REGULATION OF MITOCHONDRIAL OXIDATIONS BY INDUCTION OF MORPHOLOGICAL CHANGES I. DINITROPHENOL INITIATED CONCOMITANT TRANSITIONS IN INNER MEMBRANE-MATRIX STRUCTURE AND SUCCINATE OXIDATION

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Received August 15,1972

Summary. Dinitrophenol (DNP) induces rapid contraction of the inner membrane-matrix of rat liver mitochondria initially swollen by incubation in an iso-osmotic sucrose-phosphate medium containing succinate. Oxygen uptake is stimulated by all levels of DNP during dynamic contraction; however, once maximal contraction is approached succinate oxidation becomes inhibited. Both maximal inhibition of succinate oxidation and maximal contraction are achieved at 80μM DNP. Oxygen consumption is inhibited over 50% regardless of the concentration of DNP(80-1000μM) used to obtain maximal contraction. The magnitude of contraction (E_{650}) is essentially a linear function of concentration of DNP until maximal contraction is attained, but the rate of contraction continues to increase above the DNP level necessary to induce maximal contraction. 80μM DNP will prevent the initial mitochondrial swelling, but succinate oxidation is not inhibited until a considerably higher level of DNP is added; a certain degree of swelling seems essential to create the apparently noncompetitive inhibition of succinate oxidation at 80μM DNP.

Specific changes in mitochondrial structure accompanying induced respiratory changes have been postulated to represent primary energy capture and transduction (1,2). Although these morphological changes are coincident with changes in oxidative metabolism it now seems unlikely that oscillations between "configurations" drive the formation of ATP or other energy-linked mitochondrial reactions (3,4,5). It seems most likely that certain structural arrangements of the mitochondrial inner membrane and matrix provide the most efficient and stable environment during the specific metabolic steady states. A specific structural organization may originate and persist throughout a state of energy transduction but other forces and metabolic events can generate similar morphologies (5,6). It seems probable that morphological transformations are induced by metabolic changes and the level

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of key intermediates, e.g. phosphate and ADP but remain constant, once achieved, during steady state metabolism. These morphological "steady states" which show reciprocal volume relationships between matrix and intermembrane compartments, and possibly reflect conformational changes in the membrane and matrix components, in turn regulate utilization, flow and level of other intermediates at their particular sites of metabolism. This form of fine metabolic control may, at least, be established through changes in 1) enzyme conformation, 2) viscosity of the medium (particularly the matrix) through which the intermediates must flow, 3) concentrations of inhibitors and activators at active or allosteric sites, 4) membrane permeability to substrates and products, 5) intramitochondrial compartmentation, 6) membrane packing and 7) form and local concentrations of matrix components.

Several investigators have established the dual action of acidic uncouplers on mitochondrial oxidations, especially succinate oxidation (7,8, 9). Most experiments (10-13) suggest that the inhibitory action of dinitrophenol (DNP) on succinate oxidation at high concentrations is closely associated with competitive transport of succinate across the inner mitochondrial membrane and/or DNP may exert a direct effect on the succinate dehydrogenase.

The present investigation was undertaken to establish the role of uncoupler-induced morphological changes for control of mitochondrial processes, especially succinate oxidation. Oxygen consumption, absorbance and electron microscopic appearance were correlated whilst phosphate and dinitrophenol were used to generate swollen and contracted states during the metabolism of succinate by isolated rat liver mitochondria.

Materials and Methods. Rat liver mitochondria were isolated by the method of Johnson and Lardy (14). Protein concentrations were estimated as described by Gornall et al. (15). Mitochondria were incubated in the apparatus described previously (16) and were fixed with 1% glutaraldehyde in the specific metabolic states. The mitochondrial pellets obtained from centrifugation (2 min., Eppendorf Zentrifuge 3200) were washed in isotonic cacodylate buffer

(pH 7.2), before preparation by standard techniques (3,4,17), for examination in an AEI-EM6B electron microscope. Specific experimental details are described in legends to figures and tables.

Results. The data presented in Table I show that the addition of low concentrations (20 to 60μM) of DNP to respiring, partially swollen mitochondria stimulates oxygen uptake and promotes essentially linear increases in extinction at 650nm (E_{650}) which is a reflection of mitochondrial contraction. At 80μM DNP maximal contraction is attained in about 3min. At 1000μM DNP maximal contraction is attained in 15sec. At 80μM DNP there is a linear, relatively rapid, initial rate of contraction (ΔE_{650}/min) and a slower final phase of contraction. As the concentration of DNP is increased above 80μM the magnitude of ΔE_{650} does not increase but the initial rates of contraction increase, at least, until the sensitivity of instrumental measurements becomes limiting at 200μM DNP. The seconds to reach E_{650} (last column, Table I) indicate that the final phase of maximal contraction is considerably shortened by additional DNP.

During contraction oxygen consumption is increased by all concentrations of DNP but upon reaching maximal contraction at 80μM (or more) DNP oxygen uptake is markedly decreased (Table I, Fig. 1). Higher concentrations of DNP do not increase or measurably decrease oxidation rates during contraction nor do they consistently further inhibit succinate oxidation over that attained with 80μM DNP after reaching maximal contraction.

Dinitrophenol prevents swelling (Table II) when put into the incubation medium before introducing mitochondria. Partial swelling, the extent of which depends on the DNP level, occurs below 80μM DNP. High concentrations (1000μM) of DNP do not appear to prevent swelling as well as the intermediate levels (200μM) of DNP. Maximal stimulation of respiration is obtained at 60μM DNP, then decreases slowly as the level of DNP is increased. Mitochondrial respiration never becomes as inhibited as when swelling is allowed before adding DNP in sufficient concentration to bring about maximal contrac-

Table I. The effect of dinitrophenol concentration on the correlation between succinate oxidation and E_{650} of rat liver mitochondria.

DNP (μM)	natoms oxygen/mg protein/min during ΔE_{650}	natoms oxygen/mg protein/min after ΔE_{650}	final E_{650}	ΔE_{650}	$\frac{\Delta E_{650}}{min}$	seconds to reach final E_{650}
0	153	153	.208	0	-	-
20	210	210	.243	.035	.065	60
40	271	271	.275	.068	.160	95
60	275	275	.315	.103	.190	120
80	268	111	.353	.138	.230	195
100	275	118	.357	.142	.275	145
200	277	84	.360	.146	.570	80
500	271	101	.370	.146	.630	50
1000	279	105	.350	.135	.625	15

Oxidation rates were determined with a Clark electrode in a 4ml water jacketed cell (13 mm^2) designed for simultaneous absorbance measurements on a Rikadenki three-channel recorder. Incubations were carried out at 30° where oxygen solubility is 215nmoles per ml. The incident light was passed through a filter which transmitted wave lengths above 650nm; for brevity this is designated E_{650}. One mg of mitochondrial protein was added to the reaction mixture containing 500μM EDTA, 7.5mM succinate, 25mM phosphate (pH 7.2 with NaOH), 250mM sucrose, and 500ng rotenone per ml. The appropriate amounts of 2,4-dinitrophenol were added after the mitochondria had attained a maximally swollen state.

Table II. Oxidation rates and prevention of mitochondrial swelling by dinitrophenol.

DNP (μM)	natoms oxygen/mg protein/min	minimal E_{650}
0	172	0.222
20	210	0.251
40	305	0.277
60	324	0.297
80	324	0.313
100	286	0.319
200	276	0.329
500	229	0.311
1000	210	0.301

Conditions were the same as those given for Table I except DNP was present before starting the reaction with mitochondria.

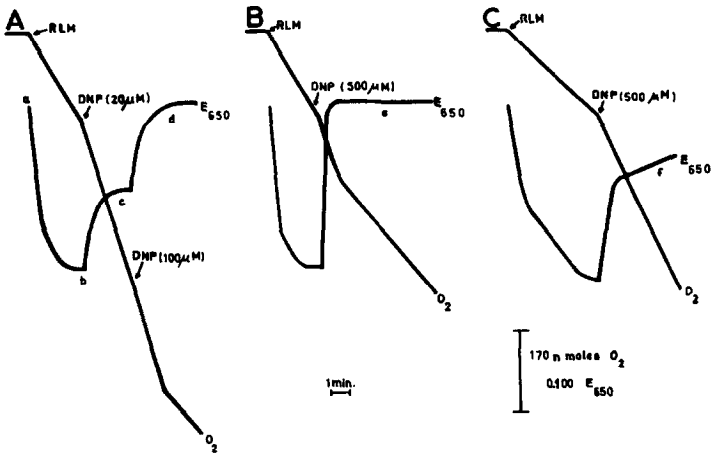

Fig. 1. Dinitrophenol-induced changes in the light scattering and succinate oxidation of rat liver mitochondria (RLM). Incubation conditions were as described for Table I except that in tracing C, phosphate was replaced by acetate. Additions are indicated by the arrows. The letters (a, b, c, d, e and f) on the Extinction trace designate the points at which preparations were fixed in similar but independently sampled experiments for electron microscopy (Fig. 2). A, B and C indicate separate recorder tracings.

tion (Table I). If additional DNP (final concentration of 140μM) is added after partial swelling in 40μM DNP the mitochondria contract maximally and respiration is inhibited maximally (over 60%). Addition of 60μM DNP to a system which is already 80μM DNP (present before adding mitochondria) does not bring about maximal inhibition of succinate oxidation.

The correlations among oxidation rates, E_{650} (swelling-contraction) and electron microscopic appearance of rat liver mitochondria (Figs. 1 & 2) illustrate the interdependence of succinate oxidation and the matrix-inner membrane morphology. Swollen mitochondria (Fig. 1A) oxidize succinate at a "controlled" rate and have a diffuse matrix (Fig. 2b). Uncoupled mitochondria (Fig. 1A) oxidize succinate rapidly and maintain a partially contracted matrix (Fig. 2c). Mitochondria which have been swollen and then maximally contracted with 100μM DNP show over 50% inhibition of succinate oxidation after contraction is completed (Fig. 1A), but uninhibited respiration during dynamic contraction, and possess a severely contracted matrix (Fig. 2d). Mitochondria which have been

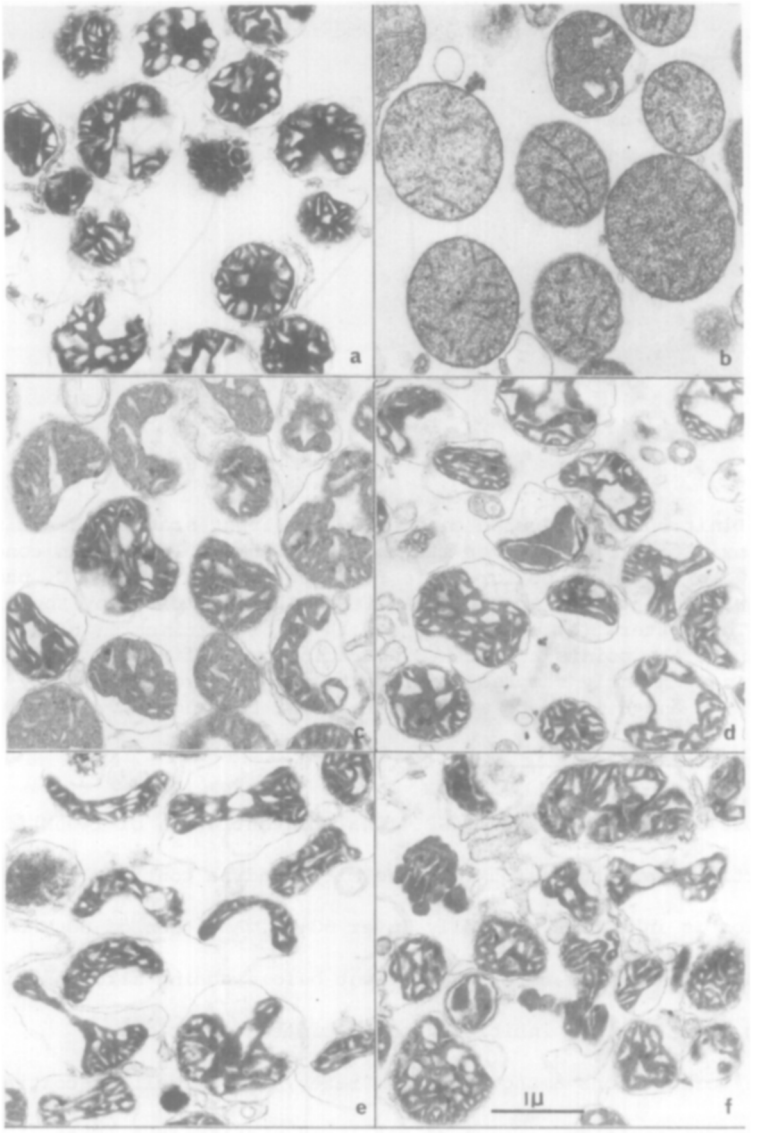

Fig. 2. Electron micrographs of rat liver mitochondria. Rat liver mitochondria were fixed with glutaraldehyde (1%) in the metabolic state indicated by a, b, c, d, e and f in Fig. 1. After a 10-15min fixation period samples were centrifuged and the mitochondrial pellets were washed once with an isotonic cacodylate buffer (pH 7.2) before a second fixation with OsO_4. Standard electron microscopy procedures were used for final processing. Specimens were examined with an AEI-EM6B electron microscope.

swollen and then maximally contracted with 500μM DNP (Fig. 1B and 2c) are indistinguishable from those shown in Fig. 2d. Again succinate oxidation is stimulated during dynamic contraction but becomes inhibited upon completion

of contraction. When acetate replaces phosphate in the incubation medium succinate oxidation is slower and a longer incubation time is required for swelling to be completed (Fig. 1C). Upon addition of 500μM DNP to these "acetate-swollen" mitochondria succinate oxidation is stimulated but only partial contraction is achieved, and at a slower rate than in the phosphate medium. This partial contraction is followed by a slow monotonous contraction but no inhibition of succinate oxidation occurs (Fig. 1C). An electron micrograph of these DNP contracted mitochondria (Fig. 2f) appears similar to the mitochondria shown in Fig. 2d.

Discussion. The interdependence of succinate oxidation (or transport), morphological changes and structural steady states exhibited by rat liver mitochondria has been correlated by utilization of the acidic uncoupler-inhibitor, dinitrophenol. At the DNP concentrations below 80μM only partial contraction was initiated by DNP and in agreement with others (7,8,9,18) succinate oxidation was always enhanced. At the DNP concentrations above 80μM succinate oxidation was stimulated during dynamic contraction but concomitant with attainment of maximal contraction succinate oxidation was inhibited. Inhibition appears to be maximal at the lowest DNP level required to attain maximal contraction. This apparent noncompetitive inhibition may prove to be modified allosteric inhibition (19) where an ultrastructural modification restricts the oxidation of succinate. The underlying mechanism(s) by which restraints are imposed cannot be ascertained by our experiments. In the introduction we pointed out at least seven possible ways in which inhibition could be accomplished by changing the structural steady state. One of these possibilities, concentration of an inhibitor, such as oxaloacetate, through the decrease in matrix volume brought about by contraction, seems unlikely because rotenone was always added with succinate and neither glutamate nor ATP relieved the inhibition consistently, though on occasion glutamate brought slight relief.

The failure to obtain 60% inhibition of succinate oxidation by pre-

vention of swelling with DNP is a dilemma but may explain why others (7-13) have not observed inhibition of oxygen uptake at the levels of DNP reported here. Another report (20) on DNP concentration and light scattering did not correlate the swelling-contraction rates and magnitudes with oxygen consumption and electron microscopic appearance. It seems possible that the swelling phase could 1) allow ions or substances from the external medium or intermembrane compartment to enter the matrix which are directly or indirectly responsible for the inhibition, 2) promote the loss of components from the matrix which restrains the inhibition, 3) change the structural characteristics of the inner membrane and matrix in such a way as to make succinate oxidation more susceptible to inhibition, and 4) make it possible for the slightly greater contractile state to occur which could be responsible for the inhibition of oxidation.

This report emphasizes the role of structural changes and steady states in the regulation of succinate oxidation and the interdependence of these parameters during mitochondrial steady state metabolism. It has been shown that a shift in steady state succinate oxidation can be forced by attainment of a new, readily observable morphological steady state which in turn results from the oxidation of succinate and perhaps intramitochondrial ion fluxes.

Acknowledgements. The authors are grateful to Miss Diana Stonebridge and Mrs. Susan Bufton for technical assistance. This investigation was supported in part by a U.S. P.H.S. Grant from NIH (AM13939-03) and a Special Fellowship Award from NIH (GM52221-01) to P.V. Blair.

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