MITOCHONDRIA IN THE FLIGHT MUSCLES OF INSECTS

III. MITOCHONDRIAL CYTOCHROME c IN RELATION TO THE AGING AND WING BEAT FREQUENCY OF FLIES

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In the historic paper which described the rediscovery of "Cytochrome, a respiratory pigment common to animals, yeasts and higher plants," Keilin (1925, page 323) summarized in the following words the status of the cytochrome system in the flight muscles of insects:

"Among all organisms examined the highest concentration of cytochrome is found in the thoracic muscles of flying insects. This has undoubtedly a connection with the peculiar activity of these muscles. We know from the old experiments of Marey (1874) that the wing muscles of insects are capable of producing very rapid contractions. . . . This peculiar property of insect muscle explains the presence in the fibrils of so high a concentration of cytochrome. . . .During the metamorphosis the concentration of cytochrome in the freshly formed thoracic muscles of the pupa increases with its development. The adult insect, however, does not contain the maximum amount of the pigment immediately after hatching; this is reached during the life of the imago, and undoubtedly depends upon the amount and composition of the food supply."

In the thirty years which have elapsed since the above was written, studies of cytochrome and of insect flight have proceeded separately, with but little further attention to the correlation between cytochrome concentration and the ability to fly. Recently, however, the matter has acquired renewed interest by virtue of the discovery that the cytochromes are localized within the muscle fibers in microscopic bodies termed "sarcosomes." The latter, as first demonstrated by Watanabe and Williams (1951), correspond to the muscle mitochondria; indeed, blowfly sarcosomes were apparently the first mitochondria to be isolated from any type of muscle and identified as such. In the case of the flight muscles of Diptera and Hymenoptera the exceptional size of the sarcosomes (up to 4 μ in diameter) has justified the use of the term "giant mitochondria."

Watanabe and Williams (1951) studied the absorption spectrum of isolated blowfly sarcosomes and noted the presence of the α-absorption bands of reduced cytochromes

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$a + a^3$, b, and c. Enzymatic activities attributable to cytochrome oxidase and catalase were measured manometrically in blowfly sarcosomes, and found to vary in a systematic manner during the 1st week of adult life. Subsequently, the structure, function, and chemistry of the sarcosomes of Diptera have been studied in further detail by Watanabe and Williams (1953), Sacktor (1953 *a*, 1953 *b*, 1954, 1955), Lewis and Slater (1954), Slater and Lewis (1954), and Chapman (1954).

These recent developments direct attention to Keilin's quotation mentioned at the outset, and suggest a more or less intimate relation between cytochromes, sarcosomes, aging, and flight ability. In the present study, such a correlation has been investigated in experiments performed on the blowfly *Phormia regina* Meigen.

Materials and Methods

1. Experimental Animals.—Blowflies were reared at a constant temperature of 25°C. according to the method of Hill, Bell, and Chadwick (1947). Freshly emerged adults were isolated at frequent intervals and stored at 25°C. in cages containing water and granulated sucrose. In experiments performed on flies less than 48 hours old, the insects' age was determined to ±0.5 hour; after the 48th hour, their age was accurate to ±2 hours.

2. Preparation of Sarcosomes.—The sarcosomes were isolated by a method modified from that of Watanabe and Williams (1951). 50 to 150 flies were anesthetized with carbon dioxide and placed in a beaker surrounded by ice. The head, abdomen, and wings were excised and the thoraces collected in a chilled mortar containing cold 0.2 M sucrose. After gentle trituration for about 2 minutes, the resulting suspension of cells and tissues was filtered by gentle suction through a sintered glass filter whose surface had been covered by four layers of fine muslin moistened with the sucrose solution. The filter's average pore size was about 20 μ. The filtrate was centrifued in the cold at *ca.* 400 $\times$ g for 3 minutes. After removal of the supernatant, the sedimented sarcosomes were resuspended in cold sucrose and collected by centrifugation at 800 to 900 $\times$ g for 3 minutes. Microscopic examination of the final sediment showed the presence of well preserved sarcosomes, and a negligibly small number of nuclei.

3. Estimation of Cytochrome c.—The turbidity of flight muscle and of isolated sarcosomes precludes the usual types of direct spectrophotometric measurements. Consequently, the spectroscopic method described by Keilin and Hartree (1946) and Keilin and Wang (1946) was utilized for measuring the concentration of cytochrome *c*. In brief, a Zeiss microspectroscope was mounted above a Ramsden ocular on the barrel of a microscope lacking objective lenses. The preparation to be examined was placed on the stage of the microscope and transilluminated by a light of adjustable intensity. By means of its comparison prism the microspectroscope presented in the same field of view the spectrum of a solution of reduced cytochrome *c*; the latter was contained in a wedge-shaped trough and also transilluminated by a light of adjustable intensity. In practice, with the preparation and trough in position, the intensities of the light sources were first adjusted until the two spectra showed equal background intensities in the region adjacent to 550 mμ. The position of the wedge-shaped trough

was then adjusted until the intensities of the 550 mμ bands of reduced cytochrome c were equal in the experimental and reference spectra. The concentration of cytochrome c could then be calculated in terms of the known concentration and optical depth of the reference standard solution.

(a) *Studies on Whole Muscle.*—A plastic ring of accurately determined depth and diameter (2 and 4 mm., respectively), and two small glass coverslips were weighed to the nearest 0.02 mg. The flight muscles were dissected from 5 to 8 blowflies and placed in the ring resting on one of the coverslips. 3 or 4 crystals of sodium hydrosulfite were then added, and the muscle cut up and evenly distributed with a microscalpel. The second coverslip was then pressed firmly onto the ring to form an optical cell, the surplus muscle which squeezed out in this procedure being carefully removed. The wet weight of the muscle was determined by a second weighing, following which the preparation was ready for spectroscopic study.

(b) *Isolated Sarcosomes.*—The cytochrome c concentration in isolated sarcosomes was determined in the same way as for whole muscle, except that a commercially available optical cell equipped with a glass plunger was utilized. In this manner the observations were performed on a precise depth of sarcosome suspension.

(c) *Cytochrome c Standard.*—Lyophilized cytochrome c (Sigma) was dissolved in 0.1 M disodium phosphate and boiled to precipitate protein impurities. After centrifugation, the supernatant solution was reduced with a few crystals of sodium hydrosulfite, placed in one compartment of the double wedge trough, and covered with a layer of mineral oil. The other compartment of the trough was filled with distilled water. Standardization of the cytochrome c was performed according to the procedure of Potter (1949).

Experimental Results

1. Calibration of the Isolation Procedure

By the procedure described in the preceding section, the sarcosomes isolated from 100 flies were rapidly resuspended in ice cold distilled water[1] and collected by centrifugation. The washing was repeated and the dry weight of the final pellet determined. The residual tissue which remained on the funnel was rinsed with 0.2 M sucrose into the original mortar and briefly reground. The filtration and washing procedure was then repeated and the dry weight of the recovered sarcosomes determined. The residual material was reworked in the same manner two further times until, after the fourth filtration, the scanty material recovered from the funnel showed only an occasional sarcosome on microscopic examination.

The above procedure was performed on two batches of flies. In the first of

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[1] Distilled water is known to have a deleterious effect, resulting in lysis, on isolated sarcosomes (Watanabe and Williams, 1953). We have endeavored to minimize such effects by maintaining aqueous sarcosome suspensions at 0°C. and working as rapidly as possible. It is nevertheless possible that an unknown fraction of the intrasarcosomal contents was lost during the washing procedure, and hence the true sarcosomal dry weight may be somewhat higher than that recorded.

these the initial filtration recovered 86 per cent by weight of the sarcosomes, in the second experiment, 92 per cent. Consequently, we conclude that the isolation of the sarcosomes was approximately 90 per cent effective. All pertinent measurements reported below have been corrected accordingly.

2. Number of Sarcosomes per Fly

Sarcosomes were isolated from 50 to 100 flies of known age, and resuspended in 0.2 M sucrose solution, the number of milliliters corresponding to the number of flies employed. After thorough mixing of the suspension, an accurately measured aliquot (5.4 μl.) was removed and pipetted onto the center of a microscope slide. Adapting the procedure of Harman (1950), a droplet of aqueous 10 per cent nigrosin solution was added and spread as evenly as pos-

TABLE I

Number of Blowfly Flight Muscle Sarcosomes as a Function of Adult Age

Age of flies	No. of sarcosomes per fly $\times 10^8$
2–3 hrs.	5.1
12 "	7.2
1 day	8.5
2 days	6.0
3 "	6.4
4 "	5.4
6 "	8.1
10 "	6.5
15 "	5.9
18 "	7.5
	Mean = 6.7 ± 1.1 (S.D.)

sible over an area of 4 cm². The slide was then rapidly heat-dried. Under the "high-dry" lens system, each sarcosome appeared as a bright spot on a dark blue-gray background. By means of a calibrated Whipple disc, the number of sarcosomes was counted in at least 30 randomly selected fields on each slide. Three slides were regularly prepared and examined from each sarcosomal preparation. The isolations and measurements were performed in duplicate on flies at each of a series of ages, and the six determinations at each age were then averaged.

The results summarized in Table I describe the number of sarcosomes in the flight muscles of individual blowflies as a function of age. The data are consistent with the view that each fly contains in its thoracic flight muscles about 7.0 $\times 10^8$ sarcosomes, and that this number is independent of age.

3. Dry Weight of Sarcosomes

The dry weights of aliquots of the sarcosome suspensions employed in the preceding section were studied as a function of adult age. For this purpose the washed sarcosomes were suspended in 0.1 to 0.2 ml. of distilled water, transferred quantitatively to a tared weighing bottle, and dried to constant weight at 110°C.

The dry weight of the sarcosomes per fly is recorded in Fig. 1 as a function of adult age, the assumption having been made that each fly contains 6.7×10^8 sarcosomes. Apparently, a threefold increase in dry weight occurs during the 1st week of adult life. A "plateau" is then reached, and no further change is evident thereafter.

4. Cytochrome c in Whole Flight Muscle

By the use of the spectroscopic technique described above under Methods, the cytochrome c content of entire blowfly flight muscle was examined as a function of age. As recorded in Fig. 2, the cytochrome c content of the muscle was found to double during the 1st week of adult life, and then to remain constant. From the time of emergence to the end of the 1st week, the cytochrome c content increased from 3.2 mg. to 6.4 mg. per gm. dry muscle. In the data recorded in Fig. 2, the muscle has been assumed to contain 72 per cent water at all stages. Actually, this figure is strictly correct only for mature flies; i.e., those aged 1 week or more from the time of emergence. Since the muscles of younger flies contain a somewhat higher concentration of water, the rate of increase in cytochrome c would be slightly greater on a wet weight basis.

During the 1st week of adult life the intensities of the α-absorption bands of all the cytochromes increase markedly, and apparently in the same ratio to one another. The estimations were in each case based on comparisons with the flight muscle cytochrome c band, which was arbitrarily assigned a value of unity. The ratios of the observed intensities were:

$$a + a_3 : b : c \quad \text{as} \quad 0.3 : 0.5 : 1.0$$

The error in these determinations is rather large (about ± 20 per cent).

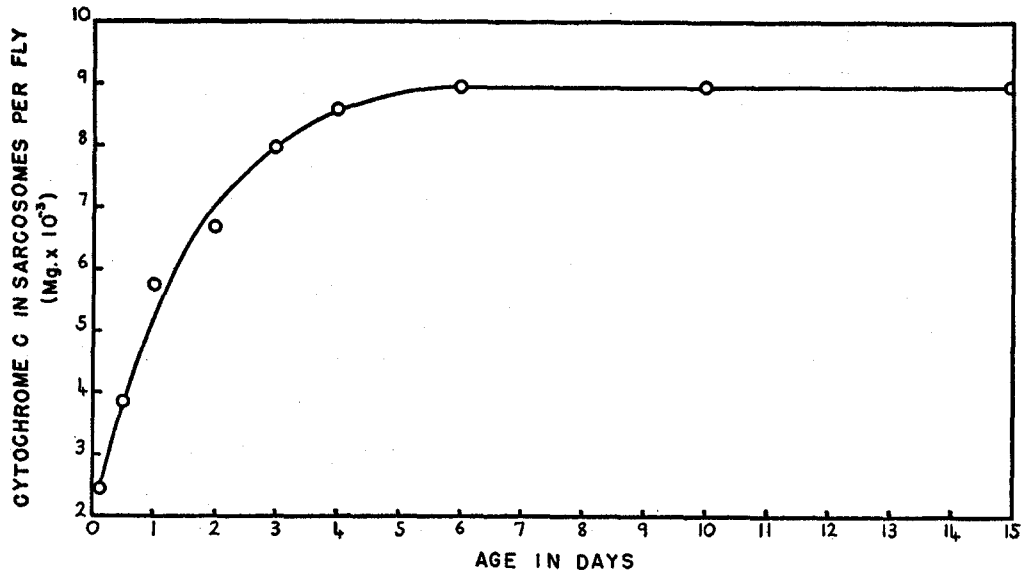### 5. Cytochrome c in Isolated Sarcosomes

Isolated sarcosomes are reddish pink in color (Watanabe and Williams, 1951), an observation which immediately suggests the possible presence of a high titer of cytochrome. This suggestion was confirmed by cytochrome c determinations on isolated blowfly sarcosomes. The relatively low titer in newly emerged flies was found to increase rapidly during the 1st week of adult life (Fig. 3). Sarcosomes in a single mature fly contain a total of about 9.0 μg. cytochrome c, corresponding to ca. 1.3×10^{-8} μg. cytochrome c per sarcosome.
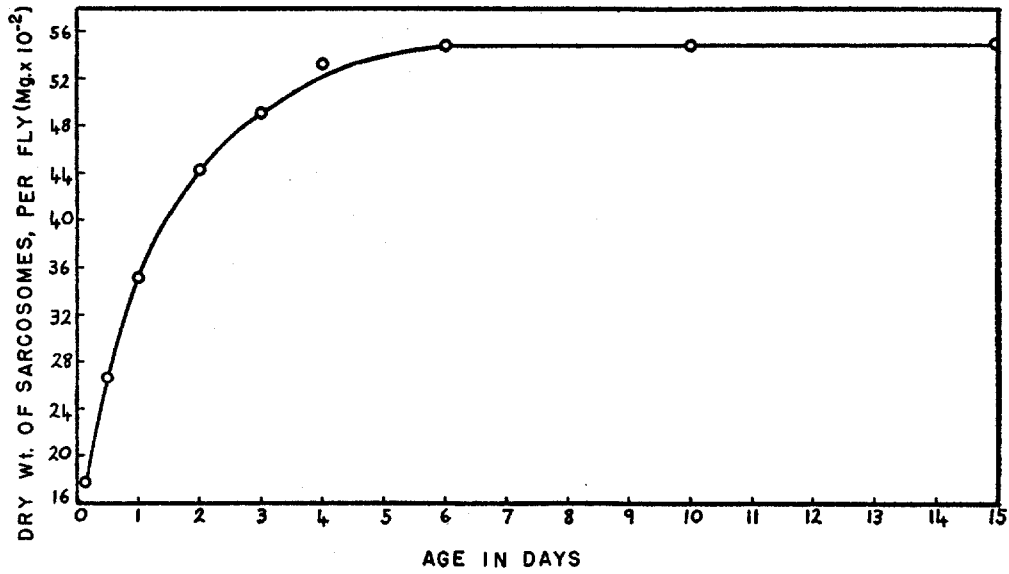
TEXT-FIG. 1. The dry weight of *Phormia* sarcomes as a function of fly age. Each point is the mean of two duplicate determinations, and is based on the estimated value of 6.7×10^8 sarcosomes per fly.
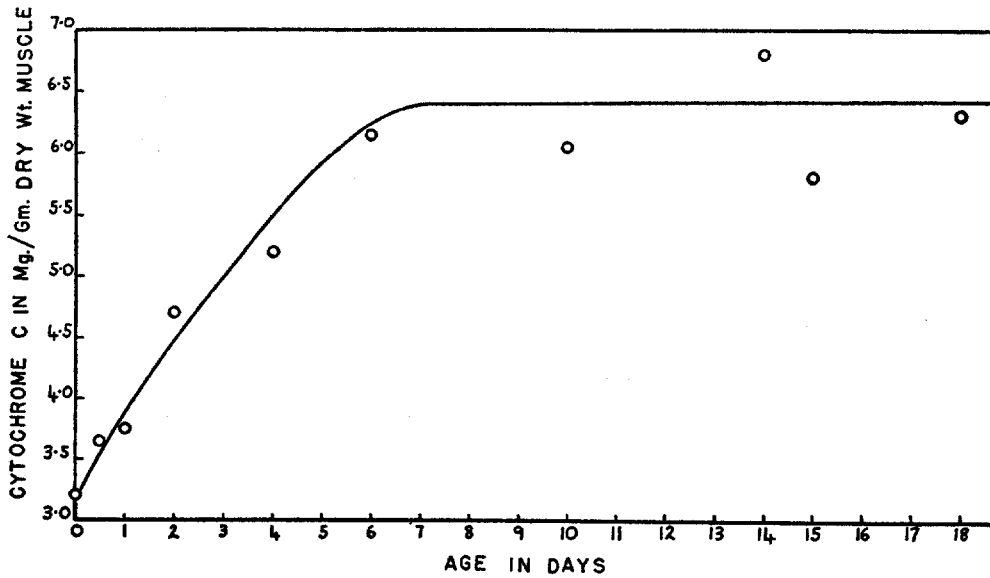
TEXT-FIG. 2. Cytochrome *c* titer of *Phormia* flight muscle as a function of fly age. Each point is the mean of two duplicate determinations.

TEXT-FIG. 3. Cytochrome c titer of isolated *Phormia* sarcosomes as a function of fly age. Each point is the mean of two duplicate determinations.
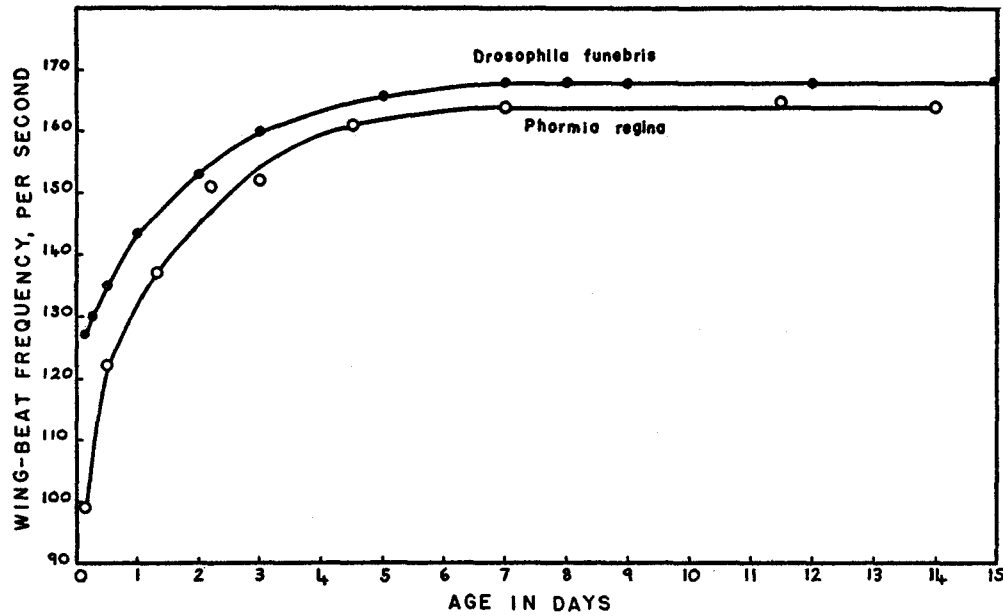
TEXT-FIG. 4. The wing-beat frequency of *Drosophila funebris* (from unpublished data of Chadwick and Williams) and of *Phormia* as a function of fly age. Each point is the mean of 10 to 20 determinations on each of at least 10 flies.

A comparison of these values with those recorded in the preceding section recalculated on the basis of the flight muscle dry weight (approximately 1.7 mg.) per fly, indicates that all the cytochrome of the flight muscle is localized exclusively in the sarcosomes. In further confirmation of this fact we can state that flight muscle fibrils, after having been freed of sarcosomes by repeated washings, showed no indication of cytochromes when examined spectroscopically.

During the 1st week of adult life both the dry weight and the cytochrome c content of the sarcosomes increase markedly and at rates which, within the limits of experimental error, are indistinguishable (see Fig. 5). This means, as is indicated in Table II, that cytochrome c makes up an unchanging proportion

TABLE II

Cytochrome c Content of Blowfly Sarcosomes as a Function of Adult Age

Age of flies	Cytochrome c, in per cent of dry weight of a single sarcosome
2-3 hrs.	1.39*
12 "	1.48*
1 day	1.63
2 days	1.58
3 "	1.64
4 "	1.57
6 "	1.62
10 "	1.69
15 "	1.64
	Mean = 1.58 ± 0.10 (s.d.)

* Due to their fragility and to other causes, cytochrome c analyses on very young sarcosomes are somewhat less accurate than for older ones.

($ca.$ 1.6 per cent) of the dry weight of the sarcosomes at all stages. Consequently, the absolute increase in sarcosomal cytochrome c during the 1st week may be regarded as a growth phenomenon of the sarcosome as a whole. This same conclusion evidently applies also to cytochrome $a + a_3$ and b, the concentrations of which at all stages show a constant ratio to that of cytochrome c.

DISCUSSION

1. Sarcosome Content of Flight Muscle

The data of Watanabe and Williams (1951) indicate that mature blowfly sarcosomes account for 50 per cent of the total flight muscle protein. The flight muscles of a mature fly weigh $ca.$ 6.0 mg. wet weight, and contain, on the average, 6.7×10^8 sarcosomes. Hence, each milligram of wet weight of muscle contains about 1.1×10^8 sarcosomes. This value is substantially the same as

the number of mitochondria (0.87×10^8) per mg. wet weight of mouse liver (Shelton, Schneider, and Striebich, 1953). However, the insect sarcosomes are much larger than the mitochondria of liver, and the displacement of the sarcosomes in flight muscle is therefore considerably greater than that of liver mitochondria. The sarcosomes have an average volume of about 4.2 μ^3, whereas liver mitochondria, as rods 2 μ long and 0.5 μ in diameter, have a volume of only 0.4 μ^3 (Claude, 1949). The dry weight of mature flight muscle is about 28 per cent of the wet weight, and the dry weight of a single mature sarcosome is *ca.* 8.5×10^{-7} μg. It may therefore be calculated that the sarcosomes make up about one-third of the muscle mass, the experimentally determined value being 32.6 per cent on a dry weight basis. Since the dry weight of a single sarcosome is about 21 per cent of its wet weight, it follows that on a wet weight basis the sarcosomes make up about 40 per cent of the total muscle mass.

2. Effect of Aging on Sarcosomes

Save for the maturation of the gonads it is a generally accepted view that the tissues of adult insects do not grow. Notwithstanding this fact, it is increasingly evident that both morphological and physiological maturation continue for several days after emergence of the fully formed insect. Watanabe and Williams (1953) reported that, during the 1st week of adult life, sarcosomes of *Drosophila* and *Phormia* approximately double in diameter. We now see that this aging of blowfly sarcosomes is accompanied by a threefold increase in dry weight and in cytochrome content. The number of sarcosomes, however, appears to remain constant and independent of adult age, evidently being determined during the period of muscle histogenesis toward the end of the pupal-adult transformation (*cf.* Agrell, 1949; Bodenstein, 1950).

During aging the different cytochrome components increase in constant ratio to one another, and make up a constant proportion of the sarcosomal dry weight. This is contrary to the finding of Watanabe and Williams (1951) that the cytochrome oxidase titer of blowfly sarcosomes, which presumably corresponds to the intensity of the α-absorption band of cytochromes $a + a_3$ (Keilin and Hartree, 1939), decreases markedly during the 1st week of adult life. This discrepancy is perhaps attributable to the fact that the manometric measurements were calculated in terms of sarcosomal nitrogen, rather than sarcosomal dry weight as in the present study. Under this circumstance it would be necessary to conclude that some nitrogen-rich component of the sarcosomes increases much faster than does the sarcosomal dry weight.

3. Cytochrome Content of Flight Muscle and Sarcosomes

It has been demonstrated that the cytochrome c content of isolated sarcosomes accounts quantitatively for the amount of the pigment present in the entire flight muscle. In all probability, the distinct reddish color of the muscle

is due in large part to its high content of cytochromes. However, it is worth recalling that other red pigments can occur in insect muscle; for example, the reddish pigment that can be extracted with boiling water from the leg muscles of the migratory locust is not due to cytochrome (unpublished observations), but is possibly insectorubin (cf. Goodwin and Srisukh, 1950). Consequently, conclusions with respect to cytochrome content can scarcely be based on muscle coloration alone, as has been suggested by Sacktor, Thomas, Moser, and Bloch (1953).

The amount of cytochrome b and c in mature *Phormia* sarcosomes exceeds that in a Keilin and Hartree (1939) heart muscle homogenate, which is itself rich in cytochromes (Chance, 1952 b). The concentration of cytochrome c in mature sarcosomes is close to 16.0 μg. per mg. dry weight, which is about ten times the concentration reported to be present in mouse liver mitochondria (Shelton *et al.*, 1953). By making certain likely assumptions with respect to molecular weight, (Chance (1952 b) has determined that in mature blowfly sarcosomes cytochromes a_3, a, b, and c are present in the molar ratios of 0.29:0.48:1.06:1.0.

4. Flight Muscle Cytochrome c in Relation to Fly Body Weight

It is known that other things being equal, a heavier insect requires a greater expenditure of energy per unit mass to sustain flight than does a lighter one. For example, the relative increase in O_2 consumption during flight of a comparatively large insect such as *Lucilia sericata* (Davis and Fraenkel, 1940) is far greater than that of a small one such as *Drosophila* (Chadwick and Gilmour, 1940). Examination of the flight muscles of *Drosophila* (body weight 1 to 3 mg.), *Phormia regina* (body weight *ca*. 34 mg.), and *Sarcophaga* (body weight 100 to 110 mg.) reveals that the average diameter of their sarcosomes is about the same; viz., 2 μ. We have also determined that in these three species the flight muscle mass makes up a constant proportion (18 to 20 per cent) of the total body weight. If it is assumed that the sarcosomes all have the same cytochrome c content, and account for the same proportion of the muscle mass as in *Phormia*, then it may be calculated that the flight muscle cytochrome c (in milligrams) equals $(2.5 \times 10^{-4}) \times$ fly body weight (in milligrams). It therefore follows that, since the flight muscle cytochrome c is directly proportional to body weight, whereas the increase in flight muscle Q_{O_2} during flight (relative to unit mass) increases with body weight, then either the cytochrome c is functionally more active in the larger insect, or, cytochrome c cannot be the limiting factor in flight muscle respiration. Evidently, the same conclusion also applies to the other components of the cytochrome system.

5. Wing-Beat Frequency in Relation to Aging

The effects of a variety of factors that influence the wing-beat frequency of insects have been admirably reviewed by Chadwick (1953). Data for a number

of genera show that the wing-beat frequency of young adult insects is lower than that of older ones. We have investigated this aging effect in *Phormia* (Figs. 4 and 5), and have included for comparison unpublished data of Chadwick and Williams on *Drosophila funebris*, in which wing-beat frequency was measured stroboscopically during very brief flights.

The wing-beat frequency of *Phormia* at different ages was determined with the kind assistance of Professor K. Roeder of Tufts University, who adapted an improved technique over his previously published method (Roeder, 1951). As shown in Fig. 4, the wing-beat frequency of *Phormia* is minimal at less than 100 beats per second at the time of emergence, and increases at a progressively decreasing rate to a maximum of 164 beats per second by the 7th day. The increase in wing-beat frequency for D. *funebris* follows a similar time sequence, although the magnitude of the change is not as great as for *Phormia*.

The rate-limiting physiological processes which determine the wing-beat frequency during aging are open to speculation. The duration of sustained flight in *Drosophila* depends upon the glycogen reserves, and these attain a maximum 4 to 5 days after emergence (Williams, Barness, and Sawyer, 1943; Wigglesworth, 1949). In the house-fly, cytochrome oxidase (Sacktor, 1950) and choline esterase (Babers and Pratt, 1950) activities become maximal after 3 and 5 days, respectively. Lewis and Slater (1954) found that isolated sarcosomes of the blowfly, *Calliphora erythrocephala*, had submaximal oxidative phosphorylation activity (*i.e.* low P:O ratios) if the flies were younger than 1 week after emergence. These changes, together with those described in the present study, undoubtedly may influence the increase in wing-beat frequency during aging. But whether this increase is dependent upon these changes, as opposed to inducing them, is as yet unknown.

6. Wing-Beat Frequency and Cytochrome c Turnover

The rate of muscular contraction responsible for the wing-beat frequency of insects, particularly of the smaller flies, is the most rapid mechanical motion known to occur in nature. The question may be asked: What is the rate of such biochemical reactions as can be correlated with the wing-beat frequency? One such reaction is the "turnover number" of sarcosomal cytochrome c. For the purpose of the present discussion, the turnover number may be defined as the number of times a cytochrome c molecule has to be alternately oxidized and reduced, per minute, to account for the oxygen uptake of a tissue in terms of the O_2 equivalence of the cytochrome c iron.

Chance (1952 a) has determined this turnover number for a "steady-state" oxidized sarcosome preparation *in vitro* to be about 300. The Q_{O_2} (succinate) of this preparation was 77.3 μl. O_2/mg. dry weight/hr. (Chance, 1952 b, and personal communication), which is low in comparison with the corresponding Q_{O_2} of 180 obtained by Watanabe and Williams (1951) for similar preparations. We may state that we have confirmed the latter value, our best prepara-

tions having a Q_{O_2} of the order of 200. If this latter figure be employed for the calculation, the cytochrome c turnover number for isolated sarcosomes is raised to about 780. However, even this value is far short of the turnover number which may be calculated for the sarcosomal cytochrome c in the intact flying insect. Thus, as described in the Appendix, the turnover number for *Drosophila virilis* flying at a wing-beat frequency of 10,000 cycles per minute, is close to 5,100. Hence, as a first approximation, it appears that the flight muscle cytochrome c turns over once for every two wing-beat cycles.
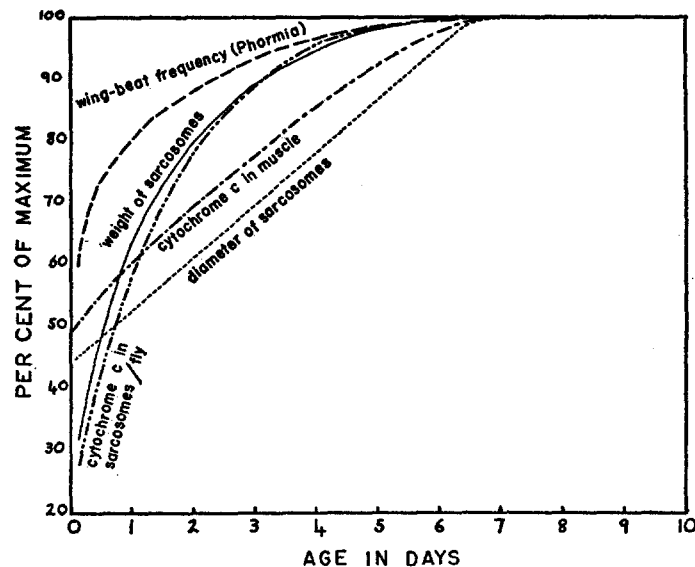
Text-Fig. 5. Summary of changes occurring during aging of *Phormia* recalculated onto common coordinates. Diameter of sarcosomes (in micra) taken from the data of Watanabe and Williams (1953) for *Drosophila funebris*. Sarcosomal dry weight, cytochrome c, and muscle cytochrome c calculated in mg. $\times$ 10^{-2}, mg. $\times$ 10^{-3} and mg. $\times$ 10^{-1}, respectively, per fly.

The approximately seventeenfold difference between Chance's (1952 a) experimentally determined value *in vitro* and that calculated *in vivo*, is too large to be attributable to experimental error, and requires an explanation. It is clear that the major reason for the discrepancy lies in the difference between the *in vitro* Q_{O_2} of 77 and the calculated *in vivo* value of about 2,000. The influence of three factors, each of which could possibly depress the *in vitro* Q_{O_2}, may be considered.

1. Absence of a suitable phosphate acceptor system. This possibility could well result in a lowered sarcosomal Q_{O_2} with many oxidizable substrates; the increase in sarcosomal O_2 consumption in the presence of 2,4-dinitrophenol

(Slater and Lewis, 1954), (which uncouples respiration from phosphorylation) was three- to fourfold.

2. Nature of the respiratory substrate. There is no reason to assume that succinate is a principal respiratory substrate *in vivo*, although it was employed in the *in vitro* systems. Sacktor (1955) has shown that a variety of probably "more physiological" substrates are oxidized considerably more rapidly by isolated sarcosomes than is succinate. Furthermore, the additional presence of the extrasarcosomal portion of the flight muscle, the respiration of which is mediated through the sarcosomal cytochrome system, resulted in up to a fivefold increase in O_2 consumption.

3. Unsatisfactory experimental technique. Under this heading we may include: damage sustained by the sarcosomes during the course of their isolation; the nature of the suspending medium; various changes undergone by the sarcosomes due to the unphysiological nature of the artificial environment. These latter factors have already been discussed by various authors (Watanabe and Williams, 1953; Sacktor, 1954; Lewis and Slater, 1954), from whose studies it is clear that these variables can have marked effects on the rate of sarcosomal respiration.

We conclude from the foregoing that, even though present knowledge does not permit a full evaluation of all the factors here involved, it appears highly probable that *in vitro* studies have so far failed to duplicate the full metabolic activity of sarcosomes in the living, flying insect.

7. Comparison of the Present Findings with Those of Keilin

The data we have presented largely confirm and considerably extend Keilin's (1925) original observations on the increase of cytochrome in blowfly flight muscle during aging. In two respects, however, our findings are not in precise agreement with those of Keilin. Thus, it was presumed by Keilin that the cytochromes were localized in the muscle *fibrils*, whereas our data indicate that the enzymes are present, perhaps exclusively, in the muscle sarcosomes. This is in agreement with current findings that the cytochrome system of vertebrate striated muscle is also localized in the sarcosomes (Paul and Sperling, 1952; Cleland and Slater, 1953). Keilin also supposed that the increase in cytochromes as a function of age was dependent upon the dietary intake. In our experience this is not so. Thus, in two experiments, groups of *Phormia* were provided with only distilled water after their emergence; 2 days later their muscle cytochrome *c* content, and their wing-beat frequency, were identical with those of control flies fed sugar and meat in addition to water. It must be concluded, therefore, that at least during the first 2 days of adult life, neither the titer of flight muscle cytochrome *c* nor the wing-beat frequency is determined by the diet, and their increase during aging is due to some as yet unknown process of maturation.

SUMMARY

1. In the present study a correlation has been sought between aging, flight muscle mitochrondria (sarcosomes), cytochrome c, and flight ability in the blow-fly, *Phormia regina*.

2. During the 1st week of adult life, individual sarcosomes increase in mass from 2.7×10^{-7} μg. dry weight at the time of emergence, to 8.5×10^{-7} μg. by the 7th day. During this period of growth, the number of sarcosomes per fly (6.7×10^8) remains constant. When mature, the sarcosomes account for 32.6 per cent of the total muscle dry weight, or close to 40 per cent on a wet weight basis.

3. It appears probable that the high content of flight muscle cytochromes is entirely localized in the sarcosomes. The cytochromes continue to be synthesized and increase in titer within the sarcosomes for 7 days after adult emergence.

4. As determined spectroscopically, the various cytochrome components at all times maintain a constant ratio both to one another and to the sarcosomal dry weight. This suggests the possibility that the cytochrome system may be synthesized as a single entity.

5. The wing-beat frequency of *Drosophila funebris* and *Phormia* varies with the age of these flies, being lowest at the time of emergence and maximum after the 6th day.

6. The relations between wing-beat frequency, respiration during flight, and sarcosomal cytochrome c content are discussed. On the basis of some likely assumptions it is calculated that the cytochrome c turnover number is over 5,000, and that the cytochrome c turns over once for every two wing-beat cycles.

APPENDIX

Derivation of the Relation between Turnover Number of Sarcosomal Cytochrome c and the Wing-Beat Frequency

In this calculation the following assumptions have been made: (1) the extra O_2 consumed by a flying insect (over and above the resting value) is utilized exclusively by the flight muscles; (2) the whole of this O_2 consumption is mediated through the sarcosomal cytochrome system; (3) there is complete oxidation and reduction of the entire sarcosomal cytochrome c; (4) the experimental data found for *Phormia* are also applicable to *Drosophila virilis*.

A flying *D. virilis* weighing 2.8 mg. consumes 550 μl. O_2/gm./min. above its resting O_2 uptake (Chadwick, 1953).

Hence, O_2 consumption per fly is

$$\frac{550\mu\text{l. } O_2 \times 2.8}{1000 \text{ mg.}} = 1.54 \; \mu\text{l. } O_2/\text{min.} \tag{1}$$

Such a fly contains close to 0.5 mg. wet weight of flight muscle, the dry weight of which is 27 per cent. Hence, if the cytochrome c content of the sarcosomes is 1.6 per

cent, and they account for 32.6 per cent of the flight muscle mass, the sarcosomal cytochrome c content per fly is

$$0.5 \text{ mg.} \times \frac{27 \text{ per cent}}{100} \times \frac{32.6 \text{ per cent}}{100} \times \frac{1.6 \text{ per cent}}{100} = 7.04 \times 10^{-4} \text{ mg.} \quad (2)$$

The iron content of cytochrome c is 0.43 per cent (Theorell and Åkesson, 1939; Keilin and Hartree, 1945), and, hence, the sarcosomal cytochrome c iron per fly equals

$$7.04 \times 10^{-4} \text{ mg.} \times \frac{0.43 \text{ per cent}}{100} = 3.02 \times 10^{-6} \text{ mg. Fe} \quad (3)$$

This is equivalent to

$$\frac{3.02 \times 10^{-6} \times 10^{-3}}{56 \text{ (atomic weight of Fe)}} = 5.4 \times 10^{-11} \text{ gm. atom Fe} \quad (4)$$

Since a single valency change of 1 gm. atom of Fe (*i.e.* the electron transfer: $Fe^{+++} \rightleftharpoons Fe^{++}$ which occurs during respiration) is equivalent to $\frac{1}{4}$ gm. mole of oxygen, or 22.4/4 liters O_2 at normal temperature and pressure, the O_2 equivalence of the sarcosomal cytochrome c iron is

$$5.4 \times 10^{-11} \text{ gm. atom} \times 10^{6} \text{ } \mu\text{l.} \times \frac{22.4}{4} = 3.02 \times 10^{-4} \text{ } \mu\text{l. } O_2 \quad (5)$$

Now, since the fly actually consumes 1.54 μl. O_2/min. (from (1)), the cytochrome c turns over

$$\frac{1.54}{3.02 \times 10^{-4}} = 5,100 \text{ times per minute.}$$

The wing-beat frequency (10,000 cycles per minute) divided by the turnover number per minute, yields the desired relationship.

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