MITOCHONDRIA IN THE FLIGHT MUSCLES OF INSECTS

II. Effects of the Medium on the Size, Form, and Organization of Isolated Sarcosomes

By MARY ISHIMOTO WATANABE and CARROLL M. WILLIAMS

(From the Pioneering Research Laboratories, United States Army Quartermaster Corps, Philadelphia, and The Biological Laboratories, Harvard University, Cambridge)

Plate 1

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The striated muscles which propel the wings of adult dipterous and hymenopterous insects are remarkable in that approximately one-third of each muscle fiber consists of discrete, spherical, intrasarcoplasmic bodies termed sarcosomes. In a previous study performed on the flight muscles of *Drosophila* and the blowfly *Phormia*, the sarcosomes were found to possess morphological characteristics and biochemical properties closely resembling those of mammalian mitochondria (Watanabe and Williams, 1951). On the basis of this evidence it was concluded that the sarcosomes are the mitochondria of this highly specialized muscular tissue—a homology of special interest since the average diameter of the sarcosomes is approximately 2.5 μ; *i.e.*, more than twice that of previously described mitochondria. This absolute difference in diameter signals a difference in volume greater than fifteenfold and brings the internal organization of the insect mitochondria within the range of resolution of the oil immersion objective. For this reason the sarcosomes promise to be useful tools in the analysis of the structure of mitochondria.

This problem, the organization of mitochondria, has in recent years become a simultaneous focus of both cytological and biochemical investigation. Biochemical studies have demonstrated that the mitochondria contain an array of respiratory enzymes whose properties appear to be markedly influenced by their spatial relationships within the intramitochondrial compartment (see Green, 1951, 1952; Schneider and Hogeboom, 1951; Holter, 1952). Indeed, it has become increasingly evident that the mitochondria provide a physical basis for the organization of these enzyme systems—a matter of considerable significance since the enzymes in question are responsible for virtually the entire series of reactions involved in the oxidative breakdown of substrate molecules. These considerations, as pointed out by Claude (1947–48), necessitate that biochemical and morphological studies of mitochondria proceed hand in hand—a goal that seems particularly accessible in the case of the sarcosomes of insects.

Materials and Methods

1. Experimental Animals.—Aside from two series of experiments performed on *Drosophila funebris*, the blowfly *Phormia regina* Meigen was utilized in all procedures. The flies were reared at a constant temperature of 25°C. by the method of Hill, Bell, and Chadwick (1947); adults were isolated at daily intervals and stored at 25°C. in cages containing water and granulated sucrose.

2. Preparation of Test Slides.—Each series of experiments was performed on a single fly of a specific adult age, usually 7 days. The head, abdomen, and appendages were excised and the thorax cut sagittally with scissors. The subdivided thorax was then temporarily stored in a covered glass dish containing filter paper moistened with insect Ringer's solution (Ephrussi and Beadle, 1936). A small drop of the solution to be tested was placed on a clean microscope slide; under the dissecting microscope several fibers of the longitudinal dorsal muscle of the thorax were removed with watchmaker's forceps and immersed in the fluid drop. The muscle was then teased with fine needles. The unteased portion was finally removed, the preparation covered with a coverslip, and the latter sealed in place with melted vaseline.

3. Examination and Evaluation of the Slides.—Observations under the phase microscope[1] were begun within 30 minutes and continued at convenient intervals for approximately 24 hours. Other methods will be described in the specific procedures reported below.

EXPERIMENTAL RESULTS

I. Sarcosome Size

When examined under the phase contrast, oil immersion lens in the presence of an optimal suspending medium, the sarcosomes in freshly teased flight muscle are dense, homogeneous, spherical bodies. The interface of each sarcosome with the surrounding medium is sharp and devoid of any visible folds or crevices (type A of Text-fig. 3; Fig. 1 A). No limiting membrane is recognizable in normal sarcosomes, although, as we shall see, a definite membrane becomes conspicuous under a variety of experimental conditions. The contents of normal sarcosomes are homogeneous, semiopaque, and non-granular, but, here again, notable changes occur in the presence of suboptimal media (Williams and Watanabe, 1952).

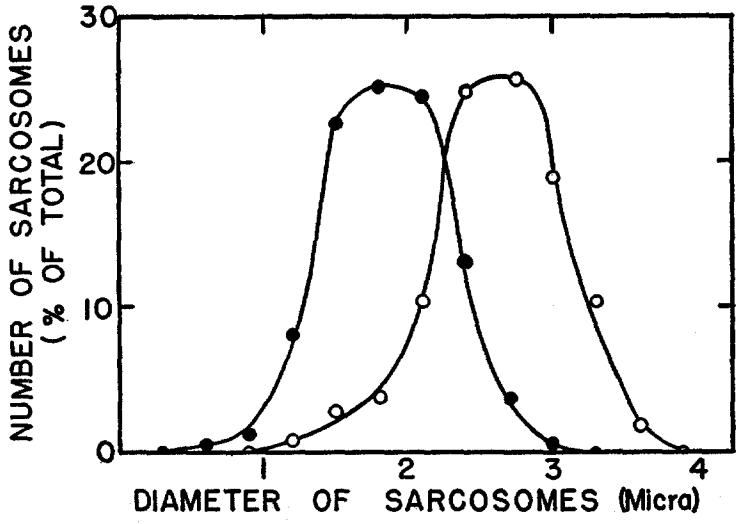
Though all normal sarcosomes, in our experience, show this same regularity of form under the phase contrast lens, one always finds considerable variation in the absolute size of the organelles within a single insect or even within a single muscle fiber. This variability was studied in detail as follows:—

A small fragment of flight muscle was removed from an adult 7 day old male *Drosophila funebris*, teased in a drop of pupal blood of the *Cecropia* silkworm, and ex-

[1] The following oil immersion, phase contrast objectives were used in the present study: 0.14 A $+ 0.25\lambda$; 0.14 A $- 0.25\lambda$; (American Optical Company), and three special Bausch and Lomb objectives whose retardations are not known to us.

amined immediately under the phase contrast lens. A similar examination was made of flight muscle from an adult 7 day old male *Phormia* in an improved medium; namely, 2.5 per cent bovine plasma albumin in 0.16 M potassium phosphate buffer at pH 7.0. Camera lucida drawings were made of all the sarcosomes in a single randomly selected field and the diameter of each sarcosome was measured from the drawings.

The results, summarized in Text-fig. 1, quantitate the variability in sarcosomal diameter for each of the insects. In the case of *Phormia,* the mean di-



TEXT-FIG. 1. The distribution of sarcosomal diameters in 7 day old male flies. Closed circles, *Drosophila funebris* sarcosomes suspended in the pupal hemolymph of the *Cecropia* silkworm; open circles, *Phormia regina* sarcosomes in 2.5 per cent bovine plasma albumin in 0.16 M potassium phosphate buffer at pH 7.0.

ameter of 105 sarcosomes was 2.6 $\mu \pm 0.046$ (S.E.). The medium utilized in the case of *Drosophila* was slightly hypertonic; the mean diameter of 163 sarcosomes was 1.9 $\mu \pm 0.033$ (S.E.). An approximately normal distribution was observed in each case.

II. Sarcosome Size as a Function of Age

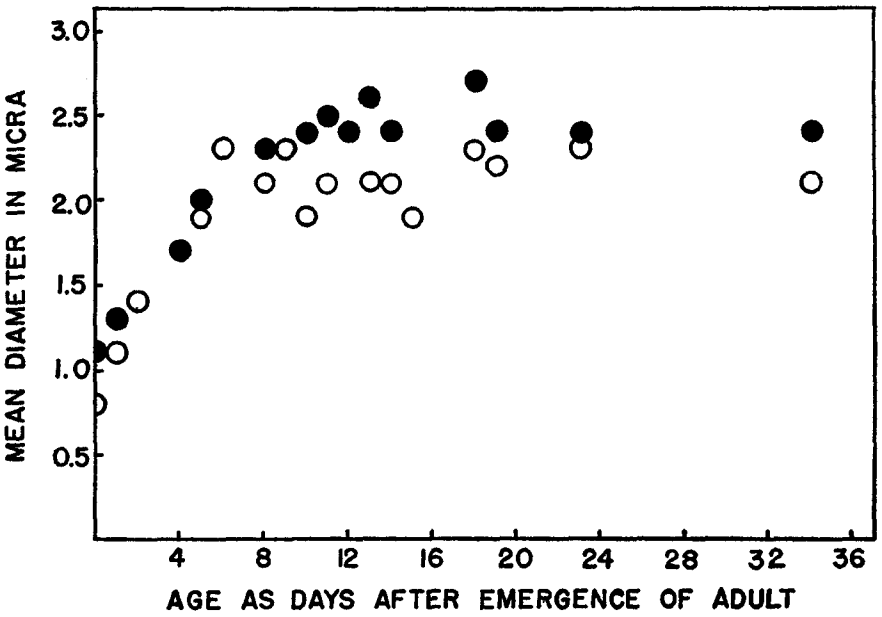
The procedure outlined in the preceding section was repeated on individual male and female *Drosophila* of specific ages. The teasing medium was slightly hypertonic and consisted of undiluted pupal blood of the *Cecropia* silkworm.

The mean sarcosomal diameters have been plotted in Text-fig. 2 as a function of the adult age of the flies. It is clear that the average diameter of *Drosophila* sarcosomes undergoes rapid increase during the 1st week of adult life and then

remains constant. Less extensive measurements indicate that the sarcosomes of the blowfly behave in precisely the same manner.

III. Changes in the Morphology of Sarcosomes

The degree to which one can preserve the sarcosomes in their initial normal state is markedly affected by the composition of the teasing and suspending medium. The vast majority of media induce more or less rapid alterations in



TEXT-FIG. 2. The mean diameters of *Drosophila funebris* sarcosomes as a function of adult age. Closed circles, female flies; open circles, male flies. The medium consisted of pupal hemolymph of the *Cecropia* silkworm.

the microscopic appearance of sarcosomes. Each medium provokes a distinctive pattern of change. As indicated in Table I and Text-fig. 3, the effects may, in general, be grouped into three categories; namely, *swelling* (with or without compaction of contents), *shrinkage*, and *fuzzy degeneration*. It should be noted, however, that among the thousands of sarcosomes in each preparation, one can ordinarily find at least a few which preserve apparently normal size and microscopic appearance. Notwithstanding this fact, the over-all direction of change in the various media under consideration was ordinarily conspicuous and easy to evaluate by appropriate sampling and counting.

1. Swelling

(*a*) *With compaction of sarcosomal contents.*—This change is best seen in

sarcosomes suspended in distilled water (Fig. 1 B). Rapid and progressive swelling occurs accompanied by the appearance of an optically empty vacuole. The latter begins in a localized area at one side of the sarcosome and gradually presses the sarcosomal contents to the opposite pole (type B of Table I and Text-fig. 3). In so doing, the vacuole elevates a discrete membrane from

TABLE I

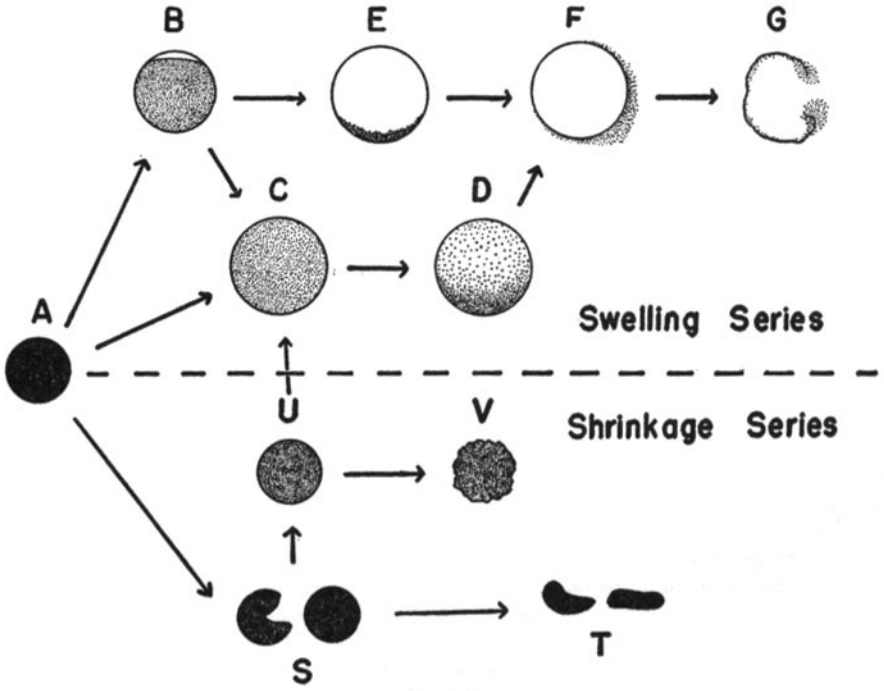
Major Types of Sarcosomes after Exposure to Various Media (Illustrated in Text-Fig. 3)

Type	Major characteristics
A. *Normal*	*Dense, homogeneous, highly refractile spheres of about 2 to 3 μ in average diameter*
Swelling series	*Abnormally pale; average size larger than normal*
B. Elevated membrane	Membrane lifted away from sarcosomal contents which remain dense and homogeneous
C. Diffusely granular	Granules diffusely distributed throughout the swollen sarcosome
D. Semicompacted contents	Internal granules partially concentrated at one pole of sarcosome
E. Compacted contents	Compaction of the solid or granular contents at one pole of the sarcosome leaving an optically empty vacuole
F. Ghosts with extruded contents	Extruded granules are attached to optically empty ghosts
G. Disrupted	Membranes ruptured and assuming odd shapes
Shrinkage series	*Average size smaller than normal; highly refractile; dense*
S. Spherical, with or without folds	Spherical in shape; homogeneous in appearance, except for folds or "cracks" in the membrane
T. Flattened, pleomorphic	Rod-like, truncate, flattened or otherwise differing from the spherical in shape
U. Densely granular	Spherical forms showing definite internal granulation
V. Fuzzy	Membrane indistinct and "fuzzy"

the retreating contents. Swelling accompanied by enlarging of the vacuole continues until the sarcosomal contents are compacted as a narrow crescent at one pole (type E). Finally, the resistance of the membrane is overcome and the contents escape as a mass of minute granules which usually aggregate at one or more points on the outside of the distended membrane (type F). The latter persists for a time as a discrete, spherical, optically empty ghost, but later collapses into various odd shapes (type G).

(*b*) *Without compaction of the sarcosomal contents.*—In 0.32 M solutions of neutral salt, such as NaCl, KCl, and in the various types of Ringer's solutions,

transient shrinkage is first observed (types S and T of Table I and Text-fig. 3; Fig. 1 D), followed by swelling as in the case of distilled water (types U and C; Fig. 1 E). However, the vacuole is less sharply defined than in distilled water and ordinarily contains numerous minute granules in Brownian motion (type D). The semicompacted sarcosomal contents also show granulation and Brownian motion. In the presence of Ringer's solution, the swelling that takes place usually occurs without vacuole formation. But, as diagrammed in Text-



TEXT-FIG. 3. The major types of sarcosomes observed in various media. The various categories are described in Table I.

fig. 3, the end-stage of swelling in all these salt solutions is the same as in distilled water; namely, an escape of the granular contents leaving the vacated sarcosome as a spherical ghost (type F).

2. *Shrinkage* is best seen after sarcosomes are teased in such fluids as mammalian blood serum or the undiluted pupal hemolymph of the *Cecropia* silkworm (Fig. 1 C). In its minimal expression the membrane is commonly thrown into one or more localized folds or invaginations without any notable decrease in the over-all diameter of the sarcosome. A "mouse-eaten" appearance is frequently observed (type S). Or the sarcosome may undergo a generalized decrease in diameter without the appearance of folds. In such cases the

spherical shape persists and the bodies become small and dense (type S). Higher degrees of shrinkage transform the sarcosomes into dense, more or less rod-shaped or pleomorphic bodies (type T).

3. Fuzzy degeneration is characteristic of sarcosomes teased in high concentrations of dextrose or sucrose. Though shrinkage and various degrees of pleomorphism are always initially observed (types S and T; Fig. 1 F), the distinctive ultimate feature is the disappearance of the sharp interface between the medium and the sarcosomal membrane (type V; Fig. 1 G). One gets the impression that amorphous material agglutinates or adsorbs on the outside of the membrane. Prior to the formation of these fuzzy forms, the compacted sarcosomes begin to lose their homogeneity and to show granulation (type U).

IV. Effects of Specific Solutions on Sarcosomal Morphology

In terms of the criteria just considered, a detailed study was made of the effects of various media on sarcosomal morphology.

Each chemical was dissolved in glass-redistilled water to yield a specific molarity—usually 0.32 M. Before dilution to final volume each solution was neutralized to pH 7.0 by the addition of KOH or KH_2PO_4.

Reagent grade chemicals were utilized with the following exceptions: AMP, 100 per cent (Bischoff); Na_3ADP, 85 per cent + 10 to 15 per cent monophosphate (Sigma); Na_2H_2ATP, 95 per cent (Pabst); glucose-1-phosphate, dipotassium salt (Schwartz); fructose-6-phosphate, monobarium salt (Bischoff); fructose-1,6-diphosphate, *ca.* 95 per cent as monobarium salt (Bischoff); DPN, 75 per cent (Schwartz); sodium glycerophosphate, 28 per cent alpha (Eastman); crystalline bovine plasma albumin (Armour); crystalline human serum albumin.[2]

The barium salts of fructose-1,6-diphosphate and fructose-6-phosphate were converted to potassium salts by the addition of potassium sulfate. In experiments utilizing lactic acid, the acid was depolymerized by boiling and its concentration determined by titration.

The procedure described under Methods was used to test the effects of each solution when utilized as a teasing and suspending medium. Microscopic observations were begun within 30 minutes and continued for several hours. The slides were then stored overnight at 5°C. Observations were continued the following day at room temperature. 20 to 24 hours after its preparation, each slide was finally studied in detail. In a series of randomly selected fields, counts were made of the number of sarcosomes in each of the eleven categories diagrammed in Text-fig. 3. Each experiment was performed in duplicate or in triplicate.

The results, recorded in Table II, have been arranged as far as possible in a sequence beginning with the least satisfactory media and ending with

[2] We are grateful to Dr. Walter Lee Hughes, of the Harvard Medical School, for supplying this highly purified crystalline human serum albumin containing less than one mole of salt per mole of protein.

TABLE II

Effects of Various Media on Sarcosomes of the Blowfly

Except where otherwise indicated, all the media were 0.32 M final concentration, and at pH 7.0. The major types of sarcosomes observable after 24 hours of exposure are indicated by the letters at the top of the vertical columns. (See Table I and Text-Fig. 3 for definition of categories.) The sarcosomes in each preparation have been scored as follows:—

+++ (75 to 100 per cent); ++ (50 to 75 per cent);
+ (25 to 50 per cent); 0 (less than 25 per cent)

An asterisk (*) indicates that Brownian movement was present. In general the various solutions are arranged in increasing order of effectiveness in preserving the sarcosomes.

Medium	Sarcosomal types									
	Normal	Swelling series					Shrinkage series			
	A	C	D	E	F	G	S	T	U	V
1. Distilled water	0	0	0	++	+	+	0	0	0	0
2. Sodium chloride	0	+*	+*	0	++	0	0	0	0	0
3. Potassium chloride	0	+	0	0	++	+	0	0	0	0
4. Calcium chloride	0	0	0	+++	0	0	0	0	0	0
5. Magnesium chloride	0	0	+*	+++	0	0	0	0	0	0
6. pH 7.0 potassium phosphate buffer	0	+*	+*	0	+	+	0	0	0	0
7. 0.64 M sodium sulfate	0	+*	0	0	+	+	0	0	0	0
8. Sodium sulfate	+	++*	0	+	0	0	0	0	0	0
9. Intracellular Ringer‡	0	0	+	0	++	+	0	0	0	0
10. Thrice concentrated intracellular Rihger	0	0	+	0	++	+	0	0	0	0
11. Twice concentrated insect Ringer§	0	0	0	0	+	+++	0	0	0	0
12. Insect Ringer	0	+*	0	0	++	+	0	0	0	0
13. Potassium acetate	0	0	+	0	+	+	0	0	0	0
14. Trisodium ethylenediaminetetraacetate	0	+*	+	0	+	+	0	0	0	0
15. Glycerol	0	+	+	+	0	0	0	0	0	0
16. Erythritol	0	++	+	0	0	0	0	0	0	0
17. Urea	0	++*	0	++	0	0	0	0	0	0
18. Aspartate	0	+*	0	+	+	+	0	0	0	0
19. Pyridoxine	0	++	0	++	0	0	0	0	0	0
20. ATP	0	+*	0	+	++	0	0	0	0	0
21. Fructose-1,6-diphosphate	0	+	0	+	+	+	0	0	0	0
22. Glucose-1-phosphate	0	0	+	0	0	+++	0	0	0	0
23. α-ketoglutarate	0	+*	0	+	+	+	0	0	0	0
24. DPN	0	+*	0	0	+	++	0	0	0	0
25. Malate	0	+*	0	+	++	0	0	0	0	0

‡ Kassel and Kopac (1949).

§ Ephrussi and Beadle (1936).

TABLE II—*Concluded*

Medium	Sarcosomal types									
	Normal	Swelling series					Shrinkage series			
	A	C	D	E	F	G	S	T	U	V
26. Succinate	0	+*	0	+	+	+	0	0	0	0
27. Aconitate	+	+*	0	0	++	0	0	0	0	0
28. Glutamate	+	0	0	+	+	+	0	0	0	0
29. Fumarate	0	+*	+	+	0	0	+	0	0	0
30. Pyruvate	+	+	0	0	++	0	0	0	0	0
31. Lactate	+	+*	0	0	+	+	0	0	0	0
32. Fructose-6-phosphate	+	+	+	+	0	0	0	0	0	0
33. Ribose	+	+	+	+	0	0	0	0	0	0
34. Xylose	+	+	+	+	0	0	0	0	0	0
35. Arabinose	0	+	+	0	0	0	0	0	+	+
36. Adonitol	0	+	+	0	0	0	0	0	0	+
37. ADP	0	+*	0	+	0	0	+	0	0	0
38. Sodium glycerophosphate	0	+	0	0	0	0	+	+	0	0
39. Citrate	0	+	0	+	0	0	+	0	+	0
40. Ascorbate	+	+	0	0	0	0	+	0	0	0
41. Glutathione	+	+*	0	0	0	0	+	0	0	0
42. Sorbitol	0	+	0	0	0	0	+	0	0	+
43. AMP	+	+	0	0	0	0	+	0	0	0
44. Mannitol	0	0	0	0	0	0	+	+	+	+
45. Galactose	0	0	0	0	0	0	+	+	0	+
46. Dextrose in pH 7, 0.32 M potassium phosphate buffer	0	0	0	0	0	0	++	++	0	0
47. Dextrose	0	0	0	0	0	0	0	+	+	+
48. Levulose	0	0	0	0	0	0	++	++	0	0
49. Sucrose	0	0	0	0	0	0	0	+	+	+
50. Raffinose	0	0	0	0	0	0	+	0	+	+
51. Nicotinate	0	0	0	0	0	0	+++	0	0	0
52. Thiamine	0	0	0	0	0	0	++	+	0	0
53. Equal parts horse serum and distilled water	+++	0	0	0	0	0	0	0	0	0
54. Equal parts pupal *Cecropia* hemolymph and distilled water	+++	0	0	0	0	0	0	0	0	0
55. 2.5 per cent bovine plasma albumin in 0.16 M potassium phosphate buffer, pH 7.0	+++	0	0	0	0	0	0	0	0	0
56. 2.5 per cent human serum albumin in 0.16 M potassium phosphate buffer, pH 7.0	+++	0	0	0	0	0	0	0	0	0

media which were optimal. It is evident that simple 0.32 M solutions of either electrolytes or non-electrolytes failed to preserve the normal architecture of sarcosomes. Prolonged preservation was attained only in more complex solutions in which protein was present in addition to crystalloids. The best "synthetic" medium consisted of 2.5 per cent crystalline serum albumin in 0.16 M potassium phosphate buffer at pH 7.0.

V. Effects of Hydrogen Ion Concentration

The effects of hydrogen ion concentration were studied by varying the proportions of acid and alkaline phosphate in the optimal serum albumin medium described in the preceding section. The concentration of protein was held constant at 2.5 per cent and the concentration of buffer at 0.16 M.

TABLE III

*The Effect of Hydrogen Ion Concentration on the Sarcosomes of the Blowfly**

pH	Sarcosomal types										
	Normal	Swelling series						Shrinkage series			
	A	B	C	D	E	F	G	S	T	U	V
4.90‡	0	0	0	0	0	0	+	0	0	+	+
6.12	0	0	+	0	0	0	+	0	0	0	+
7.00	+++	0	0	0	0	0	0	0	0	0	0
7.96	++	0	+	0	0	0	0	0	0	0	0

* The medium contained 2.5 per cent crystalline bovine plasma albumin in 0.16 M potassium phosphate buffer at the indicated pH's. The types of sarcosomes have been scored according to the system described for Table II.

‡ Sarcosomes highly refractile and clumped; normal in size but app ear fixed by the acidity.

The results, summarized in Table III, demonstrate that the normal architecture of sarcosomes was best preserved at pH 7.0. Swelling occurred when the pH was increased to 8.0. Likewise, when the pH was reduced to 6.12, the majority of sarcosomes underwent swelling, with or without disruption. At still lower pH, the mitochondria clumped together and showed disruption or changes similar to those seen after histological fixation.

VI. Effects of Freezing and Thawing

Fresh flight muscle was teased in the optimal phosphate–serum albumin at pH 7.0 and the resulting preparation frozen overnight at −30°C in a deep-freeze cabinet. The following day the slides were returned to room temperature and examined.

The normal architecture was destroyed by freezing. The sarcosomes showed fuzzy degeneration and various irregular shapes. Neither Brownian movement nor any marked swelling was observed.

DISCUSSION

The experimental results serve to characterize the sarcosomes as intracellular organelles whose average diameter, after the 1st week of adult life, is approximately one-third that of human red blood corpuscles. But, by virtue of the difference in shape, the average volume of the spherical sarcosomes is approximately the same as that of the biconcave erythrocytes.

It is fruitful to carry the comparison a stage further. Like the erythrocyte, the normal sarcosome appears to be filled with a microscopically homogeneous gel surrounded by a discrete membrane. Moreover, the maintenance of normal size and architecture, in both sarcosomes and erythrocytes, is markedly conditioned by the composition of the surrounding medium. Distilled water and numerous other solutions cause swelling and ultimate lysis of sarcosomes, thereby converting the membrane into an optically empty ghost; certain other solutions cause shrinkage, flattening, or crenation of both sarcosomes and red blood corpuscles. From these considerations we learn that the sarcosomal membrane encloses a subcellular compartment as distinctive as the cellular compartment of the red cell.

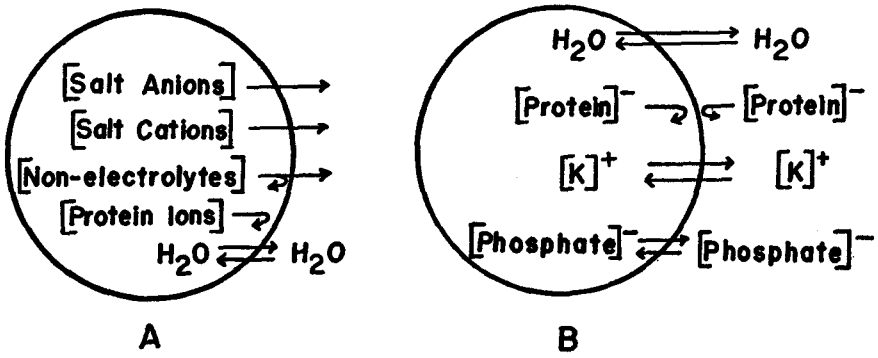
The present studies of sarcosomes are pertinent to the problem of the organization of mitochondria and to previous findings on mammalian material. Thus it is clear that Harman's (1950) admirable study recorded many of the changes which we have observed and summarized in Text-fig. 3. However, Harman was persuaded that mammalian mitochondria consist of a naked, membraneless gel—a conclusion which we attribute to difficulties inherent in microscopic studies of such small bodies and to the limits of resolution of the compound microscope. Indeed, it is worth noting that Zollinger (1948, 1950) reports the presence of a mitochondrial membrane in mammalian mitochondria of the same type studied by Harman. Moreover, the validity of this conclusion seems definitely established by the electron microscopic studies of Claude and Fullam (1945), Dalton *et al.* (1949), Mühlethaler *et al.* (1950), Palade (1952 *a*, *b*), and Sjöstrand (1953). And, as described in the present study, any further doubts concerning the reality of the mitochondrial membrane are dispelled by an examination of insect sarcosomes in distilled water.

It is a surprising fact that this latter observation on insect sarcosomes has long been buried in the literature, as is amply evident in the following statement which we quote in translation from Kölliker (1888; page 699): "A very great effect is exerted on [the sarcosomes] by water, in which the granules swell hugely and change into bubbles with a distinct though extremely delicate membrane."

If we accept the mitochondrial membrane as a demonstrated entity, then osmotic forces must necessarily condition the movement of water between the inside and outside of the mitochondria, provided that the membrane is permeable to water and impermeable to one or more solutes. That the mem-

brane possesses the postulated degree of semipermeability is, once again, suggested by the behavior of sarcosomes in distilled water, in which they respond like simple osmometers and undergo swelling and lysis.

Our interpretation of this result is diagrammed in Text-fig. 4 A. In the presence of distilled water, osmosis favors the entrance of water, while simple diffusion favors the exit of all other molecules that can penetrate the membrane. Those molecules which are trapped within the sarcosomes by the semipermeable membrane are responsible for the osmotic gradient promoting the progressive entry of water.



TEXT-FIG. 4. A is a diagram of a sarcosome immersed in distilled water. The nondiffusible contents of the sarcosome cause the osmotic entry of water. In B the sarcosome is considered in its normal situation within the muscle cell. The presence of protein outside the sarcosome is responsible for a balance of Donnan forces controlling the flux of water.

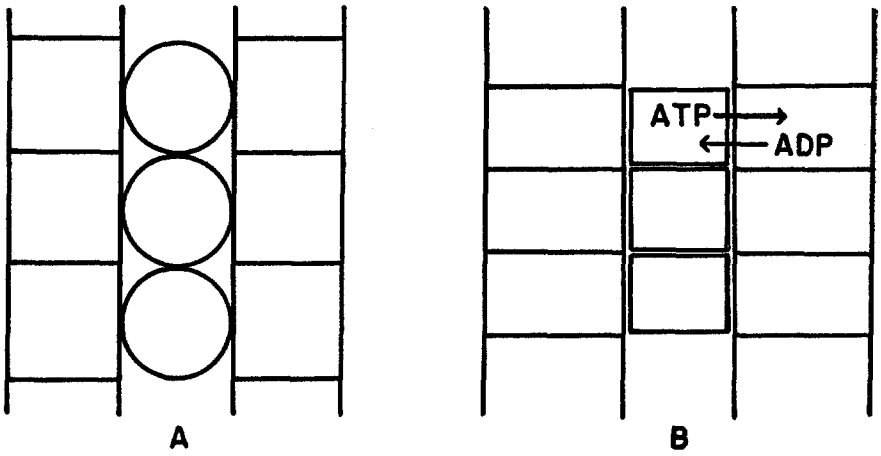
Let us now consider the situation when sarcosomes are immersed in 0.32 M solutions of simple salts. A solution of, say, sodium chloride can only be transiently hypertonic in the event that the membrane is permeable to sodium and chloride ions. When the ions have distributed themselves equally on both sides of the membrane, then the sarcosomes should behave as if immersed in distilled water and undergo swelling and lysis.

As recorded in Table II, the sarcosomes behaved in precisely this manner in all simple 0.32 M salt solutions that were tested. The same was true in twice concentrated Ringer solutions. This finding suggests that the membrane is more or less freely permeable to sodium, potassium, calcium, and magnesium cations, and to chloride, phosphate, and sulfate anions.

In this preliminary analysis of the problem, we choose to interpret the further results in Table II according to this same principle. Those 0.32 M solutes which fail to prevent the osmotic entry of water may be presumed to penetrate the membrane and thereby to lose osmotic effectiveness. Such an interpretation requires tentative acceptance of the assumptions that the solutes do not alter

the membrane itself, and that active transport mechanisms fail to camouflage the osmotic effects.

Under this analysis the data in Table II suggest that the membrane is more or less freely permeable to several intermediates in the citric acid cycle and to non-electrolytes smaller than hexoses. By a similar line of reasoning, we find the membrane to be less permeable to non-electrolytes larger than pentoses, provided that the molecules are not phosphorylated. But, as recorded in Table II, the phosphorylation of glucose or fructose vastly enhances their ability to penetrate the membrane.



TEXT-FIG. 5. Diagram of flight muscle illustrating the relationship between sarcosomes and fibrils (*hypothetical*). In the relaxed muscle A, the spherical sarcosomes occupy most of the sarcoplasm between adjacent fibrils, approximately one sarcosome to each sarcomere. In the contracted muscle B, the shortening and thickening of the fibrils is viewed as deforming the sarcosomes and thereby facilitating the ATP-ADP shuttle between sarcosomes and fibrils.

The permeability of the membrane to ATP is of special interest (Williams and Watanabe, 1952). We have previously reported that sarcosomes, like mammalian mitochondria, contain a high concentration of enzymes responsible for the oxidative breakdown of substrate (Watanabe and Williams, 1951). Indeed, the sarcosomes appear to be the sole sites within the muscle of the succinoxidase system (Levenbook and Williams, 1951, 1953) and of several flavoproteins, including $DPNH_2$ oxidase and cytochrome *c* reductase (Sacktor, 1953 *b*). Evidently, the sarcosomes are principally concerned with the oxidative phosphorylation of ADP—an inference which has recently been confirmed experimentally by Sacktor (1953 *c*). The product of this reaction, ATP, must then pass from the sarcosomes and energize the actomyosin in the adjacent muscular fibrils. As illustrated in Text-fig. 5, we suggest that the diffusion of

ATP is facilitated by the muscular contraction itself. Thus, in the relaxed muscle (A), the sarcosomes already occupy most of the sarcoplasm between the fibrils; it is difficult to see how the fibrils could shorten and broaden (B) without deforming the sarcosomes. Such a deformation, in itself, would necessarily bring the membrane of the sarcosomes into more intimate contact with adjacent fibrils.

Dr. Leigh E. Chadwick calls our attention to the astonishing fact that this conclusion was also anticipated by early workers, nearly half a century before the sarcosomes were recognized as mitochondria. Heidenhain (1911), citing the earlier observations of E. Holmgren, says (page 636, authors' translation):

"During contraction, on the other hand, the separation of the fibrils is diminished and the granules undergo a compression. . . . Besides, during contraction, they change their shape as a direct result of the compression, in that they develop wing-like appendages that force their way in among the fibrils. This 'winged' condition of the granules has already been noticed by Kölliker and Cajal."

The observed permeability of the sarcosomal membrane to ATP is consistent with the view that phosphorylation occurs *within* the sarcosomes. However, as the recent studies of Spiro (1953) indicate, it is certain that both the membrane and the contents of sarcosomes possess higher degrees of organization that can be detected by the phase contrast optical system. Moreover, as Sacktor (1953 *a, c*) has found, enzymatic reactions can apparently occur at the interface between the sarcosomal membrane and the medium. For these several reasons it would be premature to conclude that ATP formation must necessarily occur *within* the contents of the sarcosome. The relatively low permeability of the sarcosomal membrane to ADP (Table II) also argues against such a conclusion on the basis of present information.

Table II records tests of solutes which showed prolonged osmotic effectiveness in relation to the sarcosomal membrane. Most of these substances are non-phosphorylated, uncharged molecules larger than pentoses. Indeed, it will be observed that 0.32 M concentrations of these solutes are effectively *hypertonic* when balanced against the contents of the sarcosome. Exit of water leads to shrinkage and to an increase in the density of the sarcosomal contents. It is of particular interest that sucrose is one such osmotically effective substance. This fact apparently affords a rational basis for the empirical practice of isolating mitochondria in sucrose solutions (Hogeboom *et al.*, 1948).

Though sucrose and other osmotically effective sugars prevent swelling and lysis, we have observed that the microscopic properties of sarcosomes undergo pronounced alterations in such solutions. Amorphous material precipitates or agglutinates on the outside of the membrane—a state which, for want of a better term, we have called "fuzzy degeneration."

The search for media superior to sucrose (see Table IV) was aided by the

finding that proteinaceous fluids, such as mammalian blood serum or insect hemolymph, preserved sarcosomes far more satisfactorily than did any simple solutions. Thus, when diluted with an equal part of distilled water, each of these biological fluids maintained the normal form and architecture of sarcosomes for 24 hours or longer.

Efforts were made to define the blood components responsible for this effect. It was found that blood serum, deproteinized by preliminary heating to 75°C. for 5 minutes, immediately lost its stabilizing effect on the sarcosomes. Moreover, in the absence of electrolytes, the proteinaceous fraction was found to be inadequate. Thus, when suspended in a 5 per cent solution of salt-free crystalline human serum albumin, the sarcosomes underwent the same degree of swelling and lysis as in distilled water—a result intelligible in terms of the low molarity and osmotic pressure of pure protein solutions.

Although the salts of ethylenediaminetetraacetic acid (versenes) have recently been shown to be effective stabilizers of rat heart sarcosomes (Slater and Cleland, 1952; Cleland, 1952), these compounds showed no such properties with respect to the sarcosomes of flight muscle. 0.32 M solutions of versene (pH 7.0) failed to prevent immediate swelling and paling (Table II); lower concentrations, when added to salt solutions or to distilled water, only slightly delayed the appearance of the degenerative effects of these media. The optimal protein-salt medium became unsatisfactory when versene was added (see Tables II and IV). Moreover, Sacktor (1953 *c*) finds that the addition of 0.001 M versene completely abolishes the oxidation of α-ketoglutarate by isolated sarcosomes.

In summary, we can state that both proteinaceous and non-proteinaceous components of blood serum were osmotically ineffective when tested separately. But, when recombined, the solutions regained the original effectiveness. This same striking change was also achieved by the addition of a low concentration of neutral salt to the salt-free protein solution. Evidently, both proteins and salts are necessary for the prolonged preservation of sarcosomes, though it is theoretically possible that other compounds of high molecular weights can be made to substitute for the protein.

A rational basis for the significance of protein is considered in Text-fig. 4 B in which a sarcosome is diagrammed in its normal situation within the muscle cell. The sarcosome contains protein ions to which the membrane is impermeable and smaller ions to which the membrane is more or less permeable. In such a system the effective osmotic pressure is almost solely attributable to the presence of the protein and to the resulting Donnan equilibrium. The osmotic pressure arising in this manner will be proportional, approximately, to the square of the concentration of the protein and to the square of the net charge on the protein, and inversely proportional to the concentration of diffusible ions (Scatchard, 1952).

TABLE IV

*Solutions Tested in Search of an Optimal Medium for Retaining the Normal Configuration of Blowfly Sarcosomes**

Medium	Sarcosomal types						
	Normal	Swelling series		Shrinkage series			
	A	B	C–G	S	T	U	V
1. 0.01 M disodium ethylenediaminetetraacetate in insect Ringer	0	0	+++	0	0	0	0
2. 2.5 per cent bovine plasma albumin in 0.16 M potassium phosphate buffer, pH 7.0, and 0.01 M disodium ethylenediaminetetraacetate	0	0	+++	0	0	0	0
3. 0.32 M glucose	0	0	0	0	+	+	+
4. 0.5 M glucose	+	+	0	+	+	0	0
5. 1.0 M glucose	+	+	0	0	+	0	+
6. 0.15 M sucrose	+	0	0	0	0	0	+
7. 0.29 M sucrose	+	+	0	0	0	0	+
8. 0.58 M sucrose	+	+	0	0	+	0	+
9. 0.88 M sucrose	0	0	0	+	0	+	++
10. 1 part pupal *Cecropia* hemolymph in 2 parts distilled water	0	+	0	0	+	+	0
11. 1 part pupal *Cecropia* hemolymph in 1 part distilled water	+	0	0	+	0	0	0
12. 2 parts pupal *Cecropia* hemolymph in 1 part distilled water	0	0	0	0	+	0	+
13. Undiluted pupal *Cecropia* hemolymph	0	0	0	+	+	+	+
14. 1 part horse serum to 5 parts distilled water	0	0	+++	0	0	0	0
15. 1 part horse serum to 4 parts distilled water	0	0	+++	0	0	0	0
16. 1 part horse serum to 3 parts distilled water	0	0	+++	0	0	0	0
17. 1 part horse serum to 2 parts distilled water	+	+	+	0	0	0	0
18. 1 part horse serum to 1 part distilled water	0	+	0	+	0	0	0
19. Undiluted horse serum	0	0	0	+	+	0	0
20. 5 per cent crystalline human serum albumin (salt-free)[2] in distilled water	0	0	+++	0	0	0	0
21. 2.5 per cent human serum albumin in 0.02 M NaCl	0	+	+	0	0	0	+
22. 2.5 per cent human serum albumin in 0.04 M NaCl	+	+	0	+	0	0	+
23. 2.5 per cent human serum albumin in 0.08 M NaCl	+	0	0	+	+	0	+
24. 2.5 per cent human serum albumin in 0.16 M NaCl	+	0	0	+	+	0	0
25. 2.5 per cent human serum albumin in 0.32 M NaCl	+	0	0	+	+	0	0
26. 0.08 per cent human serum albumin in 0.16 M NaCl	+	0	+	+	0	0	0
27. 0.3 per cent human serum albumin in 0.16 M NaCl	+	+	0	+	0	0	0
28. 1.3 per cent human serum albumin in 0.16 M NaCl	+	+	0	+	0	0	0
29. 2.5 per cent bovine plasma albumin in 0.08 M potassium phosphate buffer, pH 7.0	+	+	0	0	+	0	0
30. 2.5 per cent bovine plasma albumin in 0.16 M potassium phosphate buffer, pH 7.0	+++	0	0	0	0	0	0
31. 2.5 per cent bovine plasma albumin in 0.32 M potassium phosphate buffer, pH 7.0	0	0	0	0	+	0	+

* The types of sarcosomes after 24 hours of exposure to specific solutions have been scored according to the system described for Table II.

But within the muscle cell the sarcosomes are imbedded in sarcoplasm which also contains protein and diffusible electrolytes. The invasion of the sarcosomes by water is thereby counterbalanced by Donnan forces acting in the reverse direction. According to this analysis, the results of the present investigation point to the Donnan equilibrium as the source of the principal osmotic forces regulating water movement between the sarcosome and its milieu.

On the basis of these considerations, it was possible to predict the composition of a "synthetic" medium which, when actually tested, proved optimal for the preservation of sarcosomes in teased preparations (Table IV) or after their isolation by filtration or centrifugation. This medium consists of 2.5 per cent bovine plasma albumin in 0.16 M potassium phosphate buffer at pH 7.0. The potassium phosphate buffer system was selected to supply the principal intracellular buffer, the phosphate system. The choice of neutral pH was based on the studies recorded in Table III. 0.16 M buffer was chosen to mimic the presumed concentration of intracellular electrolytes.

The most crucial of these components is the protein, in the absence of which the medium was scarcely more effective than distilled water in preserving the architecture of the sarcosomes. It is therefore of particular interest and importance that Sacktor (1953 *c*) has been able to demonstrate oxidative phosphorylation by isolated sarcosomes only in the presence of proteinaceous media. In retrospect, it is no surprise to find that such intracellular organelles as mitochondria are best preserved in a medium which is little short of a proteinaceous intracellular Ringer solution.

Dr. Leigh E. Chadwick and Dr. Bertram Sacktor, of the Medical Laboratories, Army Chemical Center, Maryland, and Professor W. J. Crozier were most helpful in the preparation of the present paper.

SUMMARY

1. The sarcosomes of *Drosophila* and the blowfly, *Phormia*, are dense, spherical, homogeneous bodies when isolated from flight muscle and promptly examined under the phase contrast, oil immersion objective.

2. Their average diameter in newly emerged flies is about 1 μ. This value increases rapidly during the 1st week of adult life and then becomes constant at approximately 2.5 μ. At each age the variation in sarcosome diameter conforms approximately to a normal distribution.

3. The degree to which isolated sarcosomes retain their initial size and organization is remarkably conditioned by the composition and the hydrogen ion concentration of the medium in which they are teased and suspended. In suboptimal media three major categories of change were encountered: (1) swelling, with or without compaction of the contents (as in distilled water and salt solutions); (2) shrinkage to rod-like, pleomorphic forms (as in blood serum); and (3) fuzzy degeneration (as in sugar solutions).

4. The membrane that surrounds each sarcosome becomes plainly visible in swollen sarcosomes. A continuation of swelling is accompanied by the escape of the sarcosomal contents, the vacated membrane persisting as a spherical, optically empty ghost.

5. Sarcosomes appear to behave like osmometers when suspended in various aqueous solutions. Solutes which penetrate the membrane show only transient effects in preventing the osmotic entry of water.

6. Under this analysis we find the membrane to be more or less freely permeable to the ions of sodium, potassium, calcium, magnesium, chloride, and phosphate, to non-electrolytes smaller than hexoses, to phosphorylated hexoses, and to several intermediates of the citric acid cycle.

7. The sarcosomal membrane appears to be less permeable to non-electrolytes larger than pentoses, provided that such molecules are not phosphorylated.

8. The membrane shows a higher permeability to ATP than to ADP. The significance of this observation is considered with respect to the ADP-ATP shuttle between sarcosomes and muscle fibrils.

9. Simple solutions of electrolytes or non-electrolytes cause more or less conspicuous changes in the microscopic appearance of sarcosomes. Prolonged preservation was achieved only in more complicated media containing protein. It is concluded that the Donnan equilibrium is the source of the principal osmotic forces regulating the movement of water through the sarcosomal membrane.

10. The optimal medium for the preservation of isolated sarcosomes was an intracellular Ringer solution containing 2.5 per cent crystalline bovine albumin in 0.16 M potassium phosphate buffer at pH 7.0.

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EXPLANATION OF PLATE 1

FIG. 1. *Phormia* sarcosomes isolated in various media and photographed with a phase contrast, oil immersion objective (American Optical Co. 0.14 A + 0.25λ). × 1000.

A. In 2.5 per cent bovine plasma albumin in 0.16 M potassium phosphate buffer at pH 7.0, the sarcosomes are dense, highly refractile, homogeneous spheres averaging 2.5 μ in diameter (type A of Text-fig. 3).

B. In distilled water the sarcosomes are pale and swollen, the internal contents being compacted to one side (type E of Text-fig. 3), or extruded (type F). Ghost and disrupted forms (type G) are evident.

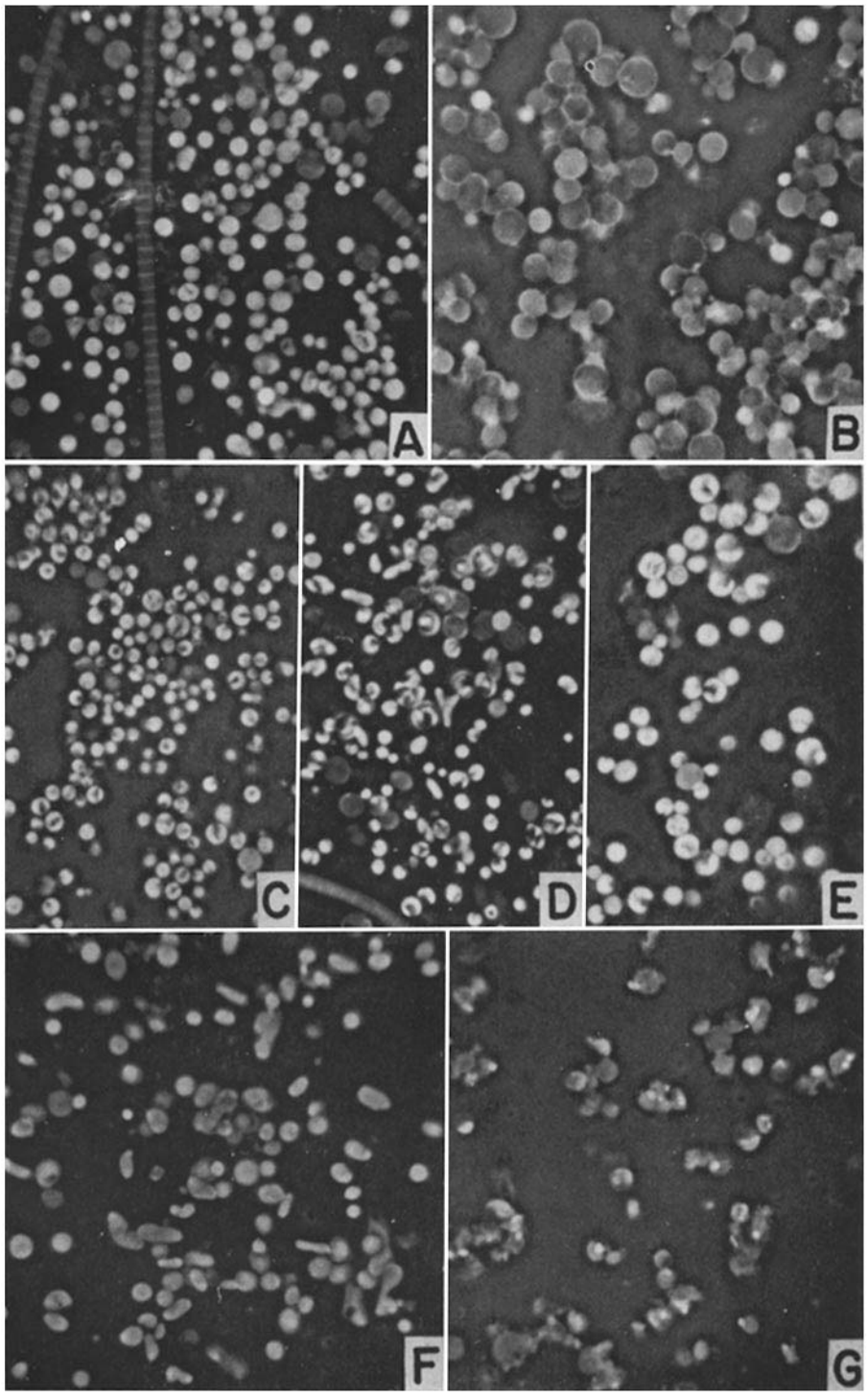
C. In 5 per cent bovine plasma albumin in 0.32 M potassium phosphate buffer at pH 7.0. In this hypertonic solution, the sarcosomes are dense, compact, highly refractile, and smaller than normal. Many show folds in the membrane (type S) or varying degrees of flattening (type T).

D. Immediately after suspension in 0.32 M NaCl solution the sarcosomes show transient shrinkage (types S and T).

E. The preceding preparation ½ hour later. Swelling, granulation, and ghost formation have already begun (types C, D, and F).

F. Immediately after suspension in 0.32 M sucrose solution, the sarcosomes are dense, homogeneous, and highly refractile. Rod-like forms are conspicuous (type T).

G. After 20 hours in 0.32 M sucrose the majority of sarcosomes show fuzzy degeneration (type V).



(Watanabe and Williams: Mitochondria in insect muscle. II)

FIG. 1