THE RELATIONSHIP BETWEEN CYTOCHONDRIA AND MYOFIBRILS IN PIGEON SKELETAL MUSCLE

By JOHN W. HARMAN, M.D., and URSULA H. OSBORNE

(From the Department of Pathology, The University of Wisconsin Medical School, Madison)

PLATES 8 AND 9

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A close mutual relationship between the processes of oxidation and oxidative phosphorylation and the maintenance of the structural integrity of mitochondria (1) has been proposed. By use of the high energy phosphate bonds generated during the metabolism, the mitochondria contribute for some time to their self preservation. It is, however, very probable that other cellular structures, which lack the means of elaborating ~P,[1] also utilize the energy provided by the mitochondrial metabolism for their structure and function. There are, however, no available studies which provide information that the mitochondria supply ~P directly to other structures in order to maintain them. The present studies deal with the occurrence of such a relationship in skeletal muscle between the cytochondria (2) and the myofibrils.

Since the pioneer physiological studies of Ranvier (3) and Bonhöffer (4), and the later metabolic work of Battelli and Stern (5), it has been realized that the difference in structure of muscles is associated with a difference in function and oxidative capacity. Stare and Baumann (6) later catalogued several of the outstanding variations among muscles and established the high Q_{O_2} of mammalian cardiac and pigeon breast muscle minces. It was with pigeon breast muscle that Krebs and Johnson (7) first obtained evidence for the citric acid cycle, although they furnished no data concerning the cytological localization of the cycle. Subsequent work showed the enzymes of the citric acid cycle to be associated with mitochondria in rabbit liver, kidney, heart, and skeletal muscles (8). More recent studies (9, 10) on the structures of insect muscles have

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[1] The following abbreviations are used in this article:—
~P—high energy phosphate
AMP—adenosine-5-phosphate
DPN—coenzyme *I*
Mg—magnesium ion
ATP—adenosinetriphosphate
DNP—2,4-dinitrophenol

shown that the oxidative systems in the wing muscles are located in the mitochondria. It has become evident also that the density of granules in muscle is roughly proportional to its oxidative capacity (11).

Experimental Methods

1. Preparation of Suspensions.—In all experiments the superficial breast muscle (musculus pectoralis major) of wild captive pigeons was used. It was removed rapidly from a decapitated, exsanguinated bird and, after making multiple transverse incisions, placed in crushed ice. The subsequent preparative procedures were performed at temperatures between 0°–4°C. From the cooled muscle either of 2 types of preparation was made. One was a whole homogenate in buffer, and the other an homogenate in sucrose which served for further fractionation of the muscle constituents into purified suspensions.

The whole homogenate was made by coarsely chopping 12 gm. of muscle into 50 ml. of 0.1 M phosphate buffer (potassium salt) of pH 7.2, contained in the monel (250 ml. capacity) Waring blendor cup. This was homogenized in the blendor for 45 seconds and used immediately for manometric and cytological studies.

The homogenate in sucrose was made the starting point of tissue fractionation (Text-fig. 1). 20 gm. of muscle were coarsely chopped into 150 ml. of 0.25 M sucrose, containing an added 1.5 ml. of 0.04 M $NaHCO_3$. This was homogenized in the blendor for 60 seconds. An additional 150 ml. of 0.25 M sucrose was then stirred in and the mixture squeezed through a single layer of gauze (type I, 44 by 36 inch mesh) into 100 ml. pear-shaped centrifuge tubes (2). It was centrifuged in the horizontal yoke of an SB-1 International refrigerated centrifuge at 200 g[2] for 2 minutes; then the force was rapidly increased to 800 g for a further 10 minutes. The (S_1) supernates were removed with automatic pipets (Alfred Bicknell Associates, Cambridge, Massachusetts, 5 ml. capacity) and stored. The buff colored layer (R_1) was resuspended in 40 ml. per tube of 0.25 M sucrose, transferred to the blendor for a 5 second dispersion, distributed into pear-shaped tubes again, and recentrifuged for 10 minutes at 800 g. The residues (R_2) were used subsequently for preparation of myofibrils and the supernates (S_2) were collected and combined with S_1 supernates.

For the isolation of the mitochondria the supernates S_1 and S_2 were centrifuged in 50 ml. lusteroid tubes in an angle head at 3,000 g for 15 minutes. The cloudy, translucent supernate (S_3) was poured off and saved for preparation of sarcosomes. To the residues 200 ml. of 0.25 M sucrose were added with mechanical stirring for resuspension. After recentrifugation at 3,000 g for 15 minutes, the supernate (S_4) was removed and added to S_3 fraction. The residue (R_4) was stirred up gently with 5 ml. of 0.25 M sucrose and used at once.

The supernates S_3 and S_4 were used for separation of sarcosomes. They were distributed into lusteroid tubes and centrifuged for 1 hour at 4,400 g in the angle head. The residue (R_5) was suspended in 2.5 ml. of 0.25 M sucrose.

Suspensions of myofibrils were prepared from the R_2 residue, removed from the tip segment of the pear-shaped tubes. After stirring up in 50 ml. of 0.5 M sucrose, it was homogenized in the blendor for 3 minutes with addition of crushed ice. The mixture was poured through a layer of gauze to remove ice particles, into pear-shaped tubes, which were then filled to capacity with 0.5 M sucrose and centrifuged for 6 minutes at 800 g. Supernates were removed and discarded; the residues were made up with a smaller volume of 0.5 M sucrose and recentrifuged at 800 g for 6 minutes. This last washing was repeated and the final residue (R_8) suspended in 3.0 ml. of 0.5 M sucrose. The nitrogen content of the various suspensions was determined by Johnson's method (12).

[2] The centrifugal forces given are maxima calculated for the distal extremities of the tubes.

2. Manometric Procedures.—The whole homogenate and the purified suspensions were examined for their capacity to oxidize intermediates of the tricarboxylic acid cycle with conventional Warburg manometric techniques. In the studies on purified particles, care was taken to maintain iso-osomotic conditions in the flasks as in earlier investigations (13).

The Warburg flasks for study of pure suspensions were prepared as follows. Filter paper and 0.2 ml. 6 N NaOH were placed in the centerwell. In the main compartment the total volume was 2.0 ml. and the sucrose concentration 0.25 M. Each flask contained per ml. fluid: 30μM potassium phosphate buffer, pH 7.20, 8μM magnesium chloride, 3.75μMAMP, 1 mg. DPN, and potassium salts of tricarboxylic acid cycle substrate to be indicated.[3] The suspensions of purified mitochondria, myofibrils, or sarcosomes were added as 0.5 ml. aliquots. Oxygen was used in the gas phase and the temperature of the Warburg bath was 37°C. After temperature equilibration in the bath for 5 minutes the stop-cocks were closed and the oxygen uptake during the first 5 minutes considered to be equal to that observed during the second 5 minutes.

For experiments employing the whole homogenate the flasks were prepared as above with the following exceptions. No sucrose was used as consideration was not given to osmotic conditons. Since the homogenate was introduced as a suspension in phosphate buffer, no additional phosphate buffer had to be present in the flask. Aliquots of the homogenate containing approximately 240 mg. of wet tissue in 1.2 ml. were used. DNP was placed in the main compartment together with the cofactors and substrate before addition of homogenate. Fluoride was added after homogenate, to obviate the precipitation of magnesium fluoride (14). Although such precipitation appeared to affect the metabolism little, the cloudiness of the suspension caused considerable difficulty with cytological studies.

To study cytological changes in parallel with oxygen uptake, several Warburg flasks with identical contents were used and a flask removed from the bath at regular intervals for cytological examination of its contents.

3. Cytological Techniques.—During preparation of all purified suspensions each definitive centrifugal step was checked cytologically (2) to ensure separation of mitochondria, myofibrils, nuclei, and sarcosomes. Although the scheme outlined in Text-fig. 1 was adhered to closely, the standard gravitational forces and times of centrifugation sometimes failed to separate the different constituents adequately, thereby necessitating additional brief centrifugations and washes. Because of this occasional difficulty, concurrent examinations by phase microscopy were never omitted.

At the beginning of the manometric experiment a sample of the suspension was examined by phase contrast and a differential count made as described previously (13). At intervals of 15 to 20 minutes flasks were removed and the contents similarly studied. In each differential count between 600 and 1,000 mitochondria were distinguished, with dark L contrast. Counts were necessarily rapid because in coverslip preparations deterioration sets in quickly.

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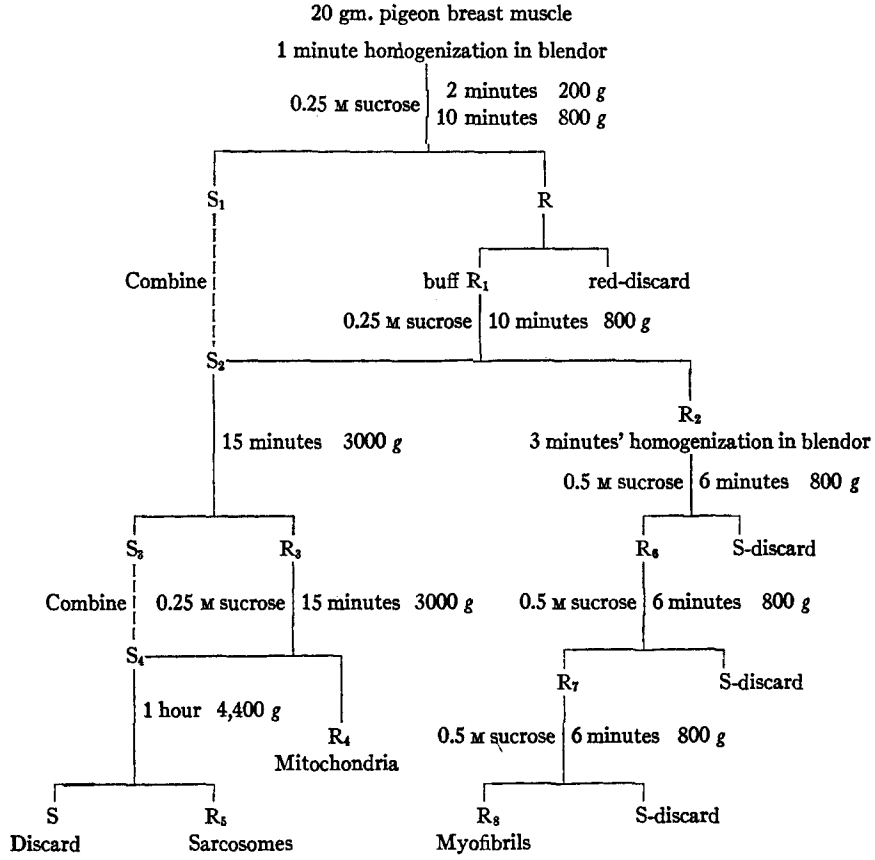
[3] Sources of materials were:—
AMP—Schwarz Laboratories, Inc., New York, New York.
DPN—Schwarz Laboratories Inc., New York, New York, 65 per cent pure.
α-Ketoglutaric acid—Nutritional Biochemical Corp., Cleveland, Ohio.
Citric acid—Merck reagent, Rahway, New Jersey.
Succinic acid—Mallinckrodt, A.R., St. Louis, Missouri.
Fumaric acid—Fisher Scientific Co., Pittsburgh, Pennsylvania.
Oxalacetic acid—Krishell Laboratories, Inc., Portland, Oregon.
Pyruvic acid—purified solution kindly provided by Dr. LePage of the McArdle Memorial Laboratory, Madison, Wisconsin.
DNP—Eastman Kodak Co., Rochester, New York, product, recrystallized.

Flowing movement and clumping sometimes interfered. But the movement of particles was usually temporary. Clumping was obviated by exerting gentle pressure on the coverslip which dispersed the clumps sufficiently. As in the previous studies the forms enumerated included rodlet, spherical dense, target, and the crescent or swollen mitochondria (Figs. 1 to 8). The sarcosomes underwent no structural alterations (2) and were not susceptible to such counts.



TEXT-FIG. 1.

In experiments with whole homogenates the structure of the myofibrils was also studied. With dark L contrast the sarcomeric constituents were clearly distinguished and the state of relaxation or contraction of the fibrils determined according to conventional criteria (15). Before differential mitochondrial counts were performed the occurrence of contractility and syneresis were sought; the distinction between syneresis and repetitious, reversible contraction in isolated myofibrils has previously been elucidated by using phase microscopy (16). More advanced stages of supercontraction and granular degeneration (17) were recorded when found. The preservation of the myofibrils in "good condition" was assessed on both the sarcomeric structure and the observation of reversible contractions, whereas degeneration was

characterized by syneresis with formation of "C" bands, supercontraction, and granular disintegration.

RESULTS

1. Cytological Examination of the Suspensions.—The routine examinations of the suspensions by phase microscopy have established their special characteristics. The morphological peculiarities of the mitochondria and sarcosomes, as described in a previous study (2), permit clear distinction between these particles. By this means it is observed that mitochondrial suspensions usually contain a small number of sarcosomes, whereas the sarcosome suspensions are regularly contaminated by a few tiny mitochondria and a larger number of mitochondrial fragments. The approximate strength of sarcosomal population of a mitochondrial preparation is gauged by performing differential counts on samples suspended in distilled water for 30 minutes or longer. The water causes the mitochondria to swell into crescents (Fig. 7) without altering the sarcosomes and facilitates the counts. Although Brownian movement is considerable and streaming occasionally interferes, the counts are remarkably reproducible. It is found that the mitochondrial suspensions contain between 11 and 22 per cent sarcosomes. Repeated washing reduces the quantity slightly.

The fresh mitochondrial suspensions in 0.25 M sucrose are composed mainly of irregular rodlets, a few dense spheres, and a rare crescent. Rodlets are unevenly contoured, sharp edged, and very dense, with facetted areas (Fig. 1) similar to those seen in the heart mitochondria (18). Introduction of the suspension into the Warburg flask is attended by a rapid conversion of rodlets into dense spherical and target forms (Figs. 1 to 5). The latter have hitherto been closely associated in heart muscle with the process of oxidative phosphorylation (1). During the course of the experiments the spherical dense forms and targets rapidly decrease and are largely replaced by swollen crescents, especially at the times when oxidation rates are declining.

The suspensions of myofibrils also have a regular composition. The principal constituent is the myofibril, which may exist in the relaxed, partly contracted, or strongly contracted states (Figs. 9 to 12). Under the influence of added 0.05 M ATP, 0.05 M $MgCl_2$, and 0.01 M KCl they enter a condition of syneresis (Figs. 13 to 16), indicating a persistence of the actomyosin complex. Spontaneous contraction is not observed. Scattered throughout the preparation are a few intact nuclei and a very rare mitochondrion. But the last two and sarcosomes are significantly few.

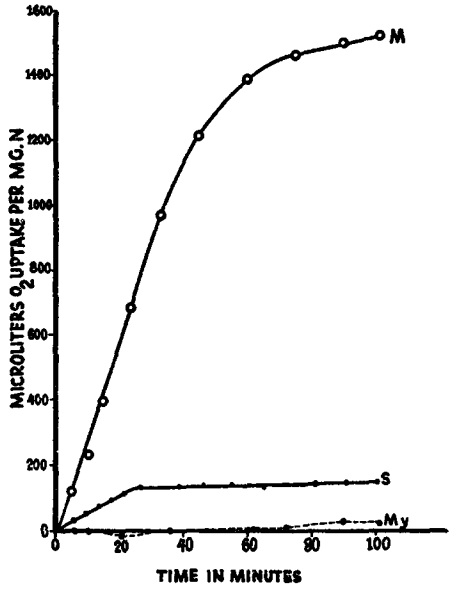
In the whole homogenates are found all the structures described above; yet, certain important distinctions are maintained. The mitochondria and sarcosomes are either free or held between the myofibrils, which remain clumped together, though many myofibrils lie freely in the medium. The pattern of change which occurs in the mitochondria during an experiment with whole

homogenates is similar to that seen in the mitochondrial experiment, except that targets are usually more numerous. The myofibrils also pursue a regular course of morphological changes. In the fresh homogenate they are in the relaxed state (Fig. 9). In actively oxidizing suspensions this state persists as long as the oxygen uptake is associated with a high target count. Such fibrils are observed to undergo repetitious reversible contractions, which are characterized by a splitting of the A discs, movement of its halves to the adjacent Z line and back again to the M line. Later in the experiments, when oxidation has declined and the mitochondria are predominantly crescent, the fibrils uniformly pass by slow, irreversible syneresis into a state of contraction with "C" bands (Fig. 14), and finally break up into amorphous granular fragments. Syneresis is a steady shrinkage, not associated with reversible contractions observed earlier in the relaxed fibrils. In presenting the results in sections 3 and 4 below, this distinction is maintained: the reversible process affecting the A disc is referred to as contraction (Figs. 9 to 12), and the slow shrinkage (Figs. 13 to 16) is regarded as a degenerative process or rigor. Although the reversible contractility is difficult to assess quantitatively and is interpreted as either present or absent, it is very conspicuous when present and occurs only in the relaxed type of fibril (Fig. 9). Hence, whenever it is indicated that contractions are absent, it is also understood that the fibrils have suffered syneresis and granular degeneration.

2. The Localization of Oxidative Activity.—The suspensions of mitochondria, sarcosomes, and myofibrils have very different oxidative capacities (Textfig. 2). Mitochondria have a considerable oxygen uptake, with a linear rate maintained for 30 to 60 minutes, followed by a rapid decline. The $Q_{O_2}^{O_2}$ N of a typical suspension is 1,080. On the contrary, the sarcosomes have a low oxygen uptake, which rapidly fails. The $Q_{O_2}^{O_2}$ N is typically around 180.0 and may be partly due to slight contamination with minute mitochondria. The purified myofibril suspensions, in which a small number of unruptured nuclei are present, have no detectable oxidative capacity with the tricarboxylic acid cycle substrates. Neither cytochrome *c* nor addition of methylene blue causes oxygen uptake. It is apparent that neither integrated oxidase systems nor isolated dehydrogenases are contained in the isolated myofibrils, whereas the mitochondria are rich and sarcosomal suspensions poor in oxidative enzymes.

In the mitochondrial suspensions there is some variation in the rate of oxidation during the first 45 minutes. As a measure of the rate of decline in oxidation of the suspensions, a value is calculated and designated as ^{t}h, which signifies the time at which the rate in oxygen uptake per 10 minutes is equal to one-half the rate observed during the first 10 minutes. There is considerable variation in ^{t}h values from one suspension to another. The breasts from pigeons kept in spacious outdoor cages which allow flight and maintain good general health yield suspensions which have a fair reproducibility in oxidative activity. On the contrary, suspensions from pigeons confined in small indoor cages,

which prevent flight, have low, irreproducible oxidative activity. Several factors were manipulated in an attempt to establish a low, constant rate of decline in



TEXT-FIG. 2. Relative oxidative capacity of the three pure suspensions. *M*, mitochondria; *S*, sarcosomes; *My*, myofibrils. 20 μM α-ketoglutarate per ml. fluid. Oxygen uptake in absence of substrate is subtracted.

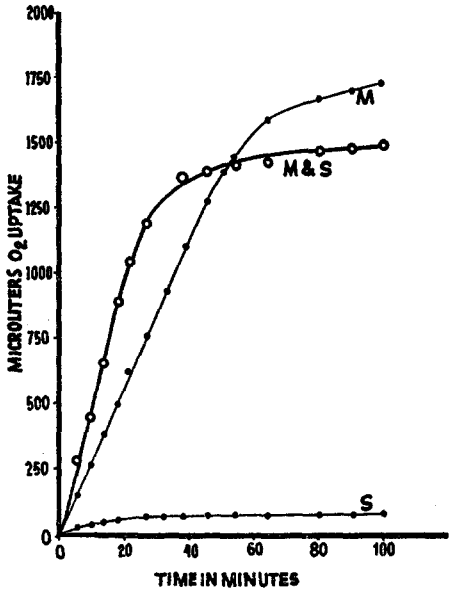
TABLE I

Sarcosome and Fluoride Effect on Mitochondrial Oxidation

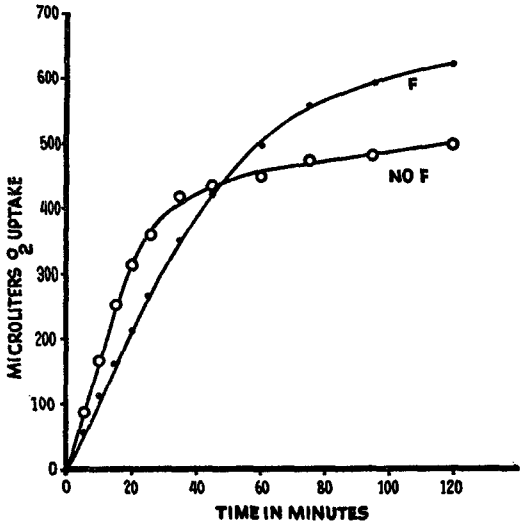
Experiment of Text-fig. No.	Condition in flask	t_h*
3	Mitochondria	65
	Sarcosomes	20
	Mitochondria and sarcomes	25
4	Mitochondria	25
	" and fluoride	60

* t_h is the time at which the rate in oxygen uptake per 10 minutes is equal to one-half the rate observed during the first 10 minutes of the experiment.

oxidation and to elucidate agencies responsible for the variation. Two factors cause considerable alteration: the addition of sarcosomes and the inclusion of fluoride (Table I). When sarcosomes are added to the mitochondrial suspensions there is a burst of oxidation which significantly exceeds the rate in the control suspensions (Text-fig. 3). The excess rate in oxygen uptake is greater



TEXT-FIG. 3. The effect on oxygen uptake of adding sarcosomes (*S*) to the system containing mitochondria (*M*). 20 μM α-ketoglutarate per ml. fluid. Oxygen uptake in absence of substrate is subtracted.



TEXT-FIG. 4. The effect on oxygen uptake of adding fluoride to the system containing mitochondria. 20 μM α-ketoglutarate per ml. fluid. Oxygen uptake in absence of substrate is negligible. 16.5 μM sodium fluoride (*F*) per ml. fluid where indicated.

than can be due to a simply additive oxygen consumption by the sarcosomes alone and is also seen under influence of stored sarcosomes, which have no oxygen uptake. In a series of experiments total uptake may be raised or lowered by addition of sarcosomes, but the ^{t}h in sarcosome-treated suspensions is always lower. Fluoride, on the contrary, in a carefully selected concentration, causes prolongation of ^{t}h and tends to depress total oxygen uptake (Text-fig. 4).

3. Oxygen Consumption of Whole Homogenates.—In the whole homogenate there is a very high endogenous respiration, which is not seen with purified mitochondria. This is augmented catalytically by inclusion of tricarboxylic acid cycle intermediates (Table II) and is similar to that described by Krebs

TABLE II

Catalytic Augmentation by Tricarboxylic Acid Cycle Intermediates of Oxygen Consumption in Whole Homogenates

Substrate	O_2 uptake	
	Theory for complete oxidation	Observed at 139 minutes
	$\mu l.$	$\mu l.$
Citrate	404	760
α-ketoglutarate	358	930
Succinate	314	950
Fumarate	269	1060
Oxalacetate	224	740
α-ketoglutarate	358	940
Pyruvate	224	460

2 μM substrates per ml. fluid.

Observed oxygen uptakes are corrected for endogenous blank.

(7) for minced pigeon breast muscle. It is apparent also that (Table III) the various substrates not only accelerate oxidation but also have an influence on maintenance of mitochondrial structure. If the target count in the flask without substrate at 24 to 25 minutes is taken as 100 per cent, it is found that in flasks containing substrates, even at 30 to 35 minutes, the number of targets is raised to 170 per cent with α-ketoglutarate, 280 per cent with succinate, 200 per cent with fumarate, 240 per cent with citrate, and to 140 per cent with pyruvate. In Text-fig. 5 is depicted the trend in target population of 11 experiments with α-ketoglutarate as substrate. The beneficial effect of the substrate is almost the same whether 2 or 20 μ moles of α-ketoglutarate per ml. fluid is used.

Not only is the presence of substrate important in the maintenance of oxidation, but the cofactors Mg, AMP, and DPN exert a beneficial action

(Text-fig. 6). A more detailed study of the individual cofactors is illustrated in Table IV. The exclusion of Mg has a profound effect: a rapid decline in oxygen uptake in association with complete disappearance of targets and transformation of nearly all mitochondria into crescents is observed. The

TABLE III

The Oxidation and Cytology of Whole Homogenates

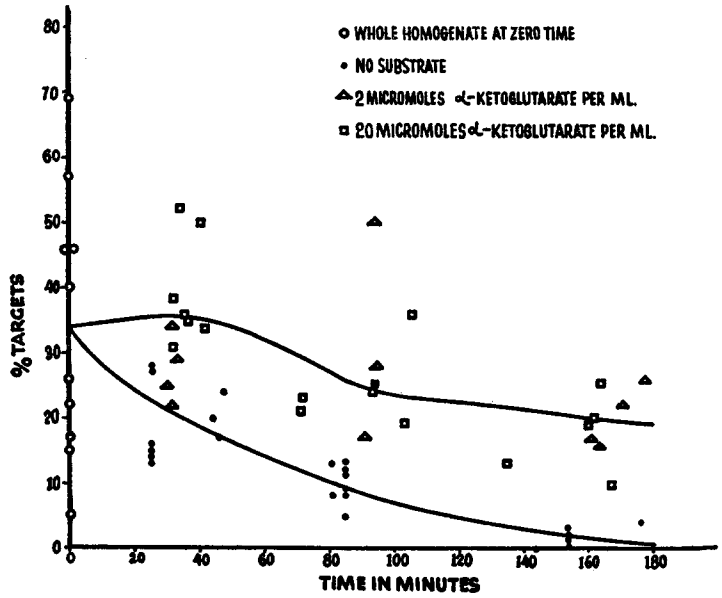
Time	Substrate	O_2 uptake	Targets	Crescents	Contraction*
min.		*μl.*	*per cent*	*per cent*	
24	—	541–659	9–16	33–57	+
36	α-Ketoglutarate	994	35	22	+
30	Succinate	1030	46	21	+
30	Fumarate	911	27	21	+
32	Citrate	1170	31	25	+
32	Pyruvate	728	38	33	+
54	—	1110–1280	3–11	63–80	+
60	Succinate	1540	19	48	0
63	Fumarate	1600	15	50	+
61	Citrate	2010	30	54	+
63	Pyruvate	1260	35	22	+
84	—	1450–1980	0–14	55–82	0, +
103	α-Ketoglutarate	2560	19	40	+
94	Succinate	2260	25	54	+
91	Fumarate	2080	14	48	+
90	Citrate	2640	18	50	+
95	Pyruvate	1830	19	57	+
114	—	1480–1810	0–8	72–89	0
121	Succinate	2680	15	63	+
120	Fumarate	2460	8	60	+
119	Citrate	3250	17	70	+
124	Pyruvate	2180	27	46	+

Data taken from 5 experiments on individual substrates.

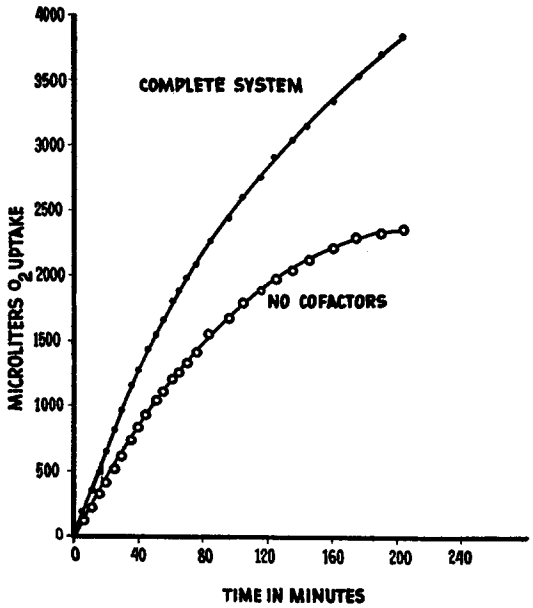
20 μM substrate per ml. fluid in all cases. Complete system is described under methods.

* This contraction signifies repetitious contractions of myofibrils.

omission of Mg is more detrimental to oxidation than omission of both AMP and DPN. When Mg is present either AMP or DPN is nearly as beneficial as their combination, and they can interchangeably augment the oxidation almost to the level of the complete system. This is interesting in view of the relationship established between ATP and DPN by Kornberg (19). Furthermore, the dependence of mitochondrial structure on AMP and DPN is shown in Table IV.



TEXT-FIG. 5. The behavior of targets in whole homogenates. Mitochondrial counts performed as indicated in the text. Time of removal of flasks from Warburg bath is shown.



TEXT-FIG. 6. The effect of cofactors on the oxygen uptake of the whole homogenate. 20 μM α-ketoglutarate per ml. fluid. To the flask without cofactors water is added to make 2.0 ml.

4. Interrelation of Oxidation and Myofibrillar Contractility in Whole Homogenates.—The data already presented in Tables III and IV indicate that the failure of oxidation and mitochondrial degeneration are concomitantly attended by disappearance of the spontaneous reversible contractions and structural degeneration of the myofibrils. Since it was demonstrated (Text-fig. 2) that the isolated myofibrils are apparently not associated with oxidase systems, the source of the energy needed for their maintenance and action is to be sought among other cytological components of the muscle fiber. It might

TABLE IV

The Effect of Exclusion of Various Cofactors on Oxidation and Cytology of Whole Homogenates

Time	Test system*	O_2 uptake	Targets	Crescents	Contraction	Relative‡ O_2 uptake
min.		*μl.*	*per cent*	*per cent*		
24	Complete	838	21	49	+	
33	−Mg	598	0	80	0	
54	Complete	1500	16	58	+	
64	−Mg	562	0	91	0	
84	Complete	1840	11	67	+	2.2
94	−Mg	840	0	92	0	1.0
24	−AMP, DPN	606	12	72	0	
32	Complete	1050	17	60	+	
54	−AMP, DPN	966	1	84	0	
62	Complete	1560	11	69	+	
84	−AMP, DPN	1080	0	90	0	1.0
92	Complete	1690	10	70	0	1.5
120	No cofactors	1500				1.0
"	Complete	2250				1.5
"	−AMP	2000				1.3
"	−DPN	2090				1.4
"	−Mg	1810				1.2
180	No cofactors	1360				1.0
"	Complete	2870				2.1
"	−AMP, DPN	2100				1.5
"	−Mg, DPN	1530				1.1
"	" AMP	1470				1.1

* 20 μM α-ketoglutarate in all flasks, otherwise, complete system as described in the text.

‡ The O_2 uptake of the most deficient system is taken as unity and the other more complete systems compared with it.

be expected on the basis of previous studies on cardiac muscle (1) that, since fluoride protects both oxidative phosphorylation and mitochondrial morphology, the incorporation of fluoride might protect both the mitochondria and myofibrils. The experiments in Table V illustrate the effect of adding fluoride. A considerable preservation of form and contractility is observed in the presence of fluoride when 20 μM of substrate per ml. fluid is used. With 2 μM substrate or in absence of substrate the fluoride exerts no protection. Fluoride

TABLE V

The Effect of Fluoride on Oxidation and Cytology of Whole Homogenates

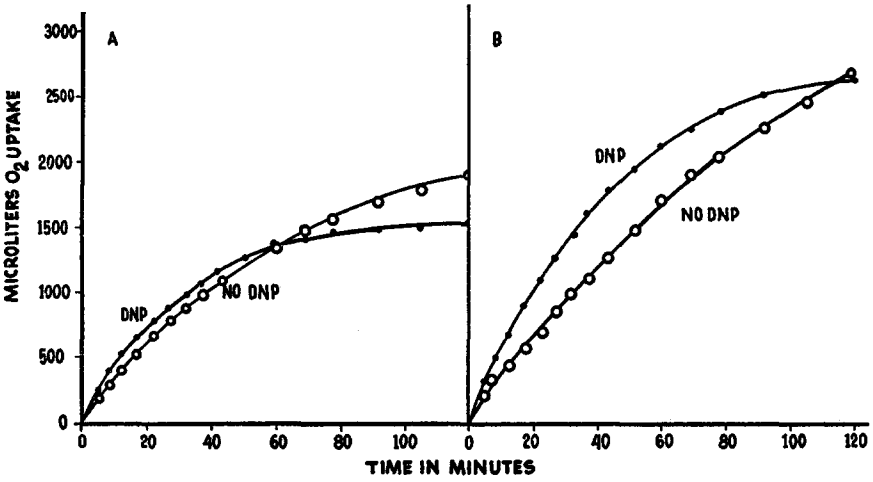
Time	α-Keto-glutarate*	NaF	O_2 uptake	Targets	Crescents	Contraction
min.	*μM/ml.*	*33μM/ml.*	*μl.*	*per cent*	*per cent*	
25	0	—	751	16	35	+
40	"	Present	246	0	53	0
85	"	—	1780	8	63	0
102	"	Present	290	0	80	0
154	"	—	2360	1	50	0
174	"	Present	327	0	80	0
33	2	—	1040	29	8	+
45	"	Present	531	16	24	+
91	"	—	2160	17	43	+
108	"	Present	789	0	70	0
164	"	—	3120	16	68	+
180	"	Present	1700	3	69	0
32	20	—	851	38	42	+
45	"	Present	826	46	32	+
93	"	—	2080	24	43	+
106	"	Present	1580	36	45	+
160	"	—	3020	19	70	+
174	"	Present	2040	29	48	+

* Complete system is as described under methods.

suppresses oxidation in all cases, particularly in the absence of any added substrate.

The evidence thus far accumulated indicates that oxidation is necessary in the preservation of structure of mitochondrial form and myofibrillar activity and implicates an additional mechanism which fluoride safeguards. In view of the known ability of fluoride to inhibit ATPase and certain phosphorolytic enzymes (20), it seems likely that oxidative phosphorylation may be the mechanism. On this account, the effect of DNP was studied, because of its well established property of uncoupling oxidation and phosphorylation (21). In Text-figs. 7A and B is depicted the stimulation of oxidation by DNP; it

accelerates the initial rate but does not appreciably affect the total uptake. The degeneration of the mitochondria is evident in Table VI and is accom-



TEXT-FIG. 7. The effect of DNP on the oxygen uptake in the complete whole homogenate system. *A*, no α-ketoglutarate; *B*, 20 μM α-ketoglutarate per ml. 1×10^{-4} M DNP is present where indicated.

TABLE VI

The Effect of DNP on Oxidation and Cytology of Whole Homogenates

Time	α-Keto-glutarate	1×10^{-4} M DNP	O_2 uptake*	Targets	Crescents	Contraction
min.	*μM./ml.*		*μl.*	*per cent*	*per cent*	
29	0	Present	927	10	59	0
46	"	—	1220	17	44	+
67	"	Present	1400	0	74	0
81	"	—	1770	13	45	0
22	20	Present	1160	28	23	0
35	"	—	1100	36	31	+
60	"	Present	2170	3	70	0
72	"	—	1930	23	44	+

* See Text-fig. 7 for further details of oxygen uptake in these experiments.

panied by a rapid loss of myofibrillar contractions and an ensuing granular degeneration.

DISCUSSION

In the present study it has been shown that the mitochondria in muscle, as in other viscera, contain the bulk of the Krebs cycle oxidative enzymes,

and that the sarcosomes have a slight, transient capacity for oxidation, which may be due to contamination with minute mitochondria. But the most interesting feature of the sarcosomes is their ability to increase the rate of oxidation by the mitochondria. The precise mechanism of the stimulation is not apparent; several are possible. It may be that this is similar to the observation of Pressman and Lardy (22), who found in the submicroscopic particles of liver a factor which accelerates mitochondrial metabolism. However, the sarcosomes are much larger than microsomes and cannot be identified morphologically with either the minute mitochondria of Laird *et al.* (23) or microsomes. The influence of the sarcosomes on mitochondria indicates that their interaction plays a role in the regulation of muscle metabolism.

The demonstration that the oxidative system is predominantly located in the cytochondria is in agreement with the observation of Williams and Watanabe (10), that the succinoxidase system is localized exclusively in the giant mitochondria of striated muscles which propel the wings of higher insects. On the contrary, Szent-Györgyi (24) has pointed out that succinoxidase and citric oxidase systems are linked with water-insoluble structure in washed rabbit muscle residues. On the basis of parallel effects exerted by ATP and ions on both oxygen uptake and the physical state of actomyosin, he concluded that the oxidase systems and actomyosin formed a single functional unit. The attempts to separate dehydrogenase activity and actomyosin were unsuccessful. In view of the present separation of oxidative enzymes and the myofibrils, it is apparent that preliminary purification of the myofibrils from cytochondria is prerequisite to analysis of enzyme distribution. It is manifest that Szent-Györgyi and coworkers thoroughly extracted the myofibrils and granules simultaneously and consequently found both actomyosin and oxidases in the extract. Furthermore, the acceleration of oxygen uptake by ATP, Mg^{++}, K^{+}, and Ca^{++} is peculiar to the behavior of the integrated enzyme system in the mitochondria of many tissues. It is fortuitous that these factors also induce syneresis of actomyosin.

The segregation of oxidative enzymes in the mitochondria and their absence from the myofibrils indicates that the morphological and functional state of myofibrils is dependent on the cytochondria. Proof of such a relationship is afforded by the behavior of the myofibrils under conditions which alter the structure and activity of the mitochondria. The connection between the mitochondrial oxidation and functional behavior of the myofibrils is drawn closer by the action of DNP and fluoride on both systems. From the previous studies on oxidative phosphorylation in mitochondria (1), it is established that DNP impairs the phosphorylation and destroys structure, whereas fluoride protects phosphorylation and mitochondrial form. The present results demonstrate a deleterious effect of DNP on mitochondria and myofibrils synchronously, and a protective action of fluoride on them. It seems that the

link between the preservation of mitochondrial function and myofibrillar function is the maintenance of an adequate level of ATP by oxidative phosphorylation.

The use of fluoride and DNP in the homogenate provides suggestive evidence of the efficiency of the oxidative and glycolytic processes in maintaining the mitochondrial-myofibrillar system. It is clear from the work of Slater (25) and of Borei (26) that fluoride has complex and manifold effects on the oxidative chain, as well as suppressing glycolysis and impeding ATPase. It is probably because of the interference with oxidation, as well as the inhibition of glycolysis, that higher levels of substrate are required to maintain morphology and oxidation in the presence of fluoride. But if it is assumed, as Clowes and Keltch (27) indicate, that glycolytic phosphorylation is more sensitive to fluoride than is aerobic phosphorylation, the beneficial action on the muscle homogenate may be interpreted as showing the ability of oxidative phosphorylation mainly to sustain the system. On the other hand, the same authors (28) have shown that dinitrocresol, closely related to DNP, can effectively suppress aerobic phosphorylation and leave unimpaired the anaerobic glycolytic phosphorylation mechanism. Glycolytic phosphorylation, if its efficacy can be assessed with DNP, appears ineffective in maintaining preservation of mitochondria and myofibrils in muscle homogenates.

SUMMARY

In pigeon breast muscle the mitochondria are the principal site of oxidative metabolism, whereas the myofibrils are incapable of oxidizing intermediates of the Krebs cycle. The mitochondria contain the oxidative enzymes, and the sarcosomes are associated with a factor which accelerates the mitochondrial oxidative rate. The maintenance of myofibrillar contractility and structure is closely correlated with preservation of mitochondrial oxidative capacity and structure. By use of fluoride and dinitrophenol the connection between mitochondrial metabolism and myofibrillar behavior is shown to occur through the process of oxidative phosphorylation.

BIBLIOGRAPHY

1. Harman, J. W., and Feigelson, M., *Exp. Cell. Research*, 1952, **3**, 509.
2. Kitiyakara, A., and Harman, J. W., *J. Exp. Med.*, 1953, **97**, 553.
3. Ranvier, L., *Arch. Anat. u. Physiol., Physiol. Abt.*, 1874, 1.
4. Bonhöffer, K., *Arch. ges. Physiol.*, 1890, **47**, 125.
5. Battelli, F., and Stern, L., *Biochem. Z.*, 1912, **46**, 317.
6. Stare, F. J., and Baumann, C. A., *Cold Spring Harbor Symp. Quant. Biol.*, 1939, **7**, 227.
7. Krebs, H. A., and Johnson, W. A., *Enzymologia*, 1937, **4**, 148.
8. Harman, J. W., *Exp. Cell. Research*, 1950, **1**, 382.
9. Watanabe, M. I., and Williams, C. M., *J. Gen. Physiol.*, 1951, **34**, 675.

10. Williams, C. M., and Watanabe, M. I., *Science*, 1952, **115**, 488.
11. Paul, M. H., and Sperling, E., *Proc. Soc. Exp. Biol. and Med.*, 1952, **79**, 352.
12. Johnson, M. J., *J. Biol. Chem.*, 1941, **137**, 575.
13. Harman, J. W., and Feigelson, M., *Exp. Cell. Research*, 1952, **3**, 47.
14. Potter, V. R., *J. Biol. Chem.*, 1947, **169**, 17.
15. Jordon, H. E., *Physiol. Rev.*, 1933, **13**, 301.
16. Hanson, J., *Nature*, 1952, **169**, 530.
17. Harman, J. W., and Gwinn, R. P., *Am. J. Path.*, 1949, **25**, 741.
18. Harman, J. W., and Feigelson, M., *Exp. Cell. Research*, 1952, **3**, 58.
19. Kornberg, A., *J. Biol. Chem.*, 1950, **182**, 779.
20. Judah, J. D., and Williams-Ashman, H. G., *Biochem. J.*, 1951, **48**, 33.
21. Judah, J. D., *Biochem. J.*, 1951, **49**, 271.
22. Pressman, B. C., and Lardy, H. A., *J. Biol. Chem.*, 1952, **197**, 547.
23. Laird, A. K., Nygaard, O., Ris, H., and Barton, A. D., *Exp. Cell. Research*, in press.
24. Szent-Györgyi, A., Chemistry of Muscular Contraction, New York, Academic Press, Inc., 1947, 80.
25. Slater, E. C., and Bonner, W. D., *Biochem. J.*, 1952, **52**, 185.
26. Borei, H., *Ark. Kemi Mineral. och Geol.*, 1945, **20A**, No. 8.
27. Clowes, G. H. A., and Keltch, A. K., *Proc. Soc. Exp. Biol. and Med.*, 1952, **81**, 356.
28. Clowes, G. H. A., and Keltch, A. K., *Proc. Soc. Exp. Biol. and Med.*, 1951, **77**, 369.

EXPLANATION OF PLATES

PLATE 8

FIG. 1. A suspension of pigeon breast mitochondria. Two rods are seen, with light, refractile facets on their surfaces. To one side is a target, with its central refringent area. Phase contrast, dark L. × 900.

FIG. 2. A single target type mitochondrion is seen, composed of the dense periphery and central refringent area. Refocusing does not eliminate this distinction as occurs with surface reflection. Phase contrast, dark L. × 900.

FIG. 3. A target mitochondrion is seen, with the surface bosselations in the central refractile area. Phase contrast, dark L. × 2,700.

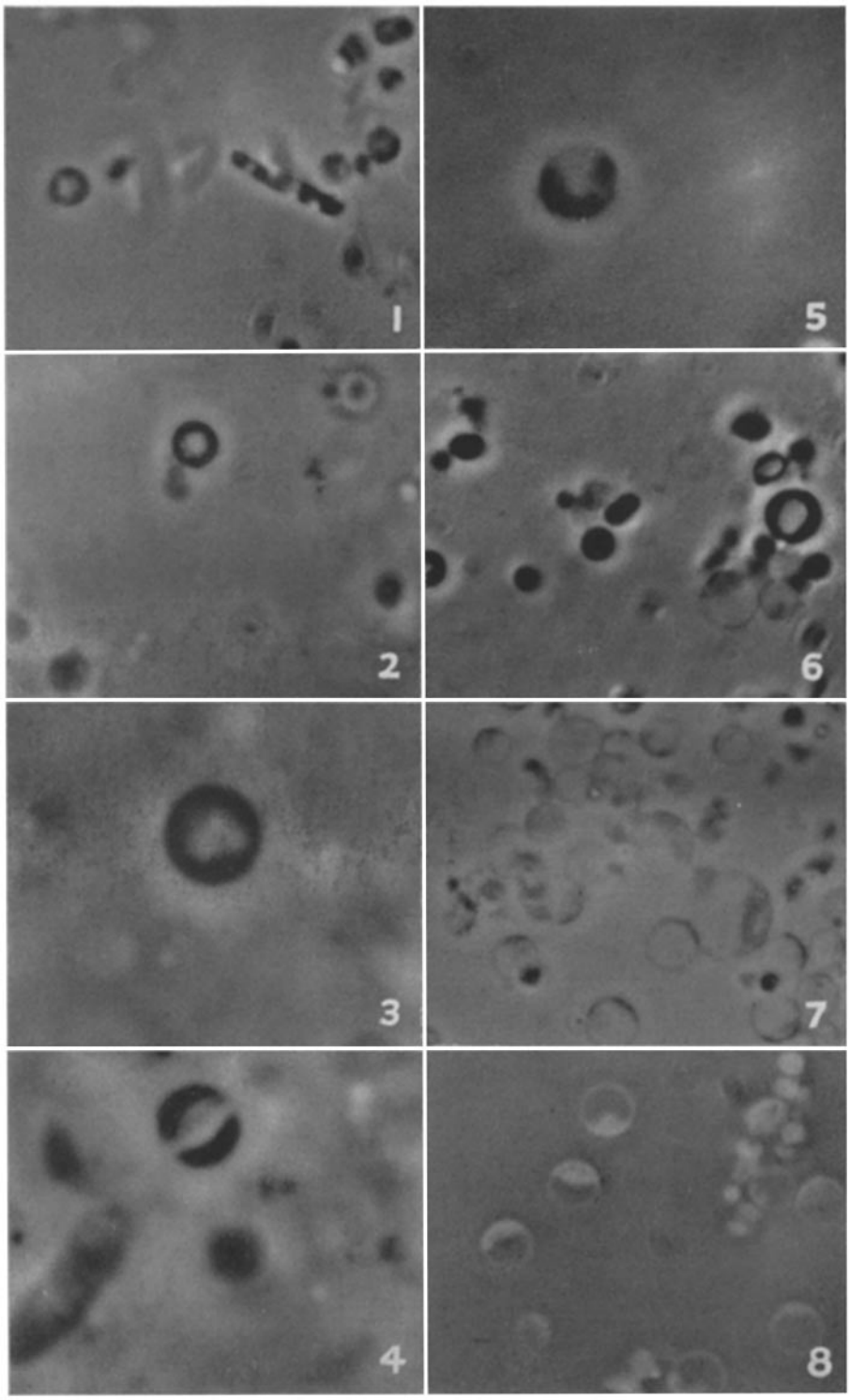
FIG. 4. A target mitochondrion is seen, in which the central area is in the concavity of the body and resembles a visor. To one corner is a rod, containing areas of varied density. Phase contrast, dark L. × 2,700.

FIG. 5. A target has rolled over on its side, showing that the distinction between areas depends on density and not surface effect. Phase contrast, dark L. × 2,700.

FIG. 6. The population of mitochondria consists of small rods, spherical dense, target, and two crescent mitochondria. With refocusing it is not possible to elicit refringency in the spherical dense forms. Phase contrast, dark L. × 900.

FIG. 7. A suspension of mitochondria and sarcosomes has been treated with distilled water at 0°C. for 180 minutes. All mitochondria are crescents, with a polar, marginal demilunar density. Several tiny, dense sarcosomes are seen. Phase contrast, dark L. × 900.

FIG. 8. A water-treated suspension of mitochondria and sarcosomes. A single, dense, bright sarcosome is seen among the swollen mitochondria. Phase contrast, light L. × 900.



(Harman and Osborne: Cytochondria and myofibrils in skeletal muscle)

PLATE 9

FIG. 9. A pair of myofibrils in an homogenate of pigeon breast muscle, suspended in phosphate buffer. The relaxed state is evident by the broad A disc and narrow I segment with the vague Z line. Phase contrast, dark L. × 1,700.

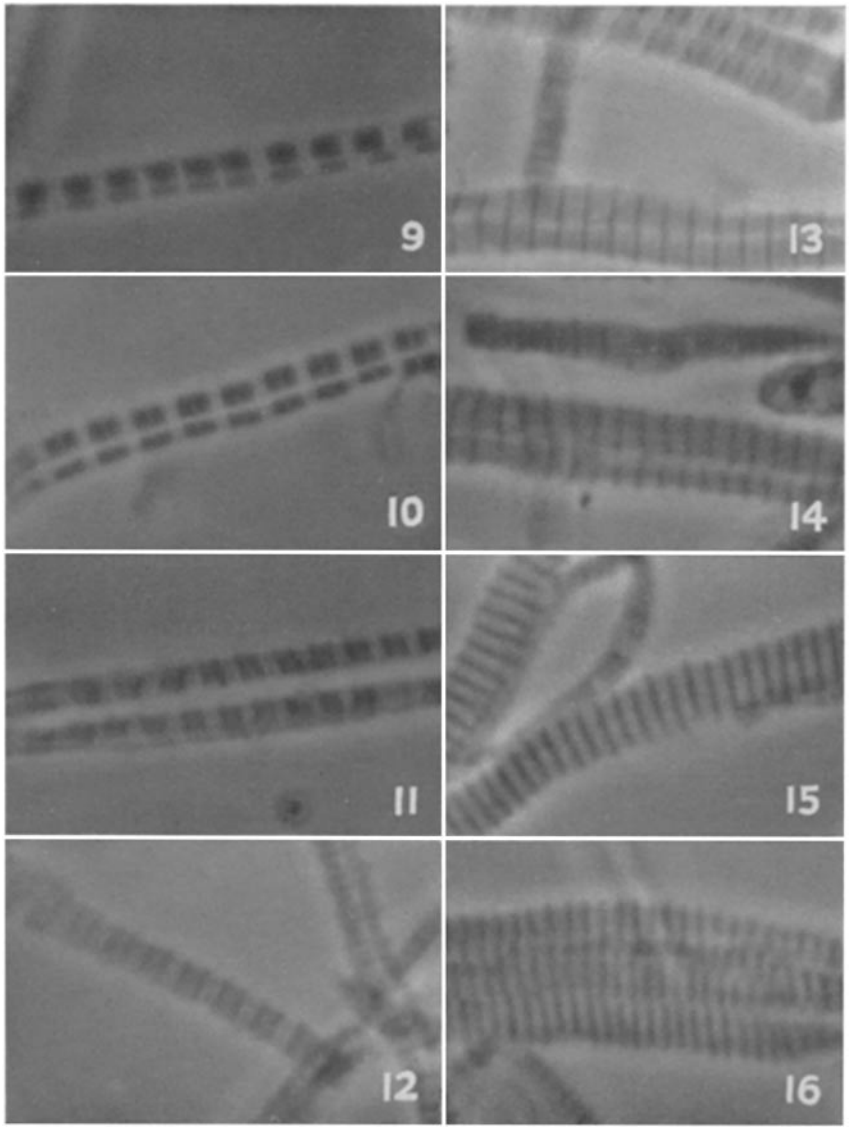
FIG. 10. The pair of myofibrils, in initial contraction, show splitting of the A disc, which is now composed of two discs separated by a median isotropic zone. Phase contrast, dark L. × 1,700.

FIG. 11. A further stage of contraction is illustrated in this pair of fibrils, where separation of the A disc fractions is wider. Phase contrast, dark L. × 1,700.

FIG. 12. In another fibril the contraction is caught at a slightly later stage, when the A disc fractions are closer to the Z lines and are separated by a very wide zone. This extent of contraction is the approximate limit of the reversible, rhythmical contraction. Reversal consists in passing back through the changes illustrated in Figs. 11, 10, and 9 in that order. Phase contrast, dark L. × 1,700.

FIG. 13. The lower fibrils contain contraction bands (C bands) formed by the fusion of two adjacent A fractions at the Z line. The light area between the very dense contraction bands is bisected by a vague M line. The upper fibrils are in mid-contraction, with the A fractions moving either toward or away from the Z lines. Phase contrast, dark L. × 1,700.

FIGS. 14 to 16. The myofibrils are in progressive states of syneresis, with the contraction bands approximating more closely and the intervening isotropic zone undergoing obliteration. The fibrils are also thicker than in the preceding figures. Phase contrast, dark L. × 1,700.



(Harman and Osborne: Cytochondria and myofibrils in skeletal muscle)