ASSAYS OF THE METABOLIC VIABILITY OF SINGLE GIANT MITOCHONDRIA

Experiments with Intact and Impaled Mitochondria

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ABSTRACT

Single giant mitochondria isolated from mice fed cuprizone were assayed for their metabolic viability. Two tests were devised. One test optically detected the accumulation of calcium phosphate within the mitochondria under massive loading conditions (including the presence of succinate and ATP). The accumulation corresponds to a test of energy coupling from either electron transport or the hydrolysis of ATP since it is blocked by either antimycin A or oligomycin. The other assay tested for the production of ATP from ADP and P_i, using myofibrils. Myofibrils prepared from glycerinated rabbit psoas muscle contract only in the presence of ATP and not in the presence of ADP. Myofibrillar contraction is unaffected by the presence of antimycin A or oligomycin. However, myofibrils in the presence of mitochondria that are phosphorylating ADP to ATP do contract. This contraction is blocked by antimycin A and/or oligomycin. Hence, the ATP which causes myofibrillar contraction is produced by oxidative phosphorylation. At low mitochondrial concentration, only the myofibrils in close proximity with mitochondria contract in the presence of ADP. Therefore the assay can be used to test the viability of individual mitochondria. Individual giant mitochondria were found to be viable, using both of these assays. Comparable results were obtained in mitochondria impaled with microelectrodes. The potentials and resistances were unaffected by concomitant calcium phosphate accumulation or oxidative phosphorylation.

KEY WORDS mitochondrial potentials · microelectrodes · calcium phosphate transport · oxidative phosphorylation

Large mitochondria are produced in the livers of cuprizone fed mice (14, 15, 20, 21). Most of the mitochondria are approximately equal in size to those isolated from normal mice. However, a variable fraction of the mitochondria range in size from 5 to 10 μm and in some exceptional cases 15 μm. Certain isolation procedures increase the proportion of the larger mitochondria (21). A study of such mitochondrial suspensions show reasonably good coupling (P/O ratios with succinate of about 1.4) and, therefore, most of the population is well coupled. In the present study the mitochondria had a P/O ratio of 1.6 ± 0.2 with succinate (15). Nevertheless, there would be

considerable advantage in assaying the transducing ability of the individual mitochondria. The large mitochondria allow for direct experimental manipulation with microelectrodes. Therefore, mitochondrial membrane potential and resistance and the transducing ability can be monitored concomitantly.

The respiratory activity of a single giant mitochondrion was measured by Suchy and Cooper (20) with a miniaturized oxygen electrode. A respiratory control ratio of 3.7 was reported. Other approaches could also be used to test the transducing ability of the mitochondria. For example, it should be possible to follow the reduction of pyridine nucleotides from succinate by the energy requiring reverse flow of electrons (4, 7). Similarly, a number of probes such as 8-anilino-1-naphthalene sulfonic acid (ANS), in the presence of mitochondria, display a fluorescence response which is energy dependent (e.g., see references 2 and 17). In fact, the technology for the microscope measurements of fluorescence already has been used effectively (e.g., to measure the reduced pyridine nucleotide fluorescence of nuclei of single cells [12]). These techniques could be adapted for studies with isolated giant mitochondria. We preferred to devise more direct assay procedures of transducing ability.

Massive amounts of calcium phosphate are accumulated by metabolically active rat liver mitochondria (10, 13). The precipitate appears in the electron microscope as large electron-dense granules (10). In the process of accumulation, the dry mass of the mitochondria increases significantly (10, 13). Conceivably, these granules could be detectable with Nomarski interference optics (1), and an increase in dry mass should be measurable with Jamin-Lebedeff interferometry (e.g., references 8 and 9). In fact, both techniques were found to serve as effective assays of transducing ability in the mouse liver mitochondria.

Myofibrils prepared from glycerinated psoas muscle of rabbit (16, 19) can be used as a sensitive detector of ATP. The production of ATP by mitochondria should then be detectable at least at the site of contact between mitochondria and the myofibrils, if the ATP production is at a rate high enough to maintain a local concentration which will cause myofibrillar contraction.

The present experiments show that the two assay procedures can be effectively used to test for the transducing ability of individual mitochondria. Application of these techniques to mitochondria impaled with microelectrodes shows that the mitochondria are functional. Neither the process of oxidative phosphorylation nor the calcium phosphate accumulation was found to alter the membrane potential or resistance.

MATERIALS AND METHODS

Chemicals

Antimycin A, oligomycin, ATP (highest purity disodium salt), and ADP (grade 1) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Antimycin A and oligomycin were used in a concentration of 1 and 100 μM, respectively.

Manipulation and Preparatory Procedures

Details of the preparation of the mitochondria, and those referring to the preparation and manipulations carried out with the microelectrodes, have been presented previously (15). For the experimental determinations, the temperature was maintained between 19° and 25°C.

Calcium Phosphate Accumulation Assay

The calcium phosphate accumulation was carried out under the following conditions. The suspension medium was 0.30 osmolal sucrose, 1 mM 2(*N*-morpholino)-ethane sulfonic acid (MES), 5 mM KCl, pH 7.4. The mitochondria were resuspended in 0.25 osmol/l sucrose, 2 mM $MgCl_2$, 2 mM NaH_2PO_4, 10 mM Tris chloride, 10 mM NaCl, 2 mM $CaCl_2$, 10 mM Na succinate and 4 mM ATP, pH 7.4. Initially, in each experiment either phosphate, Ca^{2+}, ATP, or succinate was omitted. At zero time, the omitted component was added by flooding the agar overlay with an aliquot of the medium with the missing component added at the appropriate concentration. The final concentration of the component corresponded to that of the complete medium, assuming equilibration between the solution added on top of the agar and the preparation under the agar. Generally, the granulation appeared clearly within 3–5 min after addition. The granulation was most evident when the mitochondria were focused to produce an equatorial optical section. No granulation appeared in the absence of these additions.

The accumulation underlying the granulation can be measured quantitatively with polarized normal interference microscopy. In these studies, no agar overlay was used since the irregularities of its thickness interfered with the measurements. The suspensions were placed on a conventional glass slide (1 mm in thickness) and covered with a conventional glass cover slip (0.1 mm in thickness). The measurements were carried out with the Jamin-Lebedeff system using a matched strain-free 40 × 0.65 NA objective and condenser (Carl Zeiss, Inc., New York) with the de Sénarmont method of compensation (9, 23). This system was set up on a Zeiss

Universal microscope equipped with an HBO 200 W/4 mercury burner and a KG-1 heat filter, a 546-nm interference filter and a 546-nm λ/4 plate (Carl Zeiss, Inc.). Measurements were made by eye to the point of maximum darkness (extinction) for the mitochondria vs. the background as described by Forer and Goldman (8). Since the mitochondria could not be held immobile by an agar overlay, the control (the mitochondria suspended in the accumulation medium with one component omitted) and the experimental suspensions (in the presence of the complete medium) were aliquots of the same suspension. It was not possible to compare the same mitochondria before and after the addition of the omitted component with this technique. Generally, individual mitochondria with diameters of about 6 μm were selected for analysis from a group of mitochondria in the field, or, alternatively, a single mitochondrion was the sole object in the field. There was no significant difference between the two approaches. The image was set at maximal background extinction vs. maximal brightness of the mitochondria by rotation of the polarizer with a fixed analyzer as described by Forer and Goldman (8). The analyzer was then rotated to reverse this contrast, and the degrees of rotation were recorded. The magnitude of this rotation is directly proportional to the dry mass. A similar approach has been used previously (3) to follow the deposition of calcium phosphate in the quantitative determination of alkaline phosphatase kinetics in rat kidney tubules and duodenum.

The theoretical and practical aspects of these measurements have been discussed in some detail by others (3, 5, 6). Briefly, the dry mass (M) of a component in water (in grams) per unit area, in an homogeneous object of thickness t (centimeters) is given by,

$$M = \frac{\Phi w}{\chi} \tag{1}$$

Φ is the optical path difference (in centimeters) in water, corresponding to $(\mu_0 - \mu_w)t$, where μ_0 and μ_w are the refractive indices of the object and the water respectively and χ is 100 × the specific refractive increment. Since χ is approximately constant for most substances in water (about 0.18), Eq. 1 allows for the determination of the dry weight from the optical path difference. The application in the case of deposits is not clear (see Results and Discussion) and may require a different value for χ. Assuming that the deposition is that of calcium phosphate salt, χ will be approx. 0.11 ± 0.02 (3) (see Results and Discussion).

In practical terms, path difference obtained in experiments can be used to calculate dry mass of an object (e.g., a cell) as shown by Eq. 2 (e.g., see reference 23):

$$M = \frac{A\Delta\delta}{\chi} + (n_m - n_w)\frac{V}{\chi}. \tag{2}$$

$\Delta\delta$ is the optical path difference (centimeters). A is the cross sectional area of the object, V, the volume, and n_m and n_w are the refractive indices of the medium and of water, respectively.

In the calculations discussed in the Results and Discussion section, the following assumptions were made: the mitochondrion is a perfect sphere 6 μm in diameter (in a random sample of seven, it was 6 ± 1 μm); n_m = 1.345; n_w = 1.333; λ = 546 nm = 180° = 5.46×10^{-5} cm; and χ = 0.18 or 0.11 (see Results and Discussion).

Myofibril Contraction Assay

Myofibrils were prepared from rabbit psoas muscle after glycerination as previously reported (16, 19).[1] The muscles were stored at −15°C for over six wk to reduce Ca^{2+} sensitivity (see reference 16). The myofibrils were isolated after glycerination in 40 mM KCl, 1 mM $MgCl_2$, and 10 mM piperazine-*N,N'*-bis(2-sulfonic acid) (PIPES), pH 6.8, and resuspended in 5 mM KCl, 5 mM PIPES, 5 mM $MgCl_2$, and 5 mM potassium ethyleneglycol-bis-(β-amino-ethyl ether)*N,N'*-tetraacetic acid (EGTA), pH 6.8.

After isolation, the mitochondria were suspended in 0.30 osmol/l sucrose, 1 mM MES, and 1 mM KCl, pH 7.4. The assays for phosphorylative ability were carried out after mixing isolated mitochondria and myofibrils. The assay procedure was carried out in 0.30 osmol/l sucrose, 1 mM MES, 5 mM PIPES, 5 mM $MgCl_2$, 5 mM KCl, 3 mM Na succinate, 5 mM potassium EGTA, and 5 mM $KHPO_4$, pH 7.2. ADP or ATP was added to the preparation on the agar overlay, either alone or dissolved in the assay medium. No significant differences were noted between the results obtained with these two different kinds of solutions. Occasionally, when the agar layer had small discontinuities, it was possible to perfuse the trapped mitochondrion and the myofibrils, thereby flushing out extraneous material (see Fig. 7). Therefore, the results summarized in this study (Table IV) represent two distinct experimental designs. In one, the ADP is allowed to diffuse through the agar layer. In the other, the ADP is added by perfusion of the preparation. A mitochondrial concentration was selected (below 0.01 mg protein/ml) at which the addition of ADP did not produce a contraction except when the mitochondria were in close proximity to a myofibril (usually 10 μm or less). Fields were selected to which only single mitochondria were found in proximity to the myofibrils. This required focusing through several levels since in differential interference optics the image represents an optical section about 1 μm thick. The myofibril concentration was approx. 0.5 mg/ml. A similar assay with myofibrils was used previously by others to demonstrate the viability of the contractile machinery of cells irradiated by

[1] In a study now in progress (C. Bowman and H. Tedeschi), occasionally it was found necessary to remove myokinase from the myofibrils by exposing them to 0.01% Triton X-100 for 15 s, followed by an immediate dilution and washing.

laser for microdissection (11).

Generally, contractility was judged by eye at 500 × from the shortening (in excess of about 20%) and thickening of the fibers. Contraction was verified from photographs of small samples in a few doubtful cases. In these, contraction was also verified from the shortening of the sarcomere length.

RESULTS AND DISCUSSION

The assay for ability to accumulate calcium is illustrated in Fig. 1. This figure is a micrograph taken with differential interference optics after Nomarski (1). The mitochondria shown in Fig. 1*a* represent controls which were in the calcium accumulation medium with one component omitted (in this case, ATP). Those in part *b* depict mitochondria in the same medium after the addition of the omitted component. There is no question that a change in the optical path has occurred at discrete places within the mitochondria. This change in the optical path leads to a granular appearance (see mitochondria marked with arrows). Further (see below), the granulation is accompanied by an increase in dry mass in the mitochondria as evidenced by our studies with Jamin-Lebedeff optics. The results of four experiments with control and impaled mitochondria using this technique are summarized in Table II. This table will be discussed in detail later. In all Tables and in the Discussion, the results are expressed as means ±SD. When the SD is not stated, the results are individual determinations.

The studies with interference (i.e., Jamin-Lebedeff) optics are illustrated by Fig. 2 which represents typical sets of micrographs using de Sénarmont compensation (λ/4 plate and rotation of analyzer) and the method of maximum extinction. The mitochondria shown in the first two pairs, *a* and *b*, and *c* and *d*, are controls to which the ATP necessary to complete the accumulation medium had not been added. In Fig. 2*a*, the polarizer was set for maximal background extinction with the analyzer set at zero. Clockwise rotation of the analyzer (in this case 127°) causes the mitochondrion to appear as dark as the previous background (Fig. 2*b*). Fig. 2*c* and *d* represent similar matched micrographs in which the rotation was 122°. Fig. 2*e* and *f* represent the corresponding experimental determinations, and in this case the rotation is 152°. All preparations in which mitochondria appeared to form granules with differential interference optics exhibited a change in optical path as determined with Jamin-Lebedeff optics.

Generally, with interference optics the mitochondria appear relatively uniform (e.g., Fig. 2*a* and *b*). However, irregularities and spots were noted occasionally on the photographs of both control and experimental preparations (see Fig. 2*c* and *e*). Sometimes the spots may correspond to the granulation sites noted with the differential interference optics. The error caused by eye matching in these cases is probably not serious since repeated estimates of the same mitochondrion differ only by a few degrees (note the low SD's in Table I). The difference in the image between the two microscopic systems is probably accountable by the fact that the image obtained with differential interference optics corresponds to a derivative of the optical path difference in an optical section (1). In contrast, the Jamin-Lebedeff system detects changes in optical path for the whole object. The uniformity in some of the Jamin-Lebedeff micrographs suggests that generally the granules were distributed throughout the mitochondrion. The results of three experiments are summarized in Table I.

The accumulation of calcium phosphate is probably in the form of amorphous deposits. The granules of mitochondria isolated from rat liver were found to be amorphous by either electron microscopy or electron and X-ray diffraction (10, 22). We tested whether the deposits were birefringent. The mitochondria in the complete accumulation medium and exhibiting granulation with the differential interference optics were examined with a 546-nm λ/4 plate and linearly polarized light at 546 nm with a crossed polarizer. No bright spots were observed on the mitochondria as the stage was rotated through 180°.

It is clear from the results of Table I that there is a change in the optical path when the mitochondria are in the accumulation medium. This change can only be from a change in dry mass. However, it is difficult to translate these measurements to quantitative estimates using Eq. 2 (see Materials and Methods). The reason for this difficulty resides in the fact that the constant χ (100 × the specific refractive increment), which is usually rather invariant (at about 0.18), does depend on the nature of the substance in the case of inorganic deposits. Various values of χ are reported for calcium phosphate crystals (see reference 3), and these are a function of the hydration of the crystals, ranging from 0.098 for hydroxyapatite to 0.13 for $Ca_2P_2O_7 \cdot 5H_2O$. Also, there is consider-

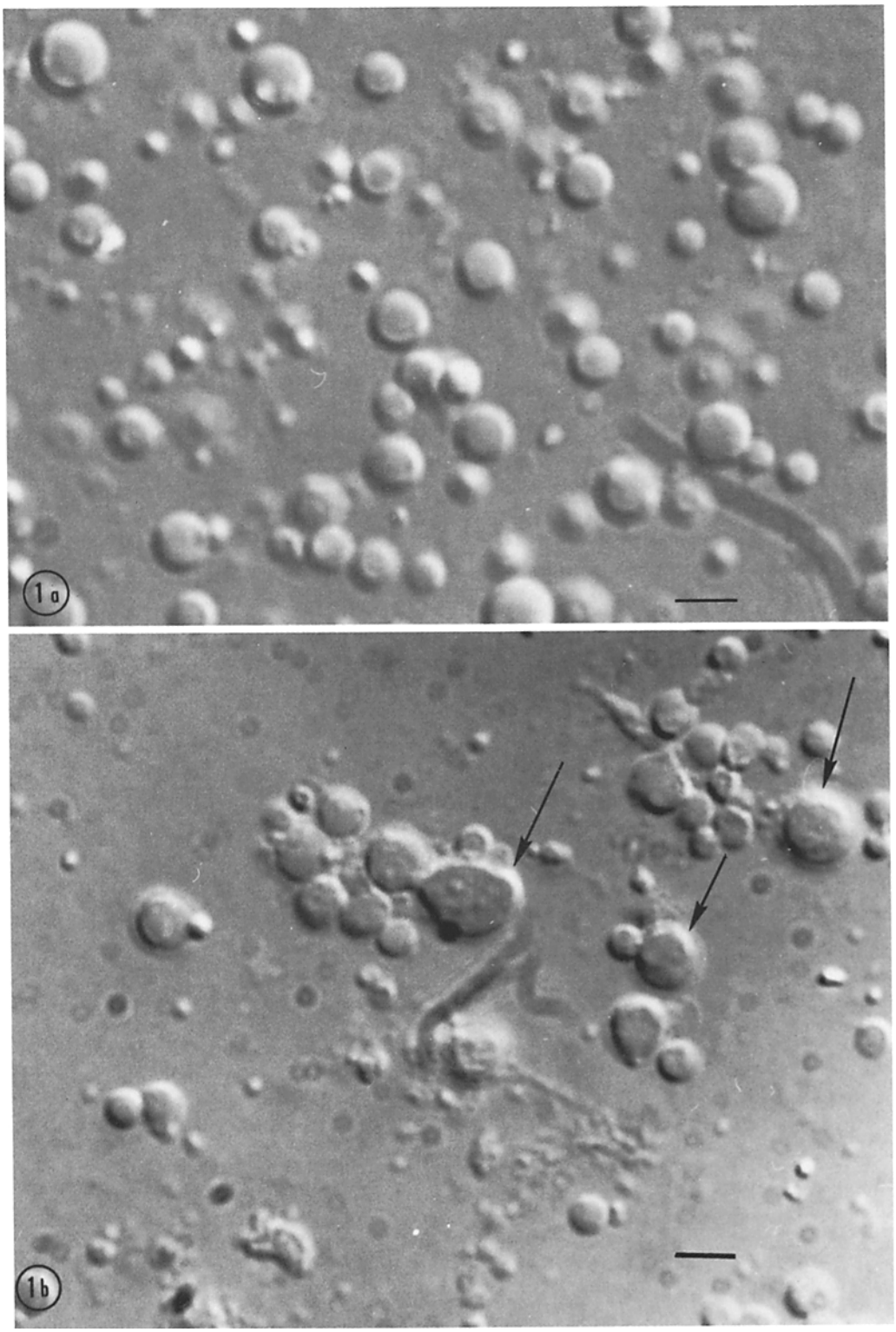

FIGURE 1 The assay of calcium phosphate accumulation. The mitochondria are viewed with Nomarski differential interference microscopy. (*a*) Control. The mitochondria are incubated in the medium in the absence of ATP. Bar, 10 μm; × 930. (*b*) Experimental. The mitochondria are incubated as in Fig. 1*a* except that 4 mM ATP was added. Note the granular appearance of the mitochondria (arrow). Bar, 10 μm; × 930.

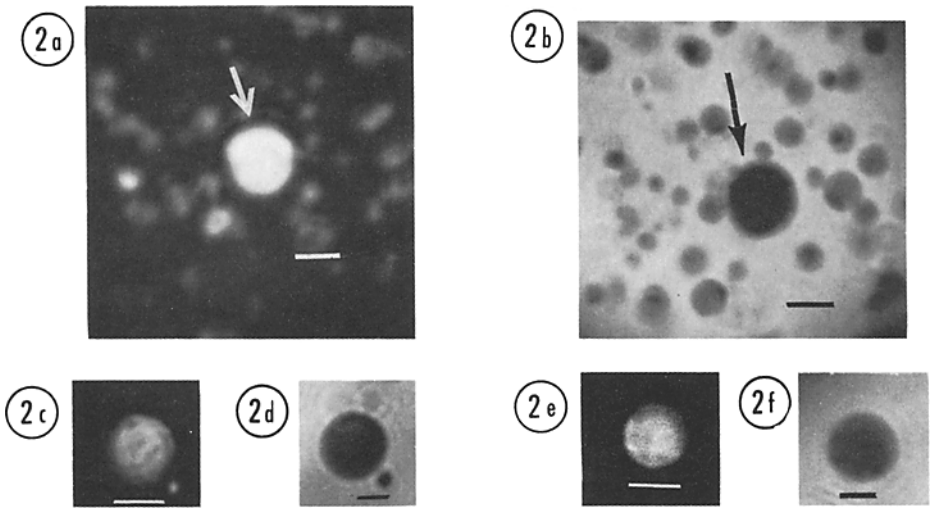

FIGURE 2 Polarized normal interference microscopy. Using the Jamin-Lebedeff system (Zeiss) with a λ/4 plate and 546 nm filter with a mercury burner, the change in mass attributed to calcium phosphate accumulation is measured. (*a*) Control. The analyzer is set to zero while the polarizer is rotated to give maximal background extinction. Bar, 5 μm; × 1,200). (*b*) Control. The analyzer is rotated 127° clockwise to match the previous background extinction. Bar, 5 μm; × 1,200. (*c* and *d*) Controls corresponding to (*a*) and (*b*). The rotation was 122°. Bar, 5 μm; × 1,400. (*e* and *f*) Experimental, corresponding to the above controls. The rotation was 152°. Bar, 5 μm; × 1,400.

TABLE I

Path Differences in Giant Mitochondria with Jamin-Lebedeff Optics

Exp.	N	Addition	Path difference	
			Control	Experimental
1	15 15	ATP	127° ± 7	149° ± 3
2	10 10	ATP	135° ± 8	161° ± 2
3	22 22	$CaCl_2$	129° ± 3	155° ± 3

Results in the absence of accumulation (control) and during accumulation (experimental). In the control, the component listed had been omitted from the medium. In the experimental determinations, the missing component had been added. Since the preparation did not have the agar overlay, the measurements were not carried out in the same mitochondria.

able uncertainty about the composition of the granules obtained from rat liver mitochondria (22). In addition to Ca^{2+} and inorganic phosphate, they contain Mg^{2+}, CO_3^{2-} and a substantial but not well determined amount of organic material (the reported values ranged from 16 to 60%, depending on the method).

Calculations were carried out with Eq. 2 and a value of χ of 0.18 for both the optical path difference of the control and the experimental mitochondria. The dry weight of the mitochondria was calculated to be 7.8×10^{-11} and 6.8×10^{-11} g for the experimental and the control, respectively, for exp. 1; 8.4×10^{-11} and 7.2×10^{-11} g for exp 2; and 8.1×10^{-11} and 6.9×10^{-11} g for exp 3. These values correspond to 15, 17, and 18% gains in dry weight. The values reported previously by Rossi and Lehninger (18) for rat liver mitochondria suggest a dry weight gain of about 17% (0.27 mg hydroxyapatite for 1 mg protein and 0.6 mg lipids). If the value of χ of 0.11 is used for the optical path difference induced by the accumulation medium, these values become 43, 45, and 47%, respectively. In view of the complex composition of the granules and the probability of hydration, we favor the use of the higher value of χ and hence the lower percentages calculated. The percent estimates that we reported previously (14) represent somewhat different assumptions and do not represent different experiments.

Impaled mitochondria also exhibit the characteristic granulation when in the appropriate complete medium. Typical results are shown in Figs. 3 and 4. The results of four experiments are summarized in Table II. Fig. 3*a* shows a control

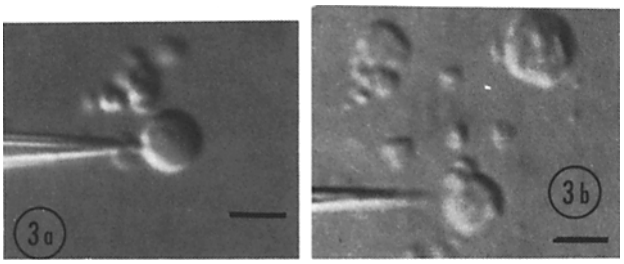

FIGURE 3 Calcium phosphate accumulation in an impaled mitochondrion. The mitochondria are visible with Nomarski differential interference optics. (*a*) Control. The mitochondrion is impaled in a medium from which $CaCl_2$ has been omitted. E = 17 mV, R = 1.9 MΩ. Bar, 5 μ; × 1,400. (*b*) Experimental. A mitochondrion from the same preparation and in the same medium as in (*a*) is impaled. At zero time 2 mM $CaCl_2$ was added. E = 16 mV, R = 2.2 MΩ. Bar, 5 μm; × 1,400.

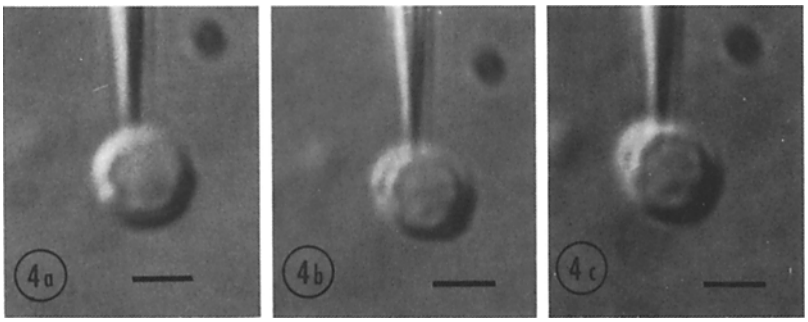

FIGURE 4 Kinetics of accumulation in an impaled mitochondrion as seen with Nomarski differential interference optics. E = 18 mV, R = 2.0 MΩ. (*a*) Zero time. At this point 4 mM ATP is added to the ATP-lacking accumulation medium by injection with a Hamilton syringe. Bar, 5 μm; × 1,600. (*b*) Approx. 30 s after the addition of ATP. Note apparent perturbation of the image due to calcium phosphate accumulation. Bar, 5 μm; × 1,600. (*c*) Approx. 60 s after the addition of ATP. Irregularity of mitochondrial image is more pronounced due to the accumulation. Typical time-course for accumulation was 3–5 min. Bar, 5 μm; × 1,600.

mitochondrion which is in a medium from which $CaCl_2$ has been omitted. Fig. 3*b* shows a mitochondrion in an aliquot of the same suspension after addition of $CaCl_2$ to the agar overlay. Fig. 4 records the changes in a single mitochondrion after the addition of ATP (originally omitted from the medium) in a similar experiment. The photographs were taken about 30 s apart, beginning right after the addition of ATP (Fig. 4*a*).

In Table II, the first column lists the component of the accumulation medium which is originally omitted. The second column lists the number of mitochondria, and the third indicates whether the mitochondria were impaled with the microelectrodes (indicated by +) or not (indicated by −). The percent responses are listed in the last column. The results indicate that 69–90% of the mitochondria exhibit this response under the appropriate conditions (part *a* and *b* of all four experiments). Similar percentages have been reported by Greenawalt et al. (10) for rat liver mitochondria. The response depends on the addition of succinate (exp 1), inorganic phosphate (exp 2), Ca^{2+} (exp 3), and ATP (exp 4). When any one of these components has been omitted, the response fails to take place. Also, the response is antimycin sensitive (exp 1*c*, 1*d*, 3*c*, 3*d*, 3*g*, 4*c*, and 4*d*) and oligomycin sensitive (exp 1*e*, 1*f*, 3*e*, 3*f*, 4*e*, 4*f*, 4*g*, and 4*h*). It is completely abolished when the two inhibitors are added simultaneously (exp 1*g*, 2*c*, 3*g*, 4*g*, and 4*h*). These findings indicate that the accumulation requires both electron transport through the cytochrome chain and the hydrolysis of ATP. Therefore, it is an active process. The oligomycin sensitivity differs from that in previous reports of studies with rat liver mitochondria (e.g., reference 13) in which the accumulation was found to be oligomycin sensitive only when supported by high concentrations of ATP alone. In our study, in

TABLE II

Formation of Granules in Giant Mitochondria, while Monitoring Membrane Potentials and Resistances

Exp.	Addition	N	Impalement	Anti-A	Oligomycin	*E*	*R*	Response
						mV	*MΩ*	%
1 *a*	Succinate	22	+	−	−	16.0 ± 0.8	2.1 ± 0.1	73
b	"	10	−	−	−	−	−	80
c	"	22	+	+	−	17.7 ± 1.1	2.2 ± 0.2	14
d	"	5	−	+	−	−	−	20
e	"	20	+	−	+	17.3 ± 1.3	2.0 ± 0.1	20
f	"	5	−	−	+	−	−	0
g	"	6	−	+	+	−	−	0
2 *a*	Phosphate	14	+	−	−	16.3 ± 0.9	2.0 ± 0.2	93
b	"	4	−	−	−	−	−	75
c	"	6	−	+	+	−	−	0
3 *a*	Ca^{2+}	17	+	−	−	16.5 ± 0.6	2.1 ± 0.2	82
b	"	8	−	−	−	−	−	75
c	"	13	+	+	−	16.4 ± 0.7	2.3 ± 0.3	15
d	"	5	−	+	−	−	−	0
e	"	11	+	−	+	17.3 ± 1.3	2.0 ± 0.1	36
f	"	5	−	−	+	−	−	0
g	"	6	−	+	+	−	−	0
4 *a*	ATP	29	+	−	−	17.8 ± 0.5	2.2 ± 0.2	69
b	"	15	−	−	−	−	−	73
c	"	24	+	+	−	17.5 ± 0.6	2.2 ± 0.1	17
d	"	4	−	+	−	−	−	0
e	"	16	+	−	+	17.7 ± 0.8	2.1 ± 0.2	12
f	"	4	−	−	+	−	−	0
g	"	6	−	+	+	−	−	0
h	"	6	+	+	+	16.5 ± 0.5	2.1 ± 0.1	0

The additions listed on the second column were made to mitochondria from which this component of the medium had been omitted. The mitochondria either were controls or were impaled (as indicated by the − and + signs, respectively). *E* represents the membrane potential and *R*, the measured resistance. The inhibitors listed were either absent (−) or added (+). Percent response indicates the clear appearance of granulation.

three independent experiments, of a total of 61 mitochondria, 51 showed no response in the presence of oligomycin. The difference between the rat and the mice mitochondria is probably quantitative rather than qualitative. It is likely that the proportion of the contributions of the two pathways (that is the oxidative and the hydrolytic pathway) differs in the two systems and that the presence of ATP is also required, at least in rat liver mitochondria, for a non-energetic function. The formation of granules appears to be reversible since they disappear after the addition of antimycin.

The results of Table II show that the impaled mitochondria exhibit the same response as the control mitochondria either in the presence or in the absence of the inhibitors (see also reference 14), and the frequency of the response is about the same for impaled and control mitochondria (in the absence of inhibitors, for the four experiments, they are 79 ± 10% and 76 ± 3%, respectively). The results support the notion that the impaled mitochondria behave exactly like the intact mitochondria. Furthermore, the data for the accumulation are generally comparable to those obtained by others for rat liver mitochondria.

The ability of mitochondria to carry out oxidative phosphorylation can be tested by assaying the production of ATP. We have found the contractility of myofibrils to be a very sensitive assay for the presence of ATP. Micrographs of myofibrils in media without (*a*) and with (*b*) the addition of ATP are shown in Fig. 5. The contraction can be recognized by the shortening and the thickening of the myofibrils. The calculated sarcomere length

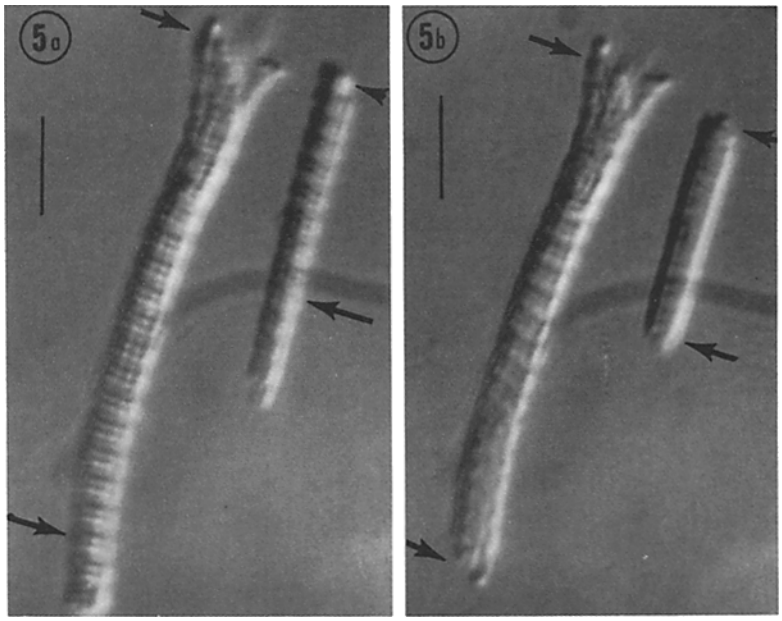

FIGURE 5 Myofibrillar assay of ATP concentration (in this case 1 mM). Nomarski differential interference microscopy. Bar, 10 μm; × 1,250. Arrows mark the contracted length. (*a*) Control. The myofibrils in the absence of added ATP. The sarcomere length for the longer fiber is approx. 3 μm and about 2.7 for the smaller one. (*b*) Experimental. The myofibrils after the addition of ATP. The sarcomere length is about 2.5 and 1.6 μm for the longer and the shorter fiber, respectively.

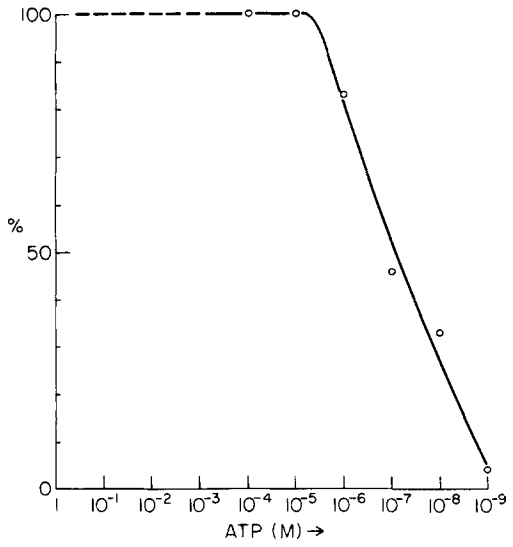

FIGURE 6 Percentage of the myofibrils contracting after the addition of various ATP concentrations. The ATP was added on to the agar overlay. Each point represents the mean percentage derived from four fields from a count of randomly selected samples of 30 myofibrils.

is about 3 μm in the resting state. This distance is considerably shortened by contraction (e.g., in the longer fiber it is approx. 2.5 μm after contraction).

The effects of various concentrations of ATP on the shortening of the myofibrils are given in Fig. 6. The percent of contracted myofibrils was recorded by examining 30 myofibrils in each observed field. In the figure, each point represents the percentage of contracted myofibrils in four randomly selected fields. The results indicate that it is possible to detect ATP concentrations as low as 10^{-8} M.

The experiments depicted in Table III show that ADP induces a contraction in the presence of mitochondria (exp 1*a*). Since the response is oligomycin and antimycin sensitive (exp 1*b*, 2*a*, and 2*b*), the contraction is the result of ATP derived from mitochondrial oxidative phosphorylation. In contrast, the contraction brought about by the addition of ATP is neither antimycin nor oligomycin sensitive (exp 2*c* and *d*).

Fig. 7 shows a micrograph of a myofibril in proximity to an impaled mitochondrion. Part *a* shows the myofibril in close promixity to a mito-

TABLE III

Contraction of Myofibrils after the Addition of ATP and ADP to Mixed Suspensions of Mitochondria and Myofibrils in the Presence and Absence of Inhibitors

Exp.	Additions	No. of determinations	No. of myofibrils	Contraction ± SD
				%
1 *a*	ADP (10^{-6} M)	3	122	80
				70
				93
b	ADP (10^{-6} M) + antimycin A	3	48	0
				0
				0
2 *a*	ADP (10^{-3} M) + oligomycin	3	90	24
				33
				15
b	ADP (10^{-3} M) + antimycin A	2	60	0
				6
c	ATP (10^{-3} M) + antimycin A	4	100	100 ± 0
d	ATP (10^{-3} M) + oligomycin	4	120	83 ± 15

The mitochondrial concentration was approx. 0.1 mg protein/ml.

chondrion in the absence of ADP. Part *b* shows the myofibril during perfusion with ADP, and part *c* shows the contraction. The length of the contracted fiber is indicated by arrows. Particles present in *a* were flushed out by the perfusion. In all cases reported in this paper, the potential was continuously monitored to insure that the individual mitochondria were impaled and to detect any possible effect of the treatment on the electrical parameters. No significant change was detected upon the addition of ADP.

A similar series is shown in Fig. 7*d–h*. Fig. 7*d* corresponds to the system before impalement. The other particles are debris from the myofibril preparation and from erythrocytes (ascertained by focusing through the preparation). In Fig. 7*e*, the mitochondrion has been impaled (E = 18 mV, R = 2.1 MΩ). The micrographs that follow (Fig. 7*f–h*) show the contraction after the addition of ADP. Fig. 7*f* corresponds to approx. 1 s after the addition of 1 mM ADP, and the remaining micrographs were taken about 1 s apart. The sarcomere length was 2.4 μm before the addition of ADP. In the final micrograph (Fig. 7*h*) the average sarcomere length is approx. 1 μm, corresponding to a supercontraction.

The results obtained with giant mitochondria in close contact with the myofibrils are summarized in Table IV. At sufficiently high concentrations of ADP (in the range of 10^{-4}–10^{-6} M), a large proportion of each one of the myofibrils in close contact with a single impaled mitochondrion contract (73% in the case of exp 1). The response is antimycin (exp 3*b*) and oligomycin (exp 4*b*) sensitive, in contrast to the concentration in the presence of ATP (see exp 3*c*, 3*d*, 4*c*, and 4*d*), which is insensitive to either inhibitor. The frequency of the contraction is about the same as for mixed suspensions of mitochondria and myofibrils (Table III). The ATP inducing the contraction does not originate from neighboring mitochondria since a concentration of mitochondria has been selected at which no contraction occurs except when the myofibrils are in close proximity to a mitochondrion. In addition, a field has been selected in which only one mitochondrion can be found in proximity to the myofibril. This requires focusing through several levels since in differential interference optics the image represents an optical section about 1 μm thick.

Recently, experiments have been carried out with single mitochondria added to myofibril suspensions (Bowman and Tedeschi),[1] with essentially the same results.

As shown in Tables II and IV, the metabolic state and the presence or absence of inhibitors had no effect on the membrane potential (E) or the resistance (R), in agreement with our previous results (14, 15). Therefore, no metabolic dependence of the mitochondrial membrane potential can be demonstrated.

In summary, the results are consistent with the

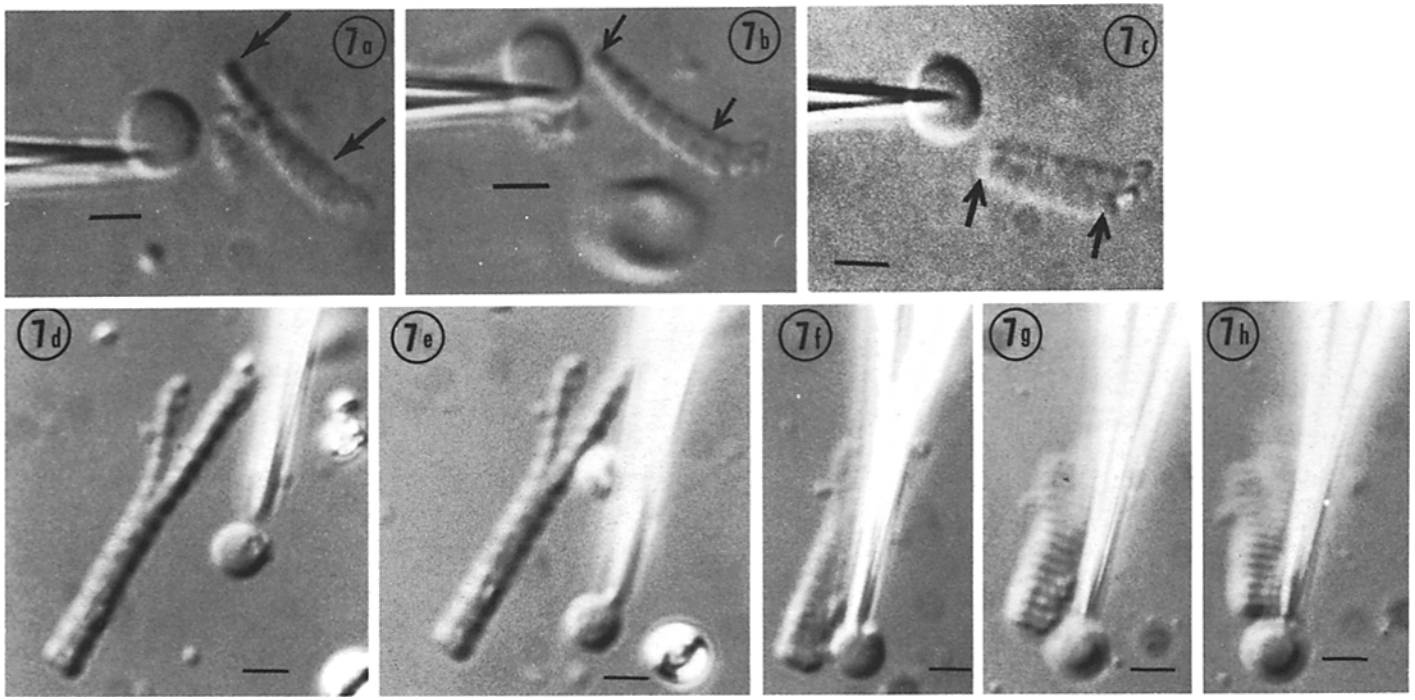

FIGURE 7 Contraction of myofibrils in close proximity to an impaled mitochondrion as viewed with differential interference microscopy after Nomarski. In both series shown (*a–c* and *d–h*), the ADP (10^{-3} M) was introduced by perfusing the mitochondrion and myofibril (see Materials and Methods). In the micrographs of the same series, the arrows are at equal distance. There was no significant change in potential or resistance after the addition of ADP. Sequence *a–c*. The photographs were taken 2–5 s apart. E = 17 mV, R = 2.1 MΩ. Bar, 5 μm; × 1,400. (*a*) Before the addition of ADP (sarcomere length approx. 2.5 μm). (*b*) During perfusion. The photograph also shows the presence of an erythrocyte. (*c*) Contraction. The contrast was enhanced in the development to demonstrate the periodicity of the fiber (sarcomere length approx. 1.8 μm). Sequence *d–h*. After impalement, the photographs were taken approx. 1 s apart. Bar 5 μm; × 1,200. The highly refractile particles are damaged erythrocytes. (*d*) Before impalement. The sarcomere length is approx. 2.4 μm. (*e*) Impalement, E = 18 mV, R = 2.1 MΩ. (*f*) Approx. 1 s after the addition of ADP. Partial contraction (sarcomere length approx. 1.5 μm). (*g*) Approx. 2 s. after the addition of ADP (sarcomere length approx. 1.2 μm). (*h*) Maximal contraction (sarcomere length approx. 1 μm, corresponding to a supercontraction).

TABLE IV

Contraction of Individual Myofibrils in Contact with a Single Impaled Mitochondrion while Monitoring Membrane Potentials and Resistances

Exp.	Additions	N	*E*	*R*	Contraction
			mV	*MΩ*	%
1	ADP (10^{-4} M)	48	15.4 ± 0.6	2.2 ± 0.3	73
2 *a*	ADP (10^{-3} M)	6	16.7 ± 0.5	2.0 ± 0.1	67
b	(10^{-6} M)	6	16.2 ± 0.4	2.0 ± 0.1	50
c	(10^{-8} M)	6	16.3 ± 0.5	2.1 ± 0.2	16
3 *a*	ADP (10^{-4} M)	3	15.0	2.2	67
			15.0	2.0	
			15.0	2.3	
b	ADP (10^{-4} M) + antimycin A	18	16.4 ± 0.5	2.0 ± 0.2	0
c	ATP (10^{-4} M)	11	17.3 ± 2.4	2.1 ± 0.2	100
d	ATP (10^{-4} M) + antimycin A	8	16.5 ± 0.5	2.0 ± 0.2	100
4 *a*	ADP (10^{-3} M)	3	17.0	2.3	67
			17.0	2.2	
			17.0	2.2	
b	ADP (10^{-3} M) + oligomycin	10	17.3 ± 0.5	2.2 ± 0.3	20
c	ATP (10^{-4} M)	3	14.0	2.0	100
			17.0	2.3	
			18.0	2.0	
d	ATP (10^{-4} M) + oligomycin	5	17.0 ± 1.0	2.2 ± 0.1	80

interpretation that the impaled mitochondria are functional since they accumulate calcium salts in the appropriate medium and, in addition, they phosphorylate ADP to ATP which causes myofibrillar contraction. Our accompanying paper (15) presents evidence that the microelectrode is in the inside space of the mitochondria since (*a*) the membrane potential shows the predicted dependence on the external concentration of K^+ in the presence of valinomycin, (*b*) the electrical parameters remain unchanged by the removal of the outer membrane and the evagination of the inner mitochondrial membrane, and (*c*) punch-through experiments, in which the microelectrode traverses through the mitochondrion, reveal a single membrane potential in the presence of valinomycin or under either energized or inhibited conditions. In these and other experiments using the giant mouse mitochondria, the evidence does not support the Mitchell chemiosmotic hypothesis which proposes a direct role of metabolism in the membrane potential and predicts a potential at least one order of magnitude higher and with an opposite sign.

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