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MITOCHONDRIA IN THE FLIGHT MUSCLES OF INSECTS

I. CHEMICAL COMPOSITION AND ENZYMATIC CONTENT

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In 1913 Warburg observed that extracts of mammalian liver showed a considerable decrease in oxygen consumption when cleared of granules by filtration or centrifugation. This was the first indication that insoluble, intracellular granules play an important role in the metabolism of living cells. Identification of the components removed in the granular fraction awaited the development of adequate techniques for isolating the various subcellular constituents. When this was accomplished by the method of differential centrifugation (Bensley and Hoerr, 1934; Hoerr, 1943; Claude, 1943, 1945), the oxygen uptake was found to be maximal in the "large granule fraction." Subsequently these large granules were shown to consist almost entirely of mitochondria (Hogeboom *et al.*, 1948; Kennedy and Lehninger, 1949; Schneider and Potter, 1949; Harman, 1950 *a*, 1950 *b*).

The metabolic activity of mitochondria has been found to depend on their high content of respiratory enzymes; these include the enzymes responsible for the complete oxidation of pyruvic acid to carbon dioxide and water (Claude, 1945; Schneider, 1946; Hogeboom *et al.*, 1946, 1948; Green *et al.*, 1948; Schneider *et al.*, 1948, 1949, 1950; Kennedy and Lehninger, 1949). Moreover, unlike the freely soluble enzymes concerned with glycolytic reactions (Le Page and Schneider, 1948), many of these oxidative enzymes are bound to the substance of the mitochondria from which their extraction is difficult or impossible. Accompanying these findings has been the recognition that the mitochondria contain a "mosaic" of enzymes whose properties are conditioned by their spatial relations within the mitochondrion itself (Green, 1949 *a*, 1949 *b*; Harman, 1950 *a*, 1950 *b*).

Thus, in the mitochondria the biochemist has encountered the first tangible basis for the organization of enzyme systems within the cytoplasm of the living cell. It is therefore not surprising that the classical problem of the mitochondria has once more become a lively contemporary subject of investigation.

Such studies have been and continue to be complicated by the extremely small size of most mitochondria whose average diameter rarely exceeds 1 μ

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(Harman, 1950 *b*). Though a variety of techniques permit one to demonstrate the mitochondria as such, their detailed structure and internal organization lie at or near the limits of resolution of most optical systems. On this account the electron microscope has recently been applied to the study of isolated mitochondria (Dalton *et al.*, 1949).

A still more direct approach to the problem seems feasible in view of the apparently universal distribution of mitochondria in animal tissues. There is no reason to suppose that the vertebrate liver and kidney are necessarily the optimal tissues for the study of mitochondria, notwithstanding the fact that most experimental work has been performed on these materials. Somewhere among the enormous assemblage of vertebrate and invertebrate species one might hope to find a tissue possessing mitochondria of exceptional size and accessibility.

From the point of view of comparative physiology, attention focusses on certain relatively large, spherical, cytoplasmic bodies termed "sarcosomes" which have long been known to occur in enormous numbers in the flight muscles of dipterous and hymenopterous insects. The possibility that the sarcosomes are, in fact, the mitochondria of this highly specialized muscular tissue has already been debated in the literature pertaining to comparative histology (Jordan, 1920; Morison, 1928).

The presence of these large sarcosomes is specifically characteristic of a unique category of insect muscle which, because of the large size of its constituent fibrils, has been designated as "fibrillar" muscle (Wigglesworth, 1939). Muscle of the fibrillar type is distinguished by an unusual organization of the individual muscle fibers. As diagrammed in Fig. 1, each fiber consists of a mass of sarcoplasm containing relatively enormous, cross-striated, longitudinal columns or fibrils. Adjacent fibrils are separated by a zone of transparent sarcoplasm in which a longitudinal row of sarcosomes is imbedded. The sarcosomes range in diameter from 1 to 4 μ.

In the apparent absence of connective tissue sheaths and sarcolemma, the muscle fiber readily separates into its constituent fibrils liberating a turbid suspension of sarcosomes. One such fresh muscle fiber, subjected to minimal teasing, is illustrated in Fig. 2. The linear arrangement of the sarcosomes between adjacent fibrils is still partially preserved.

Muscle organized in this manner and characterized by the presence of giant fibrils and sarcosomes seems to have an extremely limited distribution in animals. As previously mentioned, it is found only in insects and, even here, is encountered only in certain highly specialized muscles of the most highly evolved orders. Though adult Hemiptera and Coleoptera show thoracic muscles which approach the type under consideration, the full development of fibrillar muscles is observed only in the Diptera and Hymenoptera. Within these two orders it seems to have a general distribution, being regularly encountered in

both primitive and advanced families and species. Within the individual insect, however, fibrillar muscle occurs only in the great "indirect" thoracic muscles, which serve to move the wings during flight (Williams and Williams, 1943). Evidently the unusual structure of fibrillar muscle is functionally related to the high frequencies of wing-beat that characterize the flight of most dipterous and hymenopterous species.

Unfortunately, these morphological relationships afford no reasonable basis for deciding whether the sarcosomes are homologous to the mitochondria of

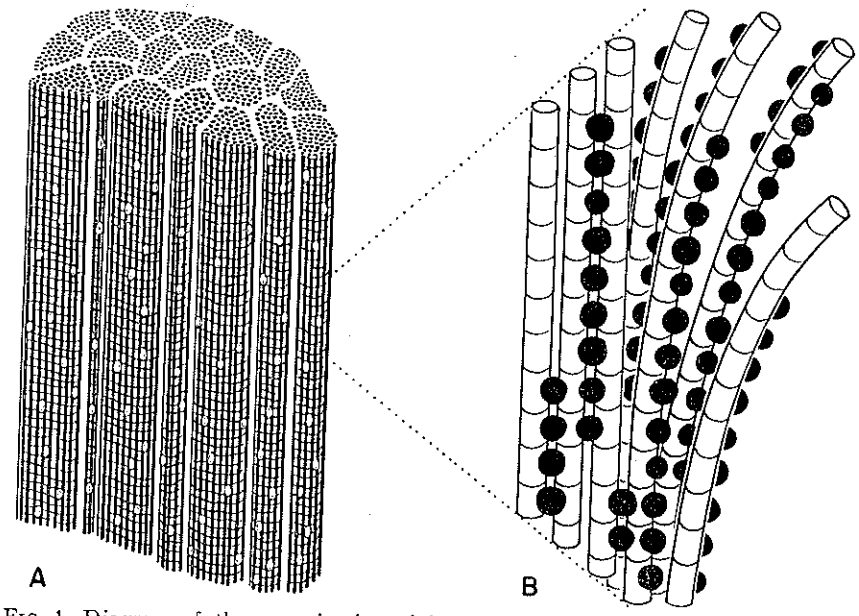

FIG. 1. Diagram of the organization of insect fibrillar muscle. A shows a small segment of a muscle mass containing twenty fibers. In B a part of a single fiber is much enlarged to show its subdivision into fibrils and sarcosomes. (Original figure by Dr. W. L. Nutting.)

other less specialized cells. As noted at the outset, however, mitochondria are now capable not only of cytological, but also of biochemical definition. Even in cytological investigations this trend has long been evident in the use of certain histochemical tests for mitochondria, such, for example, as their well known reaction with Janus green B. In addition to these empirical reactions, it now seems feasible to apply more precise characterizations of mitochondria in terms of their content of specific enzyme systems.

In the present study the sarcosomes have been considered from this point of view. By biochemical analysis of isolated sarcosomes we have sought to appraise their possible relation to the mitochondria of other tissues.

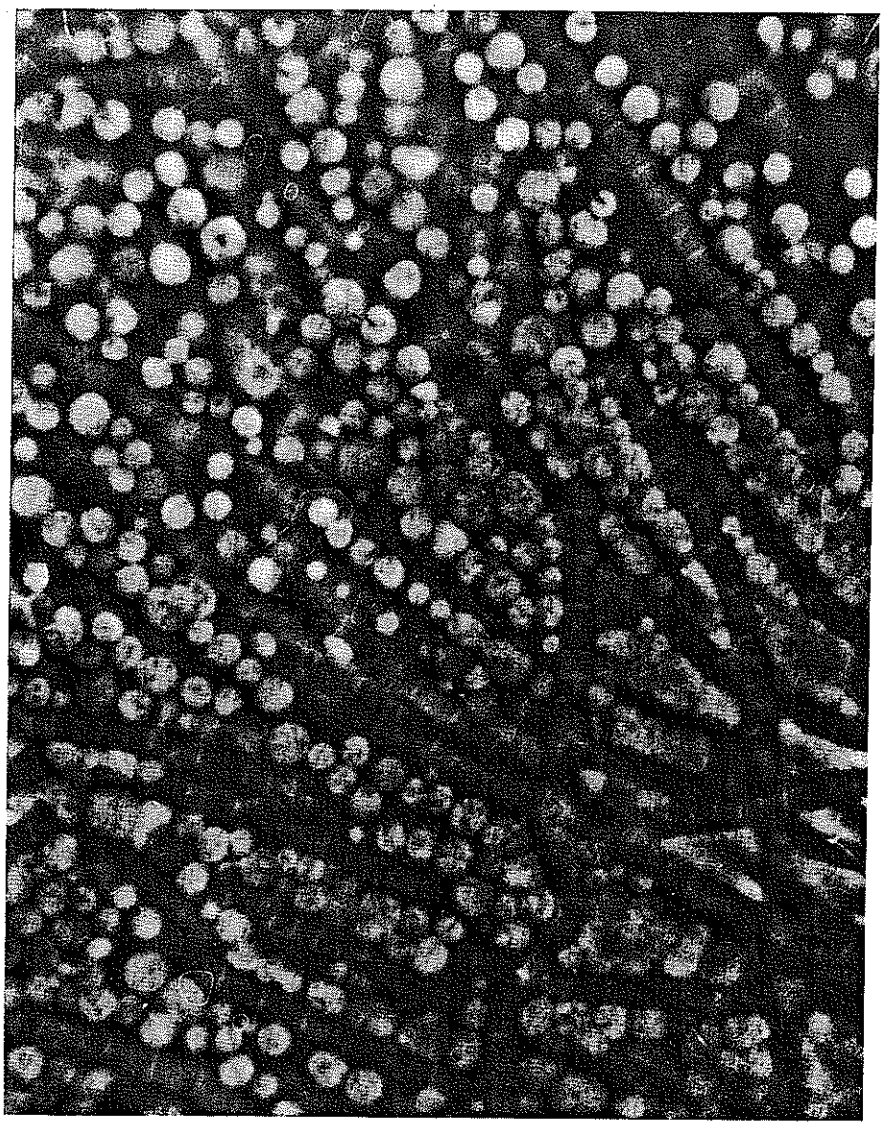

FIG. 2. Fresh fibrillar muscle of *Drosophila* after minimal teasing in a drop of pupal hemolymph of the *Cecropia* silkworm. Bright contrast, oil immersion, phase-contrast objective. × 2000.

Materials and Methods

1. Experimental Animals.—All experiments reported here were performed on *Drosophila funebris* or the blowfly *Phormia regina*. The *Drosophila* were reared and

isolated at 25 ± 0.5°C. in bottles containing a standard culture medium of agar, molasses, corn meal, and yeast. The age of adult flies was controlled to ±1 hour during the first day and ±12 hours thereafter. The flies were transferred to fresh bottles every 3rd day. The blowflies were reared at room temperature, according to the method of Hill, Bell, and Chadwick (1947). The adult flies were isolated at daily intervals and stored at 25 ± 0.5°C. in cages containing water and granulated sucrose.

2. Isolation of Sarcosomes.—Approximately forty blowflies were first anesthetized with carbon dioxide. By means of scissors the head, abdomen, and appendages were removed from the thoraces and the latter placed in a mortar containing about 0.5 ml. of ice cold 0.2 M sucrose. The material was then gently ground with a pestle and the resulting brei filtered by suction through several layers of muslin that had previously been moistened with 0.2 M sucrose. The filtrate consisted for the most part of sarcosomes and oil globules. After centrifugation (5 minutes at 500 *g*), the oil globules remained suspended while the sarcosomes were sedimented as a reddish brown pellet. Forty blowflies ordinarily yielded a pellet about 0.15 ml. in volume.

Other methods will be described in the specific procedures reported below.

EXPERIMENTAL RESULTS

1. Histochemical Studies

A small mass of fresh flight muscle was removed from the thorax of *Phormia regina* and teased on a glass slide in one of the following reagents. Janus green B, in concentrations of 1:20,000, 1:15,000, and 1:10,000, was dissolved either in insect Ringer's solution (Ephrussi and Beadle, 1936) or in 0.2, 0.4, and 1.0 M sucrose, and used according to Lillie (1948). The blood smear technique for mitochondria was applied according to Mallory (1938). Pinacyanol staining was performed by the method of Hetherington (1936), using insect Ringer's or 0.9 M sucrose as solvents. For studies of flight muscle after fixation, paraffin sections were stained according to Regaud's or Benda's methods for mitochondria (Cowdry, 1948).

Sarcosomes were selectively stained with Janus green B only when the latter was dissolved in 1.0 M sucrose solution. Most definitive results were obtained by the blood smear technique using 1:10,000 Janus green B. The staining was not homogeneous throughout the individual sarcosomes for the reason that granular material within the sarcosomes stained most intensely.

A slow but selective staining of sarcosomes with pinacyanol was obtained in slides coated with 0.1 per cent of the dye dissolved in hypertonic sucrose solution. This staining was evidently not due to non-specific absorption, for the muscular fibrils were unstained even after long periods of treatment.

Regaud's method gave differential staining of the sarcosomes in paraffin sections of fixed flight muscle.[1] With Benda's method, however, the staining was so intense throughout the muscle that no decision could be reached as to the selective affinity of the sarcosomes.

[1] We are indebted to Miss Barbara Stay for performing these staining reactions on fixed tissue.

2. *Spectroscopic Studies of Isolated Sarcosomes*

A pellet of isolated sarcosomes was placed in a depression slide and observed under the microscope by means of a Zeiss microspectroscopic ocular. The material was oxidized by adding a drop of 10 per cent aqueous solution of potassium ferricyanide and reduced by adding several crystals of sodium hydrosulfite (lycopon, dithionite), recrystallized from the Mallinckrodt product.

The isolated sarcosomes showed *alpha* absorption bands for reduced cytochrome $a + a_3$ at 595 to 611 mμ, *b* at 561 to 569 mμ, and *c* at 549 to 558 mμ, in addition to the combined *beta* bands at 518 to 535 mμ. The suspension of sarcosomes blanched to a tannish brown color when vigorously stirred in air. Simultaneously the absorption bands of the reduced cytochromes disappeared, signalling the oxidation of the several components. Addition of dithionite caused the sarcosomes to resume their reddish pink color, accompanied by the reappearance of the absorption bands of the reduced cytochromes. In like manner, the addition of ferricyanide caused the blanching and disappearance of absorption bands; the latter were promptly recovered after the addition of a few crystals of dithionite. These results indicate the presence in the sarcosomes of an intact cytochrome system including *a*, *b*, *c*, and cytochrome oxidase.

3. *Chemical Studies*

Sarcosomes were isolated from *Phormia* and freed of the sucrose medium by a series of three centrifugations (5 minutes at 15,000 *g*) and resuspensions in insect Ringer's solution. The washed pellet was then frozen in a dry-ice–cellosolve bath and lyophilized. Lipid analyses were performed by the method of Bloor (1943). Analyses for total nitrogen were made by the semimicro-Kjeldahl technique (Pregl, 1930). Non-protein nitrogen was measured by nesslerization. Riboflavin was determined by the microbiological method, using *Lactobacillus casei* as test organism (U.S.P., 1947; Difco manual, 1948).

The results recorded in Table I show that approximately 29 per cent of the dry weight of sarcosomes consists of lipids. The total protein nitrogen concentration, equivalent to about 9 per cent of the dry weight of sarcosomes, indicates a protein content of approximately 60 per cent. In these calculations no correction was applied for the trace of salts contributed by the residual Ringer's solution. However, the maximal error attributable to this source was less than 1 per cent.

Microbiological assay demonstrated the presence of approximately 1 *gamma* of riboflavin per mg. of sarcosomal nitrogen.

4. *Enzymatic Content*

(*a*) *Cytochrome Oxidase.*—

Sarcosomes isolated from *Phormia* were suspended in 0.1 M phosphate buffer at pH 7.4 and measurements of cytochrome oxidase activity made at 25°C. according to the

method of Schneider and Potter (1943). Aluminum chloride and calcium chloride were not added to the mixture, for oxygen uptakes were found to be substantially the same with or without these salts. The cytochrome *c* used in the course of the analysis was prepared by the method of Keilin and Hartree (1945) or obtained as a powder from the Sigma Chemical Company.

TABLE I

Chemical Composition of the Sarcosomes of the Blowfly

Sample No.	Dry weight of sarcosomes	Weight of lipids	Lipid content
		Lipids	
	mg.	*mg.*	*per cent of dry weight*
1	55.4	15.1	27.3
2	23.0	7.0	30.4
			Mean = 28.9 per cent
		Total nitrogen	
			per cent of dry weight
1			7.8
2			8.8
3			10.1
4			9.3
5			12.3
			Mean = 9.7 per cent
		Non-protein nitrogen	
			0.33 per cent
		Riboflavin	
			gamma/mg. N
1			1.32
2			0.99
			Mean = 1.16

As recorded in Fig. 3, the isolated sarcosomes of the blowfly contain a titer of cytochrome oxidase which varies systematically with the age of the insect. Per unit of sarcosomal nitrogen, the highest values are encountered in sarcosomes isolated from newly emerged adults. The titer then falls rapidly during the first 3 days of adult life and becomes constant at approximately one-half its initial value. Since sarcosomal nitrogen may vary with age, the data computed in terms of sarcosomal nitrogen do not necessarily imply that corresponding changes occur in the absolute concentration of cytochrome oxidase.

The titers of cytochrome oxidase in isolated sarcosomes and in whole thoracic muscle are compared in Table II. On the basis of the nitrogen content of each material, the enzyme is found to be about twice as concentrated in sarcosomes as in the entire muscle.

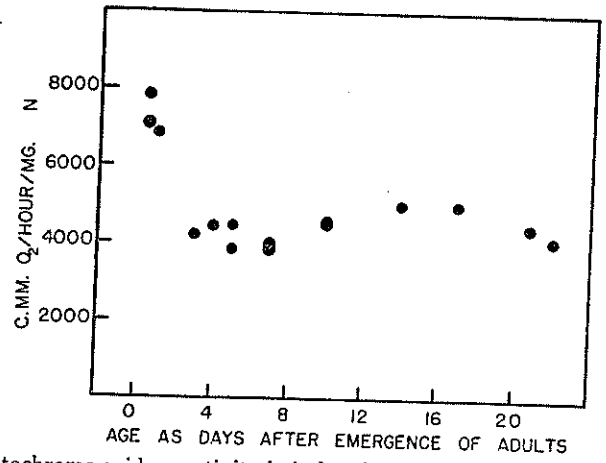

FIG. 3. Cytochrome oxidase activity in isolated sarcosomes of *Phormia* as a function of the adult age of the insect.

TABLE II

Cytochrome Oxidase Activity in Sarcosomes and in Whole Thoracic Muscle of the Blowfly

	Age of flies	Sarcosomes	Whole muscle
	days	*c.mm.* O_2 */hr./mg. N*	*c.mm.* O_2*/hr./mg. N*
Run 1	22	1855	1110
Run 2	19	1484	548

(*b*) *Catalase.*—

Sarcosomes isolated from *Phormia* were assayed for catalase by the method of Keilin and Hartree (1938). A few determinations were made with the concentrations of reactants suggested by George (1949). The measurements were made at 25°C. using the standard Warburg apparatus.

As shown in Fig. 4, the sarcosomes contain a considerable titer of catalase, which varies with the age of the insect. Per unit of sarcosomal nitrogen, the activity is highest in newly emerged insects and falls precipitously to about one-fifth the initial value during the 1st week of adult life. A continued gradual decrease is then observed during the remainder of adult life.

(*c*) *Cholinesterase.*—

Determinations of cholinesterase were made on isolated sarcosomes of *Phormia* by the method of Chadwick and Hill (1947). The results were corrected for non-enzymatic hydrolysis of acetylcholine. Comparisons were made with assays of the heads of the same flies.

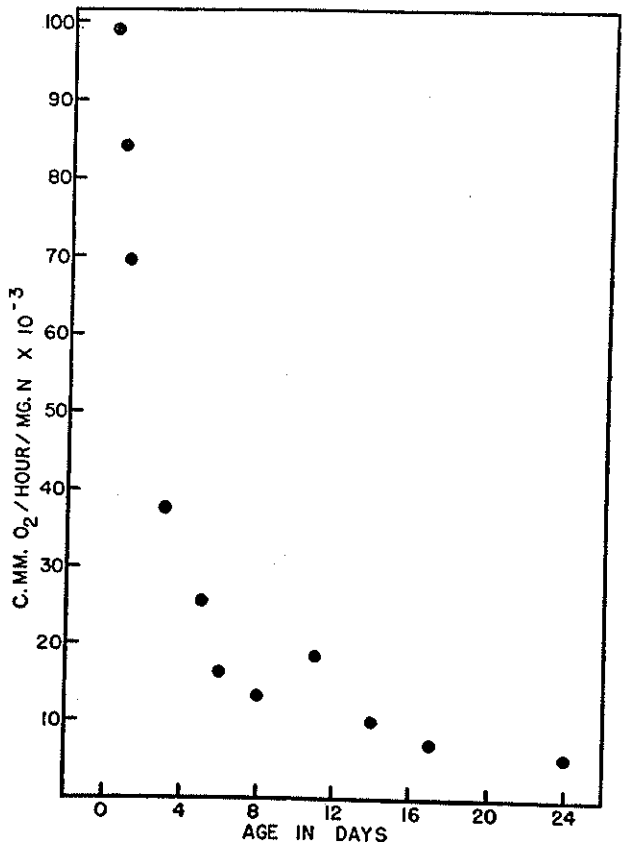

FIG. 4. Catalase activity in isolated sarcosomes of *Phormia* as a function of the adult age of the insect.

Whereas a homogenate of fly heads split 122.3 micromoles of acetylcholine per hour per milligram of nitrogen, less than 0.9 micromole of acetylcholine was hydrolyzed in the presence of a similar concentration of sarcosomal material. This value falls at or near the limits of accuracy of the assay method. Evidently, cholinesterase is either absent or is in very low titer in sarcosomes.

(*d*) *Dehydrogenases.*—Isolated sarcosomes were freed from endogenous substrates by a series of three centrifugations (5 minutes at 15,000 *g*) and resus-

pensions in glass-redistilled water. Assays for the various enzymes were made at 25°C., according to methods stated below, using the standard Warburg apparatus. 0.2 ml. of 2.0 N sodium hydroxide was placed in the center well

TABLE III

Dehydrogenases in the Sarcosomes of the Blowfly

Enzyme	Age of flies	Enzymatic activity	Assay method
	days	*c.mm. O_2/hr./mg. N*	
Succinoxidase	26	2350	Ball and Cooper (1949)
	26	1780	Schneider and Potter (1943)
	21	1172	Schneider and Potter (1943)
Succinic dehydrogenase	9	1420	Ball *et al.* (1947)
	5	708	Ball *et al.* (1947)
α-Glycerophosphate dehydrogenase	13	602	Green (1936)
Malic dehydrogenase	13	461	Potter (1946)
	19	726	Potter (1946)
Pyruvic dehydrogenase	21	282	Green *et al.* (1948)
	24	0	Pardee and Potter (1949)
	3	0	Larner *et al.* (1949)
Xanthine dehydrogenase	12	0	Axelrod and Elvehjem (1941)
Phenylalanine dehydrogenase	10	0	Stumpf and Green (1944)
Glycine dehydrogenase	13	0	Stumpf and Green (1944)
Lactic dehydrogenase	11	0	Bach *et al.* (1946).
Choline dehydrogenase	17	0	Dickens (1946)
Glutamic dehydrogenase	9, 18	0	Dewan (1938)
Alcohol dehydrogenase	13	0	Lutwak-Mann (1938)

along with the customary strip of filter paper. Each vessel contained a concentration of washed sarcosomes equivalent to 10 to 20 mg. dry weight. In very active preparations the absolute concentration was adjusted to yield pressure changes of 5 to 10 mm. of Brodie's solution per 10 minutes. Each test was run in duplicate at two different concentrations of sarcosomes.

Succinic dehydrogenase and succinoxidase were measured by the methods of Schneider and Potter (1943), Ball *et al.* (1947), and Ball and Cooper (1949). Malic dehydrogenase was measured by the method of Potter (1946); α-glycerophosphate dehydrogenase by the method of Green (1936); xanthine dehydrogenase by the method of Axelrod and Elvehjem (1941); phenylalanine dehydrogenase by the method of Stumpf and Green (1944); glycine dehydrogenase by the method of Stumpf and Green (1944); lactic dehydrogenase by the method of Bach *et al.* (1946); choline dehydrogenase by the method of Dickens (1946); *l*-(+)-glutamic dehydrogenase by the method of Dewan (1938); alcohol dehydrogenase by the method of Lutwak-Mann (1938); pyruvic dehydrogenase by three different methods, those of Green *et al.* (1948), Pardee and Potter (1949), and Larner *et al.* (1949).

The results recorded in Table III show that isolated sarcosomes contain significant titers of succinoxidase, succinic dehydrogenase, α-glycerophosphate dehydrogenase, malic dehydrogenase, and pyruvic dehydrogenase (by the method of Green *et al.*, 1948). The following dehydrogenases could not be demonstrated: xanthine, phenylalanine, glycine, lactic, choline, glutamic, and alcohol.

DISCUSSION

As discrete cytoplasmic bodies, the sarcosomes of insect fibrillar muscle show morphological resemblance to the mitochondria of other tissues. In view of the distinctive chemical properties already recognized for the latter, the results of the present study afford a new and biochemical basis for appraising the relation of sarcosomes to mitochondria.

As one such basis of comparison, the histochemical studies show that sarcosomes are slowly but selectively stained by several reagents known to have special affinity for mitochondria. It is worth noting, however, that the affinity of sarcosomes for mitochondrial stains is not as avid as that of mitochondria. Evidently, the component which combines selectively with such dyes as Janus green B is present in lower titer in sarcosomes than in mitochondria.

The spectroscopic studies of isolated sarcosomes demonstrate the presence of high titers of cytochromes *a*, *b*, and *c*. Since the bands of the reduced components disappeared on aeration, the presence of cytochrome oxidase is also indicated. These qualitative results are essentially identical to those reported for isolated mitochondria of the vertebrate liver and kidney (Schneider, 1946; Hogeboom, Schneider, and Pallade, 1948; Schneider and Hogeboom, 1950).

Of further interest are the quantitative chemical similarities between sarcosomes and mitochondria. Thus, the lipid and protein distribution in sarcosomes is similar to that reported for the liver mitochondria of rats, guinea pigs, and rabbits (Goerner, 1938; Bensley, 1943; Lazarow, 1943). Moreover, the riboflavin content of sarcosomes, when recalculated in terms of dry weight, is 95 to 127 μg. per gm. of sarcosomes, as compared to 88 to 211 in the mitochondria of rat liver (Price, Miller, and Miller, 1948).

The high titers of cytochrome oxidase and succinoxidase in isolated sarcosomes are found also in vertebrate mitochondria. A quantitative comparison of the activities of these enzymes in the mitochondria of mammalian liver and in sarcosomes is presented in Fig. 5. The data for mitochondria have been calculated from Schneider (1946). The two analytical methods were identical except that the sarcosomes were assayed at 25°C., a temperature which is physiologically optimal for the insect in the same sense as 37°C. is optimal for the mammal.

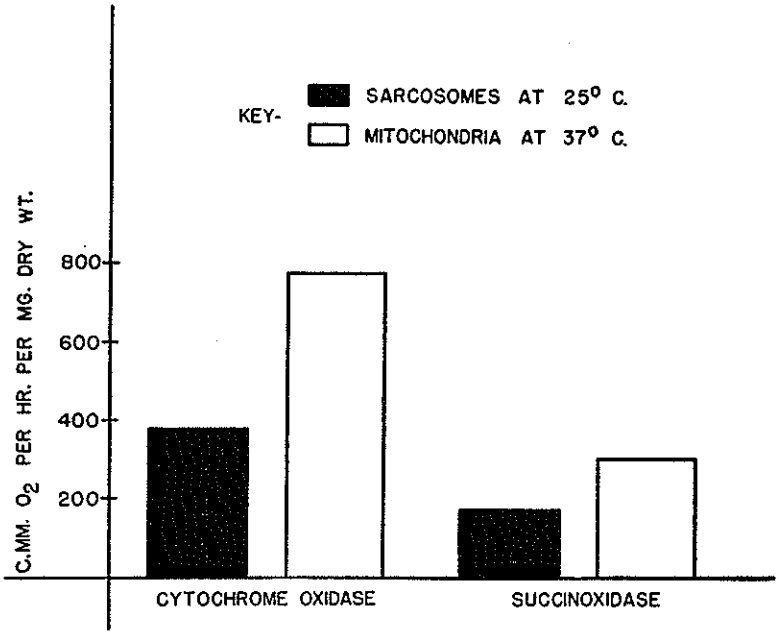

FIG. 5. Comparison of the cytochrome oxidase and succinoxidase activities of sarcosomes isolated from *Phormia* and mitochondria isolated from rat liver.

The dehydrogenases demonstrated in sarcosomes (Table III) are specifically those found in mitochondria (Claude, 1945; Graffi and Junkmann, 1946; Green, Loomis, and Auerbach, 1948; Green, 1949 *a*). Moreover, the dehydrogenases listed in Table III as absent from the sarcosomes are water-soluble enzymes which are apparently not bound to the mitochondria of vertebrate tissues (McShan, 1949).

Surveying the total evidence available at the present time, it is clear that sarcosomes fall well within the limits of biochemical definition of mitochondria. In terms of their morphological characteristics, histochemical reactions, spectroscopic properties, chemical composition, and enzymatic content, we are there-

fore persuaded to homologize the sarcosomes of insect fibrillar muscle with the mitochondria of other tissues and other animals.

SUMMARY

1. The "indirect" thoracic muscles of adult dipterous and hymenopterous insects consist of a unique type of muscle characterized by the presence of numerous spherical, intracytoplasmic bodies termed "sarcosomes."

2. When the muscle is teased or ground, the sarcosomes are liberated as a turbid suspension of bodies ranging from 1 to 4 μ in diameter. A method is described for the isolation of sarcosomes by a simple differential centrifugation.

3. The cytochemical, chemical, and enzymatic properties of sarcosomes were examined for the purpose of appraising their relation to the cytoplasmic bodies of other tissues.

4. Fresh sarcosomes are slowly but selectively stained by the mitochondrial reagents, Janus green B and pinacyanol. Fixed sarcosomes give a positive reaction with Regaud's mitochondrial stain.

5. Chemical analyses show that approximately 29 per cent of the dry weight of sarcosomes consists of lipids and 60 per cent of protein. Microbiological assay indicates the presence of about 1 *gamma* of riboflavin per milligram of nitrogen. These values resemble those reported for isolated mitochondria of vertebrate liver and kidney.

6. When examined spectroscopically the sarcosomes, like the vertebrate mitochondria, show a high titer of cytochromes *a*, *b*, and *c*.

7. The titer of cytochrome oxidase varies systematically with the adult age of the insect. A similar relation is observed for the enzyme catalase.

8. Isolated sarcosomes show significant titers of succinoxidase, α-glycerophosphate dehydrogenase, malic dehydrogenase, and pyruvic dehydrogenase. The following dehydrogenases could not be demonstrated: xanthine, phenylalanine, glycine, lactic, choline, glutamic, and alcohol. These results are compared with those previously reported for vertebrate mitochondria.

9. In view of their manifold points of biochemical similarity, it is concluded that the sarcosomes are the mitochondria of this highly specialized muscular tissue.

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