BBA 45703

THE ACTION OF DIO-9
ENERGY-LINKED SWELLING OF RAT-LIVER MITOCHONDRIA

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(Received April 1st, 1968)

SUMMARY

1. The antibiotic Dio-9 initiates a large-amplitude swelling of rat-liver mitochondria which has an absolute requirement for an energy-supplying reaction.

2. The large-amplitude Dio-9-induced swelling supported by oxidation of the Group I substrates (succinate, α-oxoglutarate, malate, pyruvate, isocitrate, pyruvate–malate and TMPD–ascorbate) requires low concentrations of phosphate (or arsenate). The requirement for this anion cannot be replaced by acetate, formate, or sulfate.

3. The large-amplitude Dio-9-induced swelling supported by oxidation of the Group II substrates (glutamate and β-hydroxybutyrate) occurs independent of the addition of phosphate (or arsenate).

4. ATP (but not ADP) will support Dio-9-induced mitochondrial swelling in the absence of added oxidizable substrate. The Dio-9-induced swelling in the presence of an oxidizable substrate is not reversed or prevented by ATP, ADP, Mg^{2+} or bovine serum albumin in any combination. The initial swelling rate is enhanced by EDTA.

5. Cyanide, Na_2S and 2,4-dinitrophenol inhibit the Dio-9-induced large-amplitude swelling with all substrates tested. Total dinitrophenol inhibition of the Dio-9-induced succinate-supported swelling is dependent upon the presence of amytal. Antimycin completely inhibited swelling supported by all substrates except for TMPD–ascorbate.

6. The Dio-9-induced large-amplitude swelling is accompanied by an inhibition of respiration and a solubilization of about 20% of the total mitochondrial protein. The addition of cytochrome *c* reconstitutes Dio-9-inhibited respiration with succinate as a substrate. NAD^+ is required in addition to cytochrome *c* for reconstitution of NAD^+-linked Dio-9-inhibited respiration. Phosphorylation is not reconstituted.

7. Sr^{2+} inhibits the large-amplitude swelling and the respiratory inhibition brought about by Dio-9 with Group I substrates and with Group II substrates in the presence of phosphate. Swelling initiated by Dio-9 and Group II substrates in the absence of phosphate is not inhibited by strontium.

Abbreviations: TMPD, tetramethyl-*p*-phenylenediamine; P_i, inorganic phosphate; As_i, inorganic arsenate.

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INTRODUCTION

Active swelling of rat-liver mitochondria may be induced by an increasingly large number of agents, among which are inorganic phosphate (P_i), arsenate (As_i) Ca^{2+}, thyroxin, glutathione and various polypeptide hormones[1]. LEHNINGER AND REMMERT[2] have proposed that the release of free fatty acids (U-factor) induces swelling. Changes in bound NAD^+ have been implicated in the initiation of mitochondrial swelling[3]. The action of inhibitors and uncouplers of oxidative phosphorylation indicates that the presence of high-energy intermediates of energy conservation during coupled respiration are required for swelling[4]. CONNELLY AND LARDY[5] consider the initiation of swelling to be related to a high-energy intermediate off the main pathway of oxidative phosphorylation.

A previous report[6] showed the antibiotic Dio-9 to be a potent inhibitor of rat-liver mitochondrial State 3 respiration* and in addition to act as an uncoupling agent in a medium lacking P_i. Dio-9 is as well a potent inhibitor of the $^{32}P_i$–ATP (ref. 8) and [^{14}C]ADP–ATP isotopic exchanges and an inhibitor of the magnesium stimulated ATPase of rat-liver mitochondria[6]. The inhibition of succinate respiration in a medium containing P_i is not influenced by the subsequent addition of 2,4-dinitrophenol, however, addition of 2,4-dinitrophenol prior to addition of Dio-9 prevents respiratory inhibition. With succinate as a substrate the antibiotic induces a large-amplitude swelling dependent upon the presence of P_i (ref. 6).

This paper represents further investigations of the conditions required for the Dio-9-induced swelling and of the relationship of this swelling to the respiratory inhibition and to the inhibition of the phosphorylation mechanism.

MATERIALS AND METHODS

Rat-liver mitochondria were isolated by the method of HOGEBOOM[9] as described by MYERS AND SLATER[10]. Mitochondrial protein was determined by the biuret procedure following solubilization with deoxycholate[11].

Oxygen uptake was measured polarographically using an oxygraph (Gilson Medical Electronics), the solubility of oxygen being taken as 0.25 μmole per ml at 25°, or with Warburg manometers having narrow capillaries and small reaction flasks (gas volume, 6–8 ml).

Mitochondrial swelling was monitored at room temperature (25°) by the change in absorbance at 520 mμ (ref. 12) using a Cary-14 recording spectrophotometer (Applied Physics Corp., Monrovia, Calif.). The initial rate of large-amplitude swelling was calculated on the basis of the decrease in absorbance for the first 30 sec after the addition of mitochondria (usually 500 μg) to the incubation medium and then the rate calculated on the basis of an initial change per min. Under the conditions of experimentation described in this paper, the large-amplitude Dio-9 swelling was complete within 90 sec. Total "swelling" was estimated on the basis of the absorbance value found after 5 min at which time no further changes in absorbance occurred.

For the analysis of protein released during Dio-9 incubation the mitochondria

* Respiration in a phosphate medium with added phosphate acceptor as defined by CHANCE AND WILLIAMS[7].

were incubated at 25° in a total volume of 20 ml. Following centrifugation (10 min at 27000 × *g* and 0°) the supernatant was carefully decanted from the mitochondrial pellet and lyophilized. The total lyophilized protein residue was made up to 4 ml with water and the protein precipitated by the addition of 0.2 ml of $HClO_4$ (final concentration of 0.55 M). The $HClO_4$ precipitate was washed once with 2 ml of 1 M $HClO_4$, taken up in water and assayed by the biuret method[11].

Nucleotides, oligomycin and cytochrome *c* (Fraction V) were purchased from the Sigma Chemical Co., St. Louis, Mo., Dio-9 was obtained from the Royal Netherlands Fermentation Ind., Ltd., Delft (The Netherlands). All other reagents were of commercial origin.

RESULTS

Swelling in the presence of succinate

Fig. 1 shows the critical requirement for P_i in the Dio-9-induced succinate-supported swelling. In the absence of P_i, Dio-9 (22 μg per 0.5 mg mitochondria) fails to induce the large-amplitude swelling which is seen in a medium containing as little as 16.7 μM P_i. At phosphate concentration in excess of 300 μM the rapid-swelling phase is complete within 30 sec–1 min after mitochondrial addition. At lower P_i concentrations this phase is in all cases greater than 50 % complete within the first min. In addition to being a function of the phosphate concentration, the rate and total magnitude of swelling is as well dependent upon the level of Dio-9. Maximum initial rate and maximal total swelling was found at 36 μg Dio-9 per mg in the presence of 1.2 mM P_i.

Arsenate is as effective as P_i in initiating the Dio-9-induced large-amplitude

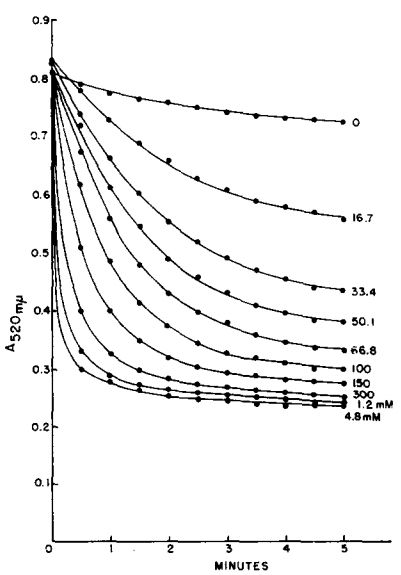

Fig. 1. Succinate-supported Dio-9-induced large-amplitude swelling as a function of added phosphate. Tris–HCl buffer (pH 7.4), 32.5 mM; KCl, 8 mM; EDTA, 1.25 mM; Dio-9, 22 μg; sucrose, 6.3 mM; $MgCl_2$, 2.5 mM; amytal, 1 mM; succinate, 16.5 mM; rat-liver mitochondria, 0.5 mg; total vol., 2 ml. P_i concentration (μM) given next to each experimental curve.

swelling. Swelling by both these anions in the absence of the antibiotic accounts for less than 15 % of the maximum amount of swelling in its presence. At 44 μg Dio-9 per mg, half-maximum rate of swelling occurred at less than 20 μM for both P_i and As_i. With 1.2 mM P_i half maximal swelling occurred at 20 μg Dio-9 per mg. Acetate, formate, or sulfate could not substitute for the P_i or As_i requirement in the Dio-9-supported swelling.

Effect of uncouplers and inhibitors of oxidative phosphorylation on succinate-supported Dio-9 swelling

2,4-Dinitrophenol inhibits Dio-9-induced swelling completely in all cases investigated except for that of the succinate-supported P_i-dependent swelling in the absence of amytal. For maximum inhibitory effect of 2,4-dinitrophenol, in the succinate case, amytal must be present. Amytal of itself does not cause swelling nor is it required for the Dio-9-induced succinate-supported swelling. Succinate-supported mitochondrial swelling (Dio-9 *plus* P_i) is the same in the presence or absence of amytal (Table I). 1,1,3-Tricyano-2-amino-1-propene (ref. 14, at 2 mM) and antimycin A (0.1 μg) completely inhibit succinate-supported Dio-9 swelling. Atractyloside up to 0.35 mM had no effect on swelling, and defatted bovine serum albumin (5 mg/0.5 mg mitochondrial protein) did not prevent Dio-9 swelling.

TABLE I

EFFECT OF AMYTAL AND ANTIMYCIN A ON SWELLING DEPENDENT ON DIO-9 AND SUCCINATE

Tris–HCl buffer, 32.5 mM; KCl, 8 mM; EDTA, 1.25 mM; $MgCl_2$, 2.5 mM; sucrose, 6.3 mM; succinate, 16.5 mM; mitochondria, 0.5 mg; vol., 2.0 ml. When added, amytal, 1 mM; P_i, 1.2 mM; 2,4-dinitrophenol, 0.05 mM; antimycin A, 0.1 μg; Dio-9, 30 μg. See text for description of swelling measurement.

Additions	*None*	*Final–$\Delta A_{520\,m\mu}$ (5 min)*	
		+ Amytal	*+ Amytal + antimycin A*
None	0.030	0.010	—
Dio-9	0.110	0.095	0.025
Dio-9 + P_i	0.480	0.495	0.065
Dio-9 + P_i + dinitrophenol	0.290*	0.120	—

* This value was still increasing at 5 min.

Effect of Mg^{2+} and EDTA on succinate-supported Dio-9 swelling

The addition of Mg^{2+} only slightly inhibits the maximal amplitude of Dio-9-induced swelling, while slowing substantially the initial swelling rate. In one experiment the addition of 2.5 mM Mg^{2+} reduced the initial Dio-9 swelling rate 76 % while the final degree of swelling at 5 min was decreased by only 32 %. The maximal initial swelling rate and maximal degree of swelling takes place in the presence of 1.25 mM EDTA without added Mg^{2+}.

The effect of β-hydroxybutyrate and glutamate on Dio-9-induced swelling

Dio-9-induced mitochondrial swelling supported by α-oxoglutarate, malate, pyruvate, isocitrate, pyruvate–malate and TMPD–ascorbate, as with succinate,

requires the presence of phosphate or arsenate. These are designated by us as Group I substrates.

While an oxidizable substrate or some energy source is a prerequisite for Dio-9-induced swelling, there is no absolute requirement for added P_i (or As_i). Swelling supported by Group I substrates requires added P_i, while glutamate and β-hydroxybutyrate support Dio-9-dependent swelling in the absence or presence of added phosphate. We have designated β-hydroxybutyrate and glutamate as Group II substrates. Amytal completely inhibits swelling by Dio-9 with glutamate and with all other NAD^+-linked substrates (Groups I and II). Glutamate will not cause a large-amplitude swelling in a phosphate-free medium containing Dio-9, succinate and amytal. The characteristic dependence of Group I-supported Dio-9 swelling on P_i addition as compared to Group II (glutamate and β-hydroxybutyrate) substrate is most clearly demonstrated in Fig. 2. The addition of succinate to a reaction medium containing Dio-9 together with mitochondria leads to large-amplitude swelling only upon subsequent addition of As_i (or P_i). Glutamate (or β-hydroxybutyrate)-supported swelling is shown to be spontaneous in the presence of Dio-9. Of the substrates tested only glutamate and β-hydroxybutyrate fall into the Group II classification.

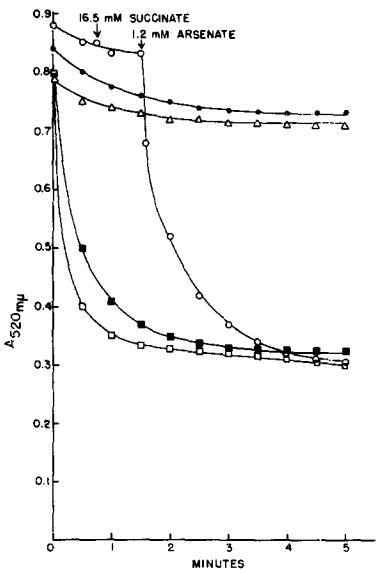

Fig. 2. Dio-9-induced mitochondrial swelling in presence of glutamate. The conditions are the same as those of Fig. 1 except that glutamate (10 mM) replaced succinate *plus* amytal. ●—●, no additions; △—△, As_i (1.2 mM); □—□, Dio-9 (22 μg); ○—○, Dio-9, amytal (mM), and additions as indicated; ■—■, Dio-9, As_i.

Swelling supported by TMPD–ascorbate

JACOBS[15] has reported that P:O ratios approaching one for the cytochrome oxidase (EC 1.9.3.1) step can be obtained by using TMPD to reduce cytochrome *c*. We have found TMPD–ascorbate to be able to sustain Dio-9-induced swelling. P_i (or As_i) is required for this swelling which is inhibited by 2,4-dinitrophenol and by cyanide. Concentrations of antimycin A (0.12 μg/ml) which effectively inhibit NAD^+-linked respiration[16] inhibits by 50 % the initial rate and total magnitude of the Dio-9 swelling

supported by TMPD–ascorbated oxidation. TMPD–ascorbate-supported swelling is not inhibited by amytal (2 mM). PACKER[17] has observed that while electron transport with TMPD–ascorbate is not antimycin A sensitive[15] the presence of antimycin diminishes P_i swelling associated with transport through the terminal energy conservation site.

Dio-9-dependent swelling supported by ATP

The substrate requirement for the support of Dio-9-induced swelling can be replaced by the addition of ATP. ATP-supported swelling takes place in the presence of amytal and is dependent upon the addition of P_i (or As_i) and electron transport. ATP will not support Dio-9-dependent swelling in the presence of Na_2S or cyanide nor will ADP or PP_i substitute for ATP.

A 1-min preincubation with low concentrations (5 μg) of oligomycin effectively inhibits Dio-9 swelling supported by ATP. Oligomycin, at these concentrations, has no influence on succinate-supported swelling. When mitochondria were preincubated with oligomycin and ATP in the absence of added substrate, Dio-9 addition had little influence on the endogenous respiratory rate. The addition of 2,4-dinitrophenol prior to succinate prevents Dio-9 swelling induced by the addition of substrate. In Fig. 3 it can be seen that the addition of succinate to a suspension of mitochondria preincubated with ATP and Dio-9 followed by 2,4-dinitrophenol results in respiratory

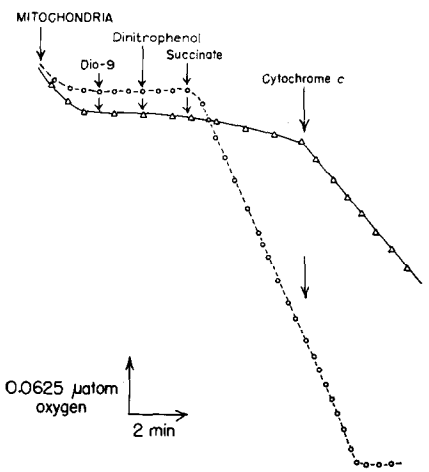

Fig. 3. Polarographic tracing showing succinate respiration following oligomycin inhibition of ATP-supported Dio-9 swelling. Tris–HCl buffer (pH 7.4), 30 mM; KCl, 8 mM; EDTA, 1.25 mM; sucrose, 6.3 mM; $MgCl_2$, 5 mM; P_i, 1.5 mM; rat-liver mitochondria, 1.5 mg. When added, Dio-9, 30 μg; 2,4-dinitrophenol, 1 mM; succinate, 25 mM; cytochrome *c*, 31 μg; ATP, 0.3 mM; total vol., 1.0 ml. O---O, oligomycin (5 μg) present from zero time.

stimulation when oligomycin is present; in the absence of oligomycin, succinate addition does not stimulate respiration. The lack of respiratory stimulation in the experiment without oligomycin is indicative of ATP-supported swelling which has taken place; a conclusion supported by the stimulation of respiration with added cytochrome *c*. It is obvious that the respiratory inhibition is a function of the ability of Dio-9 to induce a large-amplitude swelling.

Effect of oligomycin on Dio-9-dependent swelling

Concentrations of oligomycin (0.1–1.0 μg/mg protein) routinely used for the inhibition of oxidative phosphorylation in rat-liver mitochondria[18] are without effect on Dio-9-induced swelling supported by substrate oxidation. Higher concentrations of oligomycin (10–30 μg) were found to effectively inhibit the initial rapid rate of Dio-9 swelling. The β-hydroxybutyrate-supported swelling is much more sensitive to oligomycin than that supported by succinate. During β-hydroxybutyrate oxidation, 20 μg oligomycin almost completely inhibits the initial large-amplitude swelling. In this case, swelling was only 15 % that of the total Dio-9 swelling in the presence or absence of added P_i. On the other hand, while the initial rate of succinate-supported Dio-9 swelling is inhibited substantially by high concentrations of oligomycin, the total amplitude of swelling is fairly well maintained. At 20 μg oligomycin, the initial swelling rate is inhibited 86 %, while the final total swelling is reduced by only 25 % that of the control.

High concentrations of oligomycin (20–30 μg/ml) were found to accelerate succinate respiration indicating that at these concentrations it is acting as an uncoupler in addition to its classical function as a phosphorylation inhibitor. It appears as though the initial uncoupling by high concentrations of oligomycin makes Dio-9 less effective as a swelling agent and thus less effective as an inhibitor of respiration.

Influence of Sr^{2+} transport on Dio-9-induced swelling

Inhibition of the Dio-9 swelling is seen upon the addition of Sr^{2+} to the medium. The presence of Sr^{2+} has the effect of prolonging the initial onset of the large-amplitude swelling. It can be seen from Fig. 4 that increasing concentrations of Sr^{2+} inhibit the Dio-9-induced succinate-dependent swelling. At high concentrations of Sr^{2+} there

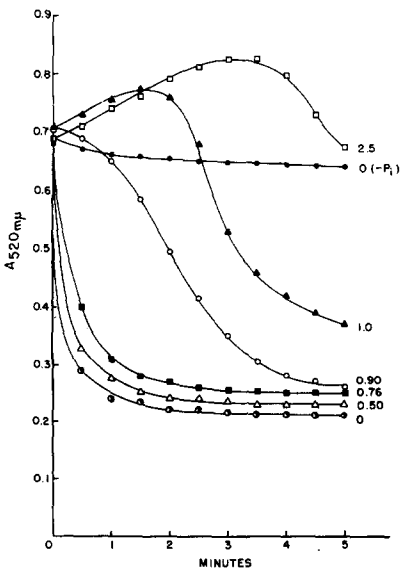

Fig. 4. Strontium inhibition of Dio-9-induced swelling. Tris–HCl buffer (pH 7.4), 32.5 mM; KCl, 8 mM; EDTA, 1.25 mM; $MgCl_2$, 2.5 mM; P_i, 1.2 mM; succinate, 16.5 mM; Dio-9, 22 μg; sucrose, 6.3 mM. Added concentrations of $SrCl_2$ (mM) are indicated adjacent to each curve.

is not only an inhibition of Dio-9 swelling, but as well a temporary increase in the absorbance of the medium. The time course of the absorbance changes with varying amounts of Sr^{2+} indicates the temporal nature of the Sr^{2+} inhibition of Dio-9 swelling.

Dio-9-induced swelling supported by glutamate and β-hydroxybutyrate is also inhibited by Sr^{2+}. In contrast to the support of swelling by the latter two substrates its inhibition by Sr^{2+} requires the presence of P_i. This requirement implicates P_i transport as a requirement for the Sr^{2+} inhibition of Dio-9-induced swelling[19].

Cytochrome c reversal of respiratory inhibition

Mitochondria oxidizing succinate in the absence of P_i are partly uncoupled by Dio-9. In the presence of P_i there is a short period in which Dio-9 acts as a respiratory uncoupler which is quickly followed by an inhibition of respiration. ADP and 2,4-dinitrophenol are unable to reverse the respiratory inhibition when added subsequent to the inhibitor[6]. The addition of cytochrome *c* to the inhibited system stimulates respiration when succinate is the oxidizable substrate (Fig. 3). This cytochrome *c*-stimulated respiratory rate is similar to that rate seen with rat-liver mitochondria in the presence of optimal 2,4-dinitrophenol concentration in the absence of Dio-9. The stimulation of inhibited respiration by cytochrome *c* occurs whether 2,4-dinitrophenol is present or not. Reversal of the inhibition of NAD^+-linked substrate oxidation requires the addition of NAD^+ as well as cytochrome *c*. The cytochrome *c* stimulation of Dio-9-inhibited succinate respiration is completely inhibited by 1 μg of antimycin A per mg protein.

Although cytochrome *c* reverses the Dio-9 inhibition of succinate oxidation it has no influence on the Dio-9-induced large-amplitude swelling in the presence of succinate and P_i (or As_i). The initial rate of swelling as well as its total magnitude are identical in the presence or absence of cytochrome *c*.

Release of mitochondrial protein by Dio-9 treatment

Dio-9 addition to a mitochondrial suspension in the presence of succinate has little influence on the quantity of protein found in the supernatant following centrifugation of the mitochondria. On the other hand, as shown in Table II, Dio-9 addition

TABLE II

DIO-9-INDUCED RELEASE OF MITOCHONDRIAL PROTEIN

Tris–HCl buffer (pH 7.4), 32.5 mM; KCl, 8 mM; EDTA, 1.25 mM; sucrose, 6.3 mM; $MgCl_2$, 10 mM; succinate, 33 mM; with amytal, 4 mM, or β-hydroxybutyrate, 25 mM. Rat-liver mitochondria, 42.5 mg. When added, As_i, 1.5 mM; Dio-9, 42 μg/mg protein; total vol., 20 ml. Incubation time, 5 min at 22° (see METHODS).

Substrate	*Additions*	*Protein released (mg)*	*% of total mitochondrial protein*
Succinate	As_i	1.7	4.0
	As_i + Dio-9	10.6	25.0
	Dio-9	2.8	6.6
β-Hydroxybutyrate	As_i	1.7	4.0
	As_i + Dio-9	6.0	14.0
	Dio-9	7.3	17.2

to this system in the presence of As_I, which of itself is ineffective, leads to a "solubilization" of some 25% of the total mitochondrial protein. Similar results were observed when P_I replaced As_I in the incubation medium.

In contrast to the system containing succinate, the Dio-9-induced release of mitochondrial protein supported by β-hydroxybutyrate does not require the presence of P_I (or As_I). In this latter case, Dio-9 alone brings about the liberation of 17.2% of the total mitochondrial protein. The ability of Dio-9 to release mitochondrial protein

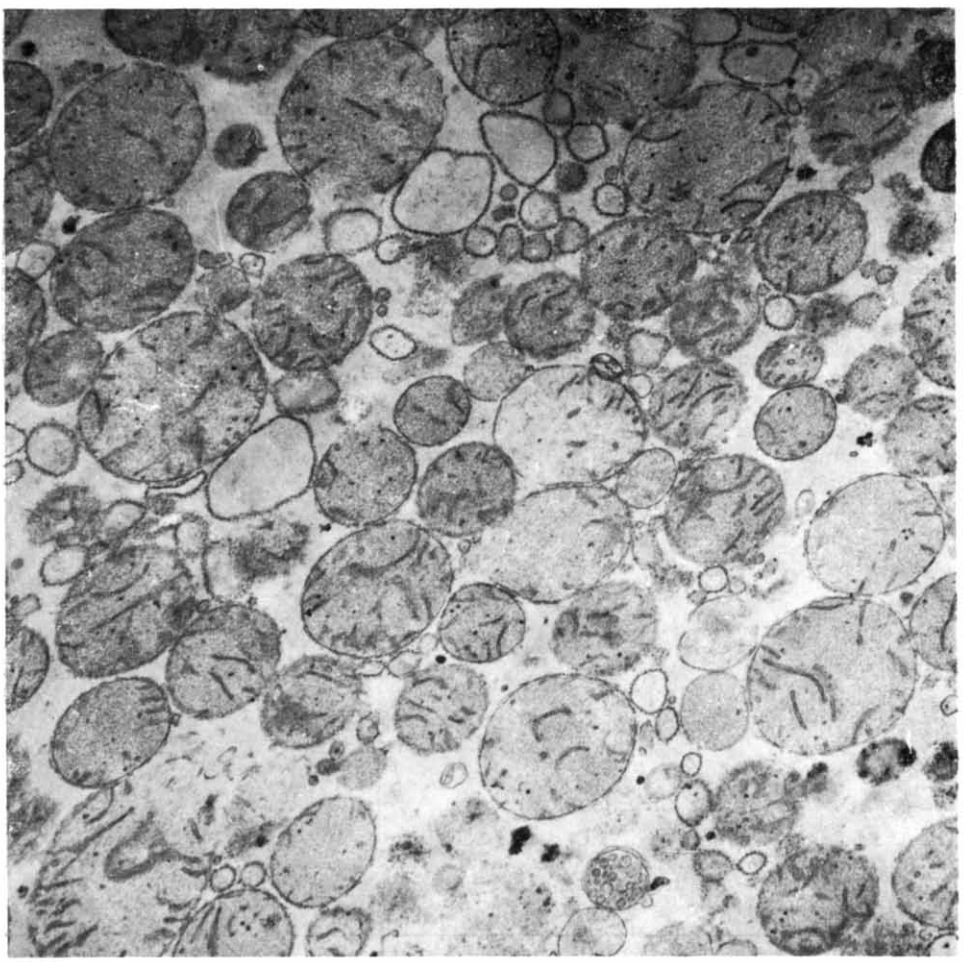

Fig. 5. Electron micrographs of treated mitochondria. Treatment of mitochondria for electron-microscopic examination consisted of the incubation of rat-liver mitochondria (35 mg) at 25° for 10 min in a reaction medium containing: succinate, 33.5 mM; amytal, 2 mM; P_I, 1.4 mM; $MgCl_2$, 5 mM; Tris–HCl buffer (pH 7.4), 32.5 mM; KCl, 8 mM; EDTA, 1.25 mM with and without Dio-9 (1.66 mg) in a total vol. of 20 ml. Following centrifugation of the mitochondria at 0°, the tightly packed pellet was resuspended in a small amount of 1% OsO_4 buffered with 0.25 M phosphate (pH 7.3) and fixed for 65 min at 4°. Following a water wash and recentrifugation the mitochondria were solidified in 2% agar and dehydrated with ethanol and propylene oxide followed by embedding in epon 812 resin (ref. 13). The epon blocks were sectioned on a MT-1 Porter Blum Microtome with glass knives. Pictures were taken with a Philips EM-100B microscope operated at 60 kV. a. Control incubation. b. Dio-9 incubation; magnification 30000 ×.

in the absence of P_i when supported by β-hydroxybutyrate oxidation has its counterpart in the Dio-9-induced swelling supported by this substrate in the absence of added P_i. The release of cytochrome *c* during the large-amplitude swelling is most probably responsible for the respiratory inhibition which follows the swelling phase.

An examination of Dio-9-treated mitochondria by electron microscopy (Figs. 5a, b) shows the Dio-9-treated mitochondria to have undergone a large-scale swelling involving gross disorganization of the internal mitochondrial membrane and dis-

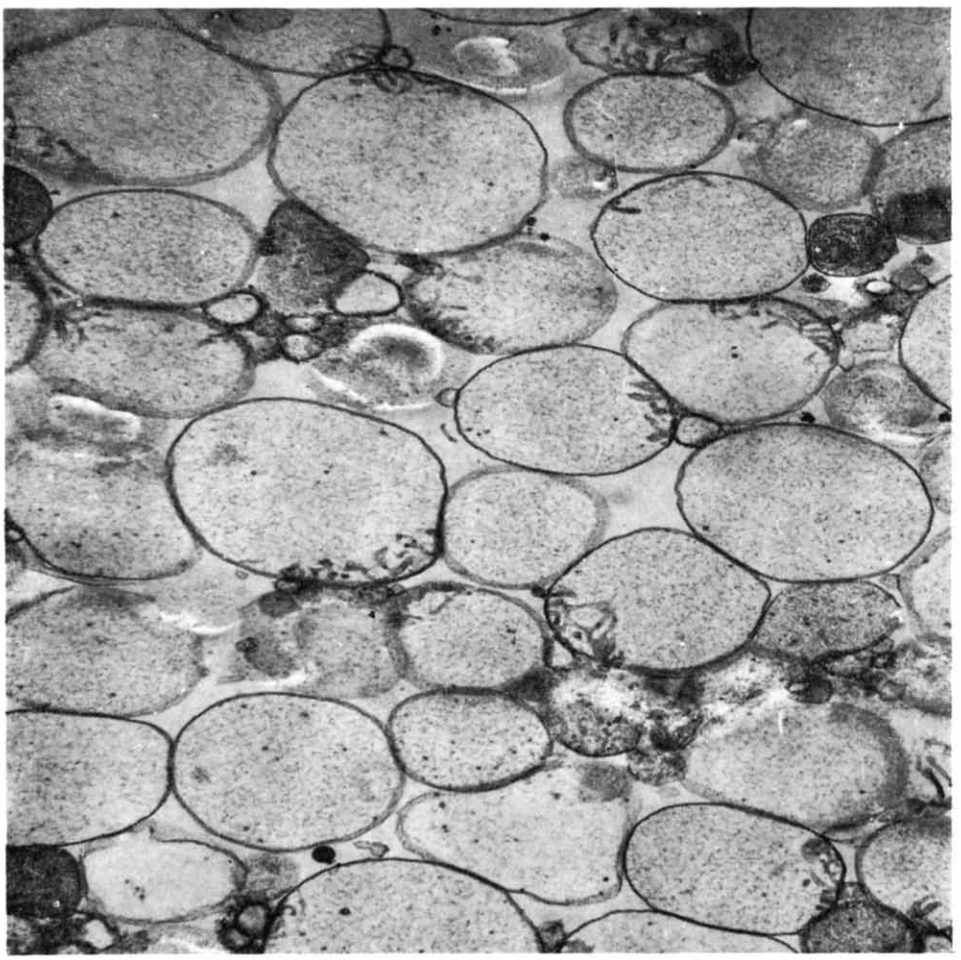

Fig. 5b.

ruption of the outer membrane. The control mitochondrial preparation incubated in the phosphate buffer lacking Dio-9 appears to have undergone only a mild degree of phosphate-induced swelling.

Temperature dependence of Dio-9-induced swelling

The initial rate of Dio-9-induced succinate–P_i-supported swelling is very sensitive to the temperature of the incubation medium. At 20° in one experiment,

Dio-9-dependent swelling occurred at an initial decrease in absorbance (520 mμ) of 1.900 units/min, at 0° this rate was reduced to 0.280 unit/min. A Q_{10} of 3.4 calculated from these initial rates is in line with the swelling induced by L-thyroxin, P_i and oxidized and reduced glutathione as reported by NEUBERT, FOSTER AND LEHNINGER[20].

DISCUSSION

The requirement of substrate oxidation for Dio-9-induced large-amplitude swelling and the inhibition of this swelling by respiratory inhibitors and uncouplers of phosphorylation indicates clearly the necessity of an energized mitochondrial state for the support of the swelling action. A further indication of such an energy requirement comes from experiments showing ATP to be capable of supporting Dio-9-induced swelling. HUIJING AND SLATER[21] showed oligomycin to be useful in testing whether the high-energy intermediates of oxidative phosphorylation can be directly utilized for energy-requiring reactions in the mitochondria, without having first to be converted to ATP. The inhibition of ATP-supported swelling, but not of substrate-supported swelling, by low concentrations of oligomycin is consistent with the involvement of a high-energy intermediate other than ATP in the swelling process[18,22]. Transport of Sr^{2+} may effectively compete with the Dio-9-induced swelling by the utilization of a non-phosphorylated intermediate. The Dio-9-sensitive region, is considered to be at either the dinitrophenol- or oligomycin-sensitive site in the reaction sequence leading to ATP synthesis during oxidative phosphorylation.

Concentrations of oligomycin normally used to inhibit oxidative phosphorylation do not inhibit substrate-supported swelling while much higher concentrations inhibit almost completely. At concentrations in excess of 20 μg/ml, oligomycin causes a slight uncoupling of mitochondrial respiration. It is this uncoupling which appears to be responsible for the inhibition of Dio-9 swelling by oligomycin in the presence of Group I or Group II substrates.

In contrast to the reversal of other types of swelling[23] attempts to reverse the large-amplitude Dio-9 swelling have been unsuccessful. This irreversibility may be due to the very rapid attainment of the fully swollen state and the loss of essential mitochondrial structural factors. From electron-microscope investigations of Dio-9-swollen mitochondria it is obvious that the swelling has led to a profound distortion and disintegration of the outer membrane with a concomitant swelling of the inner membrane.

The time course of Dio-9 swelling and the influence of uncouplers indicates a direct correlation between the swelling and the respiratory inhibition. The reversal of respiratory inhibition by cytochrome *c* for succinate and by NAD^+ and cytochrome *c* in the case of NAD^+-linked substrates supports this contention. With mitochondria which have undergone ATP-supported Dio-9 swelling, subsequent addition of 2,4-dinitrophenol followed by succinate does not result in respiratory stimulation. On the other hand when ATP swelling is inhibited by oligomycin, oxidation of added succinate is not impaired. These facts indicate that Dio-9 does not itself lead to inhibition of respiration, but that this inhibition is secondary to the large-scale morphological changes and co-factor loss which occurs during swelling.

Although there is no direct evidence, the difference between Group I and Group II substrates with respect to phosphate requirement for Dio-9 action may

represent a compartmentation of P_i (or some other potentially osmotically active ion) within the intact mitochondrion. Phosphate may be bound within a compartment containing the β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) and glutamate dehydrogenase (EC 1.4.1.2). Oxidation of these substrates could release the bound P_i, rendering it osmotically active, resulting in swelling. The possibility that β-hydroxybutyrate and glutamate may act both as oxidative substrate and as osmotically active ions in this case is unlikely, since Dio-9 does not induce swelling in a phosphate-free medium containing β-hydroxybutyrate, succinate and amytal (to prevent the oxidation of β-hydroxybutyrate). HEMKER[24], on the basis of differential uncoupling data, has suggested that the first phosphorylation site active during pyruvate-malate oxidation is physically separated from that operating during the oxidation of glutamate or β-hydroxybutyrate.

In a previous publication[6] it was postulated that Dio-9 inhibition of respiration involved antibiotic interaction with a phosphorylated high energy intermediate. It is now clear that such respiratory inhibition has as its fundamental cause the large amplitude swelling and consequent release of cytochrome *c*. On the other hand, the mechanism of action of Dio-9 is not restricted to its action as a swelling agent. The antibiotic is an inhibitor of oxidative phosphorylation and of the partial reactions in beef-heart submitochondrial particles, under conditions where there is no inhibition of respiration and presumably no swelling[8]. The antibiotic inhibits the ATPase activity of coupling factor 1 (F_1) (ref. 25) in the presence of the oligomycin-sensitizing factor isolated by KAGAWA AND RACKER[26] resembling, in some of its actions, the activity of oligomycin. Coupling factor 1 has recently been implicated as having both a structural and a catalytic role in mitochondria[27]. These observations and those reported in this communication lead to the speculation that Dio-9 is reactive with a component of the energy conservation mechanism which perform a structural as well as an enzymatic role in oxidative phosphorylation.

ACKNOWLEDGEMENTS

This work was supported by a Grant in Aid (R.J.G.) from the American Heart Association.

The very competent technical assistance of Mrs. G. KAZMI is appreciated. For the electron microscope work we are indebted to the technical skills of Miss P. BOWIE and Mr. J. SWAFFORD of the Electron Microscope Laboratory at Arizona State University.

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