic acid was used in place of palladium and hydrogen to reduce the cytochrome c and the mitochondria were incubated at 25°. Rates obtained from this assay were converted into μ l. of O_2 /hr./mg. of protein.

Dehydrogenase-DCPIP reductase (with preparations from whole insects). Succinate dehydrogenase and glycerophosphate dehydrogenase were assayed as described by Dalgarno & Birt (1963), but with the addition of PMS (0.07 mg./ml. in the cuvette) as an intermediate redox carrier (Arrigoni & Singer, 1962). The change in extinction at 600 m μ was recorded with a Beckman DK2 spectrophotometer.

Dehydrogenase-INT reductase (with preparations from thoracic tissues). The method was basically that of Lee & Lardy (1965). Modifications were the lowering of the concentration of PMS to 0.7 mg./ml. in the PMS-INT solution and the addition of bovine serum albumin (0.4% in the incubation mixture). Preliminary experiments showed that these changes increased the colour yield with both glycerophosphate (200%) and succinate (50%), and that the serum albumin permits the use of much smaller amounts (down to 8 μ g.) of mitochondrial protein. The mitochondrial preparations were treated ultrasonically for 30 sec. at 0° in a MSE Ultrasonic Disintegrator (60 w) except that those for glycerophosphate-dehydrogenase activity were assayed without this treatment. Differences in the rates of reaction were observed with different samples of INT; the sample giving the highest rates was reducible with both ascorbic acid and dithionite, whereas the less reactive samples were

only reduced with the latter compound. The manufacturers were not able to account for this variation. Each preparation was tested at two different concentrations of mitochondrial protein and each estimation was made in duplicate. Endogenous colour production was not more than 10% of the test values except in the assays for NADP-isocitrate dehydrogenase in large particles, where the value for the blanks reached 40% of the test values. This activity appears to be due to the addition of NADP rather than to the oxidation of endogenous citrate (present at a high concentration in fly sarcosomes; Van den Bergh, 1962), as it did not occur with the blank reaction mixture used for the assay of NAD-isocitrate dehydrogenase.

(a) FAD-glycerophosphate dehydrogenase. This enzyme was not very stable, the activity in isolated particles declining by 75% after 30 sec. treatment with ultrasonics at 0°, and by 70% during overnight storage at 0°. Similar instability of this enzyme in sarcosomes isolated from *Phormia* has been reported by Sacktor (1961). Assays were therefore carried out on preparations that had not been treated with ultrasonics. Contrary to the results of Estabrook & Sacktor (1958), the activity was not increased by Mg^{2+} (1-10 mM). The inclusion of EDTA in their isolation media may account for this difference.

(b) Succinate dehydrogenase. This enzyme was very stable, being unaffected by either storage at -15° for 2 days or treatment with ultrasonics for 30 sec. at 0°. The presence of a permeability barrier for this substrate (Van den Bergh, 1962) would be masked by the disruption of the mitochondrial structure in the phosphate buffer (0.125M) of the reaction mixture (Bartley & Enser, 1964). The enzyme was not stimulated by ATP (Gregolin & Scalella, 1965).

(c) NAD-isocitrate dehydrogenase. Mg^{2+} and ADP were both necessary as in the locust but the addition of glutathione to the isolation media had no effect (Gobell & Klingenberg, 1964). Concentrations used in the incubation mixtures were 8 mM- Mg^{2+} and 2 mM-ADP.

(d) NAD-malate dehydrogenase. The activity of this enzyme declined rapidly to zero during the estimation, probably resulting from inhibition by oxaloacetate. The addition of cysteinesulphinic acid, hydrazine or semicarbazide interfered with the assay. Activities were calculated from initial rates.

Determination of protein

The protein content of whole insects and homogenates of these was determined by the biuret method of Cleland & Slater (1953). The fractions isolated from homogenates of whole insects were treated with an equal volume of 10% (w/v) trichloroacetic acid and the nitrogen of the washed precipitates was determined by the micro-Kjeldahl procedure (Association of Official Agricultural Chemists, 1955), as the amount of protein available in some of the fractions was too small to be determined by the biuret method. The more sensitive Lowry-Folin method (Lowry, Rosebrough, Farr & Randall, 1951) was not used because of the presence of tyrosine or its derivatives, which varied in amount during the life cycle; it was used, however, with the thoracic preparations.

RESULTS

Protein content of insects and homogenates. Table 1 presents the amounts of protein/insect determined

with single organisms and with filtered homogenates of insects from the same batch of tissue. With pupae and flies about 90% of the protein was present in the homogenates; the lower value (53%) with larvae was due mainly to the difficulty of dispersing the extremely tough cuticle, which contained biuret-positive material and which was retained during filtration of the homogenate.

The protein content of entire larva (7.4 mg./insect) was considerably greater than that of later stages; about 20% of the decline at pupation was probably due to the formation of the puparium, which, unlike the larval cuticle, does not react with the biuret reagent. There was no significant variation (at the 5% level) in the protein content during pupal life,

the average value being 4.2 mg./insect. The adult fly contained less protein (mean value 3.1 mg./insect), the decrease being highly significant (0.1% level). The two sexes also differed in their protein content, the average values for the males being 2.6 and for the females 3.5 mg./insect. This variation agrees with the reported difference in size (Shanahan, 1965). Part of the decrease in protein content after emergence was probably due again to inability of the biuret reagent to react with the tanned cuticle of the adult.

Protein content of subcellular fractions isolated from homogenates of larvae, pupae and flies. The distribution of protein in the various fractions as a percentage of the total protein content is shown in Table 2. In the homogenates of larvae, about 75% of the protein was in the 'soluble' fraction (average content 2.9 mg./insect). At pupation, this value declined to 2.1 mg./insect, rising again as the fly developed to about 2.6 mg./insect at $\frac{1}{2}$ day after emergence. Finally it declined (1.1 mg./insect) as the fly matured.

The amount of protein in particles greater than 0.2 μ in diameter doubled at pupation (from about 1 to 2 mg./insect), as the result of increases in both the very small and, more markedly, the very large particles. As the development of the pharate adult occurred (pupae day 4 to emergent fly), 0.6 mg. of protein was lost from the large particles (greater than 3 μ in diameter) of each insect, but an increase of 0.5 mg./insect was recorded in the 'soluble' protein in this period. Conversely, during the maturation of the fly, the protein of the large-particle fraction increased by 0.7 mg./insect, whereas that of the 'soluble' fraction decreased by 1.0 mg./insect.

Table 1. *Protein content of insects and homogenates during the life cycle of L. cuprina*

Single insects were washed with 0.25 M-sucrose, crushed in 2 ml. of 5% (w/v) trichloroacetic acid, and the protein was estimated by the method of Cleland & Slater (1953). Homogenates were prepared in 0.25 M-sucrose by using between 50 and 100 insects (five insects/ml.), the resultant suspension being filtered through two layers of fine muslin. The larvae were 4 days old (third instar), pupae were selected at four stages during pupal life, and flies were 7 days old. The numbers of samples are given in parentheses.

	Protein content (mg./insect)		
	Late larvae	Pupae	Flies
Insects	7.40 (4)	4.19 (14)	3.10 (6)
Range	6.70-7.75	3.33-5.40	1.80-3.50 (males) 3.10-4.10 (females)
Homogenates	3.90 (3)	3.89 (9)	2.97 (2)

Table 2. *Protein distribution between subcellular fractions isolated from homogenates of insects at various stages of the life cycle*

Between 20 and 30 insects were cooled to 0°, washed and homogenized in 0.25 M-sucrose (five insects/ml.) and then filtered through two layers of fine muslin. Particles from the homogenates were separated into various fractions by the centrifuging method of Smith (1961). The protein in each fraction was determined by the micro-Kjeldahl method. The protein in each fraction is expressed as a percentage of the total protein in the fraction.

		Distribution of protein (%)				
Stage of life cycle	Age	Particles > 3 μ diam.	Particles 1-3 μ diam.	Particles 0.2-1 μ diam.	Sum of particles	'Soluble' fraction
Larvae	4 days	15.7	4.8	4.1	24.6	75.4
Pupae	1 day	24.2	6.8	14.5	45.5	54.5
	4 days	29.9	9.0	7.1	46.0	54.0
	6 days	16.5	11.5	10.4	38.4	61.6
Flies	$\frac{1}{2}$ day	14.3	14.0	4.0	32.3	67.7
	4 days	39.0	12.8	12.8	64.6	35.4

Fig. 2. Distribution of cytochrome *c* oxidase, succinate dehydrogenase and glycerophosphate dehydrogenase in fractions separated by method 1 during development of the insect. The values in parentheses are the sums of the activities in the fractions shown (as $\mu\text{l. of O}_2/\text{hr./insect}$).

Variations in the activity of some respiratory enzymes in tissue homogenates during the life cycle. Cytochrome *c* oxidase, succinate dehydrogenase and FAD-glycerophosphate dehydrogenase were selected as typical mitochondrial enzymes, probably present in the membrane of the particles; the sarco-somes are characterized by having a glycerophosphate dehydrogenase of particularly high activity (Birt, 1961). The activities are shown in Fig. 2, the values in parentheses being the total activity ($\mu\text{l. of oxygen/hr./insect}$) for each stage. The activity of cytochrome *c* oxidase declined rapidly from the late larval to mid-pupal stage (day 3). There was a less marked decrease in succinate-dehydrogenase activity; the low activity detected in the larvae may be due to inactivation of the enzyme during the relatively drastic treatment required to homogenize this tough tissue. A very low activity of glycerophosphate dehydrogenase

(about 1% of the final value for the mature fly) was detected early in pupal life. Unlike the other enzymes, this dehydrogenase increased during days 1 to 3 of pupal life. Subsequently all three enzymes continued to increase in activity as the fly matured.

In the fully grown larvae cytochrome *c* oxidase was localized mainly in particles less than 3μ in diameter. With the rapid histolysis of larval tissues during early pupal life, the activity declined to an extremely low value in the mid-pupae (Fig. 2). The initial increase in activity during adult development (after day 3) was predominantly in the smallest particles, 79% in this fraction ($0.2-1\mu$ in diameter) at the late pupal stage (day 6). As the fly matured distribution of the activity within the fractions changed until in the mature fly (day 4) 63% of the activity was present in particles greater than 1μ in diameter, with 53% in those of diameter above 3μ . In flies maintained for 1 month, the activity

declined to about 10% of the value in fully developed adults, with 71% of this activity being in the particles 1–3 μ diameter; by this age many of the insects were not able to fly.

Very little (average 8 μ l. of oxygen/hr./insect) succinate-dehydrogenase activity was present in the particles during the first 3 days of pupal life. The increase of this enzyme during adult develop-

ment again occurred initially in smaller and later in larger particles; thus in late pupae (day 6) all of the activity was in particles less than 1 μ in diameter; the corresponding values for flies at days 1 and 4 were 26 and 23% respectively.

FAD-glycerophosphate dehydrogenase was detected for the first time in early pupal life (day 1) and the pattern of its development was similar to that for the other enzymes (e.g. 76% of activity in particles greater than 1 μ from 4-day flies).

Table 3. *Percentage distribution of protein in the fractions separated from homogenates of thoraces of insects during the development of the adult*

Pharate adults were removed from the pupal cases. Thoraces, freed from appendages, were bisected, homogenized by hand and filtered through glass wool, and the resultant suspensions were separated into fractions by using method 2.

Tissue fraction	Distribution of protein in insects (%)			
	Before emergence		After emergence	
	1½ days	½ day	1 day	8 days
Particles > 10 μ diam.	18.0	9.7	2.3	2.5
Particles 1–10 μ diam.	23.0	14.2	36.6	46.3
Particles 0.2–1 μ diam.	7.2	11.8	16.0	6.9
'Soluble' fraction	38.2	46.5	26.0	28.7
Retained on filter	13.6	18.8	19.1	15.6
Total protein (mg.)	1.39	1.55	1.31	1.60

Changes in the distribution of thoracic protein during the development of the flight-muscles. The values reported in Table 3 were obtained from particles isolated from homogenates of thoraces by method 2. During adult development there was little variation in the protein content of the thorax. The proportion of the protein in the fraction containing particles of diameter greater than 10 μ declined during the period 1½ days before emergence to 1 day after emergence and then remained very low (2%). Before emergence there was a slight decline in the protein of particles 1–10 μ in diameter, followed by an increase of about 0.5 mg. in these particles during the 8 days after emergence. The percentage of protein in the particles 0.2–1 μ in diameter almost doubled in the 1½ days up to emergence, then declined to the original value in the mature (8-day) fly. The protein content of the soluble fraction increased before emergence and then declined to a lower value (about 0.4 mg./insect), which was maintained during adult life.

Fig. 3. Distribution of protein in sarcosomes isolated from homogenates of developing thoraces. The protein contents were obtained by (a) direct measurement, with the Lowry-Folin method (Lowry *et al.* 1951), on fractions separated by method 3 (O, particles > 1 μ diam.; Δ , particles 0.2–1 μ diam.; $\square$, sum of two fractions), and by (b) calculation from the total activity (Fig. 4) and specific activity (Table 5) of glycerophosphate dehydrogenase ($\bullet$, particles > 1 μ diam.; $\blacktriangle$, particles 0.2–1 μ diam.; $\blacksquare$, sum of the two fractions). Flies emerge from the puparium at day 0.

Changes in the distribution of sarcosomal protein during the development of the thorax. A marked increase in the amount of sarcosomal protein occurred during the development of the thorax. The results in Fig. 3 describe the distribution of this protein between the two fractions separated by method 3. The fractions were virtually free of contamination by non-mitochondrial particles (as judged by phase-contrast microscopy), but some sarcosomal material was lost. An estimate of the magnitude of these losses was obtained by calculating the amount of sarcosomal protein that could be expected in the fraction from the total (Fig. 4) and the specific activity (Table 5) of glycerophosphate dehydrogenase at three stages. Although calculated

from the data with different batches of tissue the results for the particles 0.2–1 μ in diameter agreed almost exactly. With the larger particles (greater than 1 μ in diameter) losses had occurred, especially with the preparations from insects before emergence. Thus with 6–8-day-old flies the isolation procedure gave about 73% of the calculated value, just after emergence 24%, and at 1½ days before emergence only 13%. Because of the very low activity in the large-particle fraction at this last stage (Fig. 4), the calculation of protein content was liable to considerable error; it seems probable that the value would be about 0.05 mg./insect at this stage.

When the tissue was fractionated by method 2 (Table 3) the yield of protein in the smaller-particle

Fig. 4. Activity of the respiratory enzymes in fractions isolated from homogenates of developing thoraxes by method 2: (a) glycerophosphate dehydrogenase; (b) succinate dehydrogenase; (c) NAD-isocitrate dehydrogenase; (d) NADP-isocitrate dehydrogenase; (e) NAD-malate dehydrogenase; (f) cytochrome *c* oxidase. The fractions were: ●, particles 1–10 μ diam.; ■, particles 0.2–1 μ diam.; ▲, 'soluble' fraction; ○, sum of these fractions. Flies emerge from the puparium at day 0. The activity is reported as $\mu\text{l. of O}_2/\text{hr./insect}$.

fraction was again in close agreement with the values in Fig. 3. However, the values for the large-particle fractions were greater than those derived by calculation (40% before emergence and 30% with mature flies).

These results illustrate the difficulties of isolating mitochondria early in the development of the thorax; as judged by microscopic examination most of the contamination (at all stages) was due to fragments of muscle fibres. With mature insects, most of the fibres were removed by filtration and a low-speed centrifuging (50*g* for 3 min.), as they were relatively resistant to disintegration and as they had sp.gr. greater than 1.24. However, at earlier stages the fibres appeared to fragment into smaller pieces and as they are less dense (sp.gr. about 1.21–1.22) they did not separate so readily from the mitochondria (sp.gr. 1.20). The specific gravity of the mitochondria did not change appreciably during development to 8 days.

In summary, the results in Fig. 3 show that mitochondrial protein rose from about 100 μ g./insect (1½ days before emergence) to about 600 μ g. in the mature insect (day 8); other experiments have shown that the values declined slowly thereafter. The rate of increase was greater (about two- to three-fold) during the first 2–3 days. The amount of protein in the larger particles increased linearly (at about 50 μ g./day) over the entire period (to 8 days); that in the smaller particles reached a maximum value about 2 days after emergence and then declined slowly. The initial rate of increase in protein in the small particles was high (about 100 μ g./day).

Changes in the enzymic activity of sarcosomes during the development of the thorax (Fig. 4). (a) Cytochrome *c* oxidase. The activity of cytochrome *c* oxidase in the thorax increased rapidly in the latter stages of pharate life and in the newly emerged fly (1½ days before to 1 day after emergence), but did not change appreciably in the maturing fly; thus 60% of the maximum activity was present ½ day before emergence and almost 100% 1 day after emergence. The enzyme was concentrated initially in the smaller particles (which contained about 80% of the activity ½ day before emergence), whereas in the adult it was present almost entirely (80%) in the larger particles. Activity in the soluble fraction was negligible except with mature (8-day) flies, when the high value (17%) was difficult to reconcile with the distribution of glycerophosphate dehydrogenase and succinate dehydrogenase in the same preparations. These results suggest that, during isolation, the sarcosomes of the mature fly release cytochrome *c* oxidase [and NAD-isocitrate dehydrogenase (Fig. 4), which is probably not bound to the mitochondrial membrane] more readily than the two flavoprotein enzymes.

Table 4. *Specific activities of various respiratory enzymes in the particulate fractions isolated (method 2) during the development of the thorax*
Activities of dehydrogenases were determined by the rate of formazan production and of the oxidase by the rate of oxidation of reduced cytochrome *c*. Rates were converted into μ l. of O_2 /hr./mg. of protein.

Particle diam. (μ)... Stage of adult development	Specific activity (μ l. of O_2 /hr./mg. of protein)					
	Glycerophosphate dehydrogenase	Succinate dehydrogenase	NAD-isocitrate dehydrogenase	NADP-isocitrate dehydrogenase	NAD-malate dehydrogenase	Cytochrome <i>c</i> oxidase
	0.2-1.0 1.0-10	0.2-1.0 1.0-10	0.2-1.0 1.0-10	0.2-1.0 1.0-10	0.2-1.0 1.0-10	0.2-1.0 1.0-10
1½ days before emergence	500	Nil	—	—	—	40
½ day before emergence	1360	122	83	19	190	506
1 day after emergence	1025	62	206	26	124	1082
8 days after emergence	1300	45	370	1	109	45
						83
						573
						1360

(b) Flavoprotein dehydrogenases. The pattern of development of the two flavoprotein dehydrogenases was similar to that of cytochrome *c* oxidase. The same redistribution of activity within the two particulate fractions was apparent but no activity was detected at any stage in the 'soluble' fraction.

(c) Dehydrogenases requiring nicotinamide nucleotides. The activity of NAD-isocitrate dehydrogenase increased about fivefold in the period $\frac{1}{2}$ day before to 7 days after emergence, the development being somewhat later than that for the other enzymes tested (Fig. 4). The redistribution of activity within the fractions was similar to that of cytochrome *c* oxidase. In marked contrast with all other enzymes tested, NADP-isocitrate dehydrogenase remained constant in activity and was localized mainly (85%) in the 'soluble' fraction. The increase in the activity of NAD-malate dehydrogenase was relatively small (39% from $\frac{1}{2}$ day before to 7 days after emergence). Throughout the entire period tested, the greater proportion of the activity in the mitochondria was in the larger particles (1–10 μ in diameter); the activity in this fraction increased as the fly developed, whereas that in the small particles and 'soluble' phase decreased.

Specific activities of the dehydrogenases in sub-cellular fractions of the developing thorax. Calculation of the specific activities (μ l. of oxygen/hr./mg. of protein) for the experiment described in Fig. 4 indicated that, in the period before the emergence of the fly, all the enzymes tested (except NADP-isocitrate dehydrogenase) were more concentrated in the smaller particles (Table 4). Conversely, in the mature fly the larger particles had the greater concentration of these enzymes. As the preparations described in Table 4 were known to be contaminated with non-mitochondrial protein the relation between the enzyme concentrations in the two particulate fractions during development was clarified by a more precise determination of the specific activity of glycerophosphate dehydrogenase in sarcosomes isolated by method 3. This enzyme was chosen because it was the most active dehydrogenase in the mitochondria (Table 4). At $1\frac{1}{2}$ days

before emergence, the enzyme was concentrated in the smaller particles, which were four times as active as the larger (Table 5). The specific activity of the smaller particles increased by about 40% during the $1\frac{1}{2}$ days before emergence and did not change thereafter; that of the larger particles increased very considerably (4 $\frac{1}{2}$ -fold) before emergence and continued to rise (by 60%) as the fly matured. Thus the relationship was the same as that obtained from Table 4, i.e. the enzyme was more concentrated initially in the smaller and finally in the larger particles. The somewhat higher values in Table 4 compared with those in Table 5 reflect the rapid inactivation that occurs during separation, the isolation of the particles by method 3 (Table 5) taking considerably longer than for method 2 (Table 4).

DISCUSSION

Changes in the distribution of protein during the life cycle of Lucilia. The present results were obtained from homogenates of whole insects, which contain a great diversity of subcellular particles (Wigglesworth, 1965, pp. 67–401). Nevertheless, the results do permit an evaluation of the significance of some of the changes during pupal-adult development. At pupation, the larval cuticle is tanned to form the puparium, which is unreactive to biuret reagent; there is a loss of about 2mg. of 'soluble' protein/insect (Table 2; Howells & Birt, 1964) at a time when it is possible that amino acids are providing intermediates for synthesis of carbohydrates and fatty acids and for oxidation. All these changes result in a marked fall in the measured protein content/insect during this period. However, the protein content of the pupa remains constant, despite great morphological changes and associated histolysis and histogenesis. This constancy indicates a delicate balance between degradation and synthesis, as the concentration of free amino acids varies only slightly during pupal life although there are large changes in the amount of some amino acids (Lennie & Birt, 1965).

Table 5. *Specific activity of glycerophosphate dehydrogenase in particulate preparations freed from non-mitochondrial contaminants (method 3)*

Activities were determined by the rate of formazan production and were converted into μ l. of O₂/hr./mg. of protein. The numbers of determinations are given in parentheses.

Fraction	Age	...	...	Specific activity of glycerophosphate dehydrogenase (μ l. of O ₂ /hr./mg. of protein)		
				$1\frac{1}{2}$ days before emergence	0–1 day after emergence	7 days after emergence
Particles > 1 μ diam.				164 (14)	760 (8)	1220 (4)
Particles < 1 μ diam.				690 (4)	955 (12)	980 (4)

The changes in protein distribution that take place after pupation may be interpreted as follows: during prepupal and early pupal life, a store of protein (about 0.5 mg./insect) is laid down in particle form at the expense of some of the soluble protein of the larva; the lack of respiratory enzymes and the presence of enzymes catalysing an amino acid (especially tyrosine)-dependent ATP-pyrophosphate exchange reaction in these particles (Lennie & Birt, 1965) is consistent with this storage function. The increase in the amount of phospholipid/insect after pupation (D'Costa & Birt, 1966) may reflect the formation of the membranes of these particles. Their function may be to control the availability of amino acids for adult development and the osmotic balance within the pupal system. Thus, towards the end of pupal life, the storage proteins are released in soluble form and the amino acids therefrom are utilized for the synthesis of the specific proteins of the adult (amino acid-activating enzymes increase in activity in late pupal life; Howells & Birt, 1964). This process produces a pronounced decrease in the soluble protein as the adult matures. A similar sequence of changes (rise, fall, rise and fall) in the amount of soluble protein in the haemolymph of the moth, *Samia cynthia*, during its transformation from larva to adult, has been described by Laufer (1960).

The changes that occur in the distribution of protein during pupal life are in general agreement with those reported by Evans (1932) for *L. sericata*; the difference at the prepupal stage probably results from the removal of the larvae of *L. sericata* from their food supply while they were still capable

of feeding. Such treatment amounts to starvation, and in *L. cuprina* resulted in a fourfold decline in the free amino acid concentration (Lennie & Birt, 1965).

Activities of cytochrome c oxidase and some dehydrogenases during the life cycle of Lucilia. The change in activity of cytochrome c oxidase and succinate dehydrogenase showed the U-shaped curve typical of the changes reported for many of the enzymic activities during the metamorphosis of holometabolous insects (Agrell, 1949; Jones, 1964). A similar pattern has been reported for oxygen uptake (Finch & Birt, 1962). The very low activity for cytochrome c oxidase in the moribund flies is no doubt related to the general decrease in physiological activity due to senescence.

Glycerophosphate dehydrogenase was not detected in larval tissues; it appeared initially in particles early in pupal life and probably represents the very early formation of thoracic tissues. This enzyme is very active in adult flies, having a special role in the catabolic cycles that supply the large amounts of chemical energy necessary for flight (Sacktor, 1965, p. 505).

In the larval tissues, distribution of enzymic activity within the fractions is skewed towards the fractions containing the smaller particles. In the adult the distribution is skewed towards the fraction containing the larger particles, reflecting the appearance of the large flight-muscle mitochondria in the adult.

Relative rates of development of respiratory enzymes in the sarcosomes. The development of enzymic activity during the metamorphosis of the locust

Table 6. *Activities of respiratory enzymes relative to that of glycerophosphate dehydrogenase during the development of the thorax*

The values are expressed as the ratio of the activity of each enzyme to that of glycerophosphate dehydrogenase taken as unity.

Enzyme	Relative activity		
	Age ½ day before emergence	1 day after emergence	8 days after emergence
Glycerophosphate dehydrogenase	(1.00)	(1.00)	(1.00)
Succinate dehydrogenase	0.14	0.09	0.10
NAD-malate dehydrogenase			
Total	0.58	0.30	0.23
Particulate	0.33	0.23	0.20
'Soluble'	0.25	0.07	0.03
NAD-isocitrate dehydrogenase			
Total	0.57	0.62	0.91
Particulate	0.45	0.55	0.75
'Soluble'	0.12	0.07	0.16
NADP-isocitrate dehydrogenase	0.18	0.08	0.05
Cytochrome c oxidase	0.47	0.36	0.23

appears to involve the almost synchronous formation of groups of enzymes, the so-called 'constant-proportion groups' of Brosemer *et al.* (1963). When the activities of the enzymes of *Lucilia* are compared with the activity of glycerophosphate dehydrogenase at each stage of development (Table 6) it is seen that certain of the enzymes form a similar group. On this basis, succinate dehydrogenase, three of the NAD-dependent dehydrogenases (the malate dehydrogenase and isocitrate dehydrogenase associated with particles and the 'soluble' isocitrate dehydrogenase) and possibly cytochrome *c* oxidase appear to develop synchronously. (The decline in the ratio of cytochrome *c* oxidase may be related to the fragility of the mature sarcosomes with resulting loss in activity for this enzyme.) Thus in the fly and locust, developing under uniform external conditions, it is possible to detect 'constant-proportion groups' of respiratory enzymes. However, it is apparent that the components of such groups are not necessarily identical during the formation of the flight-muscle mitochondria of all insects, as Herold & Borei (1963) have reported that cytochromes *a* and *c* are synthesized at appreciably different rates during the development of the thoracic mitochondria of the adult bee. Moreover, in other organisms that can survive considerable variations in their external environment (e.g. yeast; Polakis & Bartley, 1965; Polakis, Bartley & Meek, 1965), the activities of many respiratory enzymes may alter in isolation or in different groupings.

Specific activities of dehydrogenases during adult development. During development the specific activity of glycerophosphate dehydrogenase increased particularly in the large particle, the highest value obtained being 1530 $\mu\text{l. of oxygen/hr./mg. of protein}$. From the protein content and the specific activities of the small-particle and large-particle fractions (Fig. 3 and Table 4) it was calculated that the increase in the specific activity of the entire mitochondrial fraction between emergence and day 8 is about 25% of the value for emergent flies. As this compares very closely with the increase (26%) reported by Van den Bergh (1962) for the Q_{O_2} ($\mu\text{l. of oxygen/hr./mg. of protein}$) of sarcosomes isolated from 1-day-old and 8-day-old *Musca*, it seems likely that the same pattern of mitochondrial development occurs in both these flies. The high activity of this enzyme gives further support for the important role of glycerophosphate as a source of energy in *Lucilia*.

Work with sarcosomes of *Locusta* led Gobell & Klingenberg (1964) to regard NAD-isocitrate dehydrogenase as 'the regulatory enzyme' of the tricarboxylic acid cycle. The high values for the specific activity of this enzyme and its dependence in experiments *in vitro* on the concentration of

ADP makes it likely that it is important in the control of the tricarboxylic acid cycle in *Lucilia* also. Thus it is clear that inactivity of isocitrate dehydrogenase cannot be considered as an impediment to the operation of the tricarboxylic acid cycle (Sacktor, 1965, p. 545); moreover, the accumulation of citrate in flight-muscle may be related to the control of the enzyme by the concentration of ADP, rather than to an inability to utilize isocitrate.

The low values for the specific activity of succinate dehydrogenase may be a reflection of an inactivation of this enzyme either from the action of phospholipase A or because of the loss of an essential phospholipid during preparation (Abdulla & Davison, 1965). The activity was not increased by treatment with ultrasonics, but, as the mitochondria were suspended in a medium containing 0.1M-phosphate, which, even at 1mM, disrupts mitochondrial structure (Bartley & Enser, 1964), these experiments do not contradict Van den Bergh's (1962) suggestion of a permeability barrier in the sarcosomes.

Development of the flight-muscle sarcosomes. The results described in the present paper may be used to formulate a scheme for the development of the thoracic mitochondria, though not for their ultimate origin. The description of this scheme is based on a diagrammatic representation of the appearance of 'respiratory' and 'non-respiratory' protein in a single particle (Fig. 5). The 'respiratory' protein includes the primary dehydrogenases and proteins of the respiratory chain, whereas the 'non-respiratory' protein will be predominantly 'structural' protein (Criddle, Bock, Green & Tisdale, 1962; Gilby, 1964) together with enzymes not concerned directly with primary redox reactions.

An increase in the 'respiratory' protein takes place throughout development of the sarcosome and is almost linear. The amount of 'non-respiratory' protein present initially is very small and this component decreases as a proportion of the particle protein during the first stage of enlargement (stage A). Thus the specific activity of 'respiratory' enzymes in the particles increases. In this stage the particle may be similar in appearance to the particles almost devoid of internal structure detected by Herold (1965). The next stage (B) is characterized by a rapid increase in the amount of 'non-respiratory' protein so that the specific activity of the 'respiratory' enzymes of the particle declines rapidly. In the final stage (C) the rate of formation of 'non-respiratory' protein decreases considerably so that the specific activity of the 'respiratory' enzyme again increases. The curve describing the changes in the total protein reflects the increasing mass of the particle.

The diagram may also be used to explain the changes in specific activity of the respiratory

Fig. 5. Proposed scheme for the development of sarcosomes. The 'respiratory' protein (curve 1) includes the primary dehydrogenases and protein of the respiratory chain, and the 'non-respiratory' protein (curve 2) will be predominantly 'structural' protein (Criddle *et al.* 1962; Gilby, 1964) together with enzymes not concerned directly with primary redox reactions. Curve 3 represents the total protein of the particle. The developmental period is divided into three stages, the limits of which are determined by the rate of formation of the 'non-respiratory' protein. The point at which a growing particle reaches a diameter of 1μ is indicated by the arrow.

enzymes of the sarcosomal fractions isolated at various times in the life cycle. Thus about 1 day before emergence the small-particle fraction will contain sarcosomes in stage A and stage B up to a diameter of 1μ ; the distribution of particle diameters in this fraction is skewed towards a value less than 0.5μ . After emergence (0–1 day) the mode of the distribution shifts to the right and the specific activity of the fraction increases somewhat (about 50%). Further development does not alter the composition of this fraction appreciably.

The small number of large particles (greater than 1μ in diameter) present before emergence are in stage B and consequently the respiratory enzymes are of lower specific activity than those in the smaller particles. Just after emergence this fraction contains sarcosomes in stages B and C with a corresponding increase in the specific activity of these enzymes. This increase continues at least until day 8, when most of the larger particles will have a composition represented on the right-hand side of the diagram. During this period, phase-contrast microscopy showed a considerable increase in the number of very large mitochondria ($5\text{--}7\mu$ in

diameter). Electron micrographs of thoracic sections of 1-day-old flies (R. W. Lennie, D. W. Gregory & L. M. Birt, unpublished work) contained a number of multi-lobed mitochondria with membranes stretching across part of the junction of the lobes. Thus it seems possible that the formation of the giant sarcosomes of the mature fly involves a process of fusion of smaller mitochondria, as suggested for sarcosomal enlargement in the silkworm (Michejda, 1963) and the bee (Herold, 1965). The physiological advantage of the formation of very large mitochondria, which can surround bundles of muscle filaments (Smith, 1962), may lie in achieving the maximum contact between mitochondrial surface and the muscle fibres (cf. the 'ATP-ADP shuttle'; Watanabe & Williams, 1953) with the minimum requirement for external mitochondrial membrane, and in decreasing the number of barriers to the movement of substrates within the mitochondrial mass.

Some further considerations may be added. First, this scheme is consistent with data indicating that all the NAD of the mitochondrion is present at an early stage in its development (at or before the

beginning of stage A) and the suggestion that there is a continued formation of the smaller particles in the adult fly (Birt, 1966). Secondly, about a day before emergence (i.e. just before the rapid increase in non-enzymic protein) there is a rapid increase in the amount of DNA in the small-particle fraction, which also contains considerable RNA (R. W. Lennie, unpublished work).

These data, and the evidence for mitochondrial synthesis of structural protein (Bücher, 1965; Beattie, Basford & Koritz, 1966), suggest that in the blowfly the synthesis of this protein, at least, is directed by sarcosomal DNA. That such a development should occur during the period of emergence may be related to the need to assemble the respiratory potential of the developing particles into a functional form capable of coupling oxygen uptake with the phosphorylation processes essential for adult flight.

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