PASSIVE INDUCTION OF THE "ENERGIZED-TWISTED" CONFORMATIONAL STATE IN BOVINE HEART MITOCHONDRIA

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It is demonstrated that hypotonically-swollen bovine heart mitochondria undergo transformation into the "energized-twisted" conformational state (Green et al. Arch. Biochem. Biophys. 125:684, 1968) when simultaneously exposed to glutaraldehyde fixative and induced to contract by passive osmotic means. It is suggested that this passively-induced transformation and energy-dependent transformation of mitochondria into the "energized-twisted" state occur by similar mechanisms.

Green and coworkers (1,2,3) have recently described three conformational states in isolated bovine heart mitochondria: (1) "non-energized", (2) "energized", and (3) "energized-twisted" - observed by means of electron microscopy on mitochondria fixed initially at room temperature with glutaraldehyde-acrolein subsequent to incubation at 30° C in 0.25 M sucrose media (1) in the absence of ATP or oxidizable substrates, (2) in the presence of ATP or oxidizable substrates, and (3) in the presence of oxidizable substrates and Pi, respectively. Mitochondria in the "non-energized" state are characterized by extremely high matrical densities and extremely low ratios of inner compartment space to outer compartment space - characteristics typical of highly contracted mitochondria. Upon supplying ATP or oxidizable substrate to mitochondria in this state, inner compartment space increases slightly concomitant with a slight decrease in outer compartment space, matrical density, and light scattering ability (about 15%), forming mitochondria in the "energized" state. Upon being exposed to Pi, mitochondria "energized" with oxidizable substrate rapidly accumulate Pi and undergo a further decrease in light-scattering ability (about 30% relative to "non-energized" mitochondria). Concomitantly the inner mitochondrial membranes undergo remarkable transformations, breaking

up to form masses of twisted-appearing tubules: the "energized-twisted" state. Since inhibitors of energy utilization (e.g., antimycin, rotenone, rutamycin) and substances known to discharge high-energy intermediates of oxidative phosphorylation (e.g., ADP, uncouplers, divalent metal ions) are capable of reversing the above-described transformations, a functional relationship between these transformations and oxidative phosphorylation is indicated. Thus, Green and coworkers (3) conclude that there are no high-energy intermediates of oxidative phosphorylation and that the "energized" and "energized-twisted" conformational states are the functional equivalents of these intermediates.

Finding it difficult to accept a theory of mitochondrial energy conservation involving disruption of the inner mitochondrial membrane, we have examined the possibility that the "energized-twisted" state is an artifact of fixation. That mitochondria in this state can be formed artifactitiously was indicated by results obtained in the early stages of a detailed investigation of the ultrastructural changes associated with osmotically-induced swelling of bovine heart mitochondria. In this study we noted that under certain conditions of glutaraldehyde fixation the inner membranes of swollen mitochondria tend to break up to form tube-like structures with bulbous ends, similar to those of "energized-twisted" mitochondria except that the tubules and bulbous ends are relatively large in diameter and relatively untwisted in appearance. In this study we also noted that (1) glutaraldehyde fixation occurs very rapidly, (2) glutaraldehyde rapidly inactivates the mitochondrial enzymes involved in respiration and energy transfer, and (3) glutaraldehyde-fixed swollen mitochondria can undergo osmotically-induced contraction. It seemed possible, therefore, that the "energized-twisted" state could be induced artifactitiously by rapid breaking up of the inner membranes of swollen mitochondria upon being exposed to glutaraldehyde, followed immediately by osmotically-induced shrinkage of the tubules thereby reducing their size and giving them a more flattened and twisted appearance. That a fairly high de-

gree of mitochondrial swelling is associated with induction of the "energized-twisted" state and that contraction ensues when energy utilization is blocked are indicated by the light-scattering data of Green et al. (3).

This communication summarizes the results of our efforts to determine whether the "energized-twisted" state can be induced according to the mechanism suggested above. In designing the experiments it was assumed that mitochondrial swelling in the 0.25 M sucrose media of Green et al. (3) occurs in response to increased inner compartment osmotic pressure resulting from active accumulation of substrates and Pi, and that contraction occurs in response to decreased inner compartment osmotic pressure resulting from net outward diffusion of the accumulated substrates and Pi. Active mitochondrial swelling and contraction occurring by this mechanism can be simulated simply by lowering and raising the ambient concentration of nonpenetrating solutes; therefore, it was possible to simulate with non-energized mitochondria the conditions suggested above to be essential to the energy-dependent induction of the "energized-twisted" state. Thus, heart mitochondria were induced to swell in hypotonic media and then exposed to glutaraldehyde either at the same time or slightly before they were induced to contract by raising the ambient tonicity gradually over a period of about 1 min [approximately the time it takes the actively swollen mitochondria of Green et al. (3) to contract after energy utilization is blocked - as indicated by the light-scattering data]. By this means it is demonstrated that the "energized-twisted" conformational state can be passively induced.

Methods. Bovine heart mitochondria were isolated according to a slight modification (4) of the Nagarse procedure of Hatefi et al. (5). Conditions of incubation and preparation for electron microscopy are presented with the results. Glutaraldehyde was prepared by vacuum distillation of 50% glutaraldehyde (Biological Grade, Fisher Scientific Co.) and quantitated spectrophotometrically, as described by Anderson (6).

Results and Conclusions. Fig. 1 shows heart mitochondria fixed in various

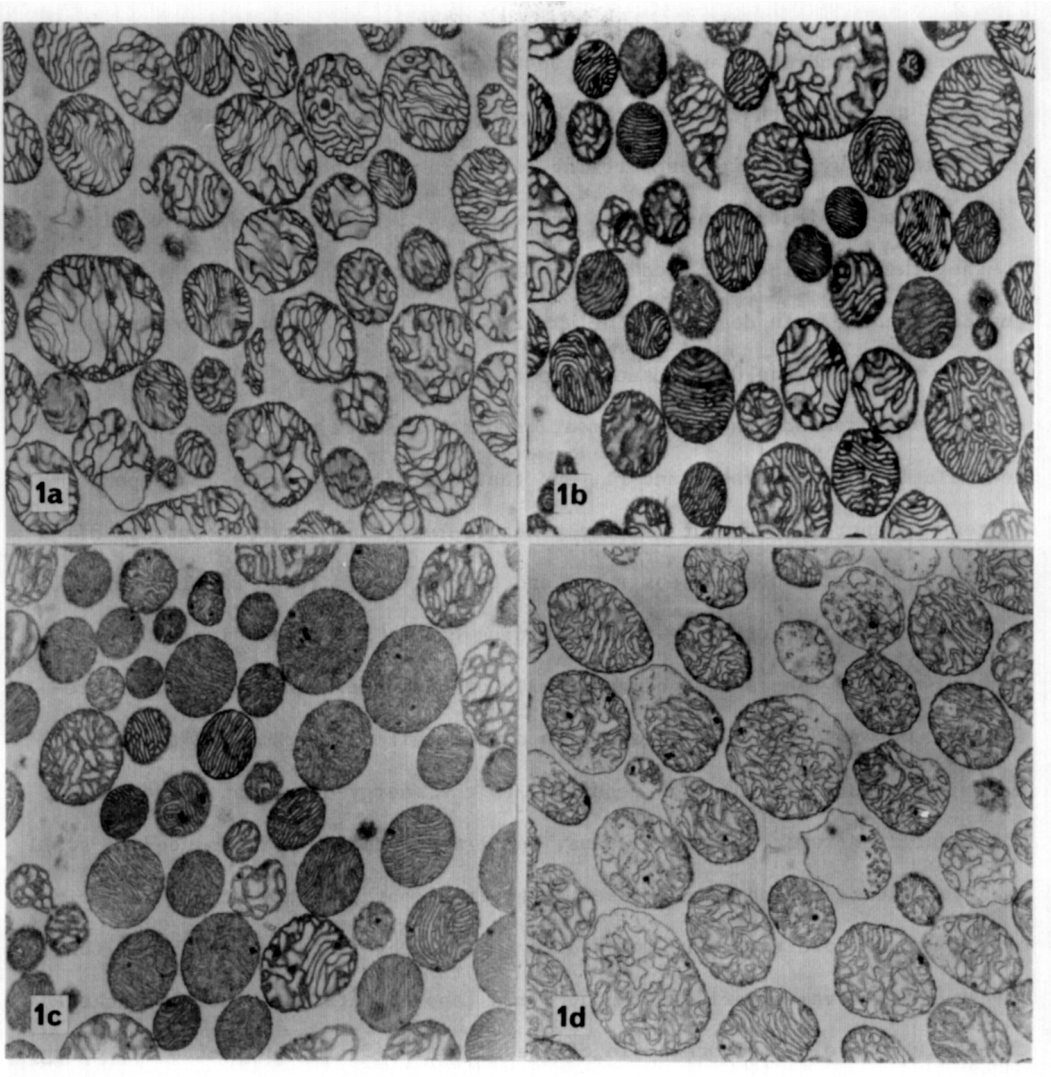

Figure 1. Ultrastructural changes associated with osmotically-induced swelling of bovine heart mitochondria. Mitochondria were (1) suspended at a concentration of 0.5 mg protein/ml in sucrose solutions containing 5 mM of the potassium salt of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (K-HEPES), pH 7.6, (2) incubated at 0° C for 5 min, (3) fixed initially with 0.15% glutaraldehyde (added to the suspensions as 15% solution) for 5 min, and (4) fixed secondarily with osmium (added to the suspensions as 4% OsO_4 in two consecutive aliquots spaced 15 min apart, each giving a final concentration of 0.125%) for 30 min. Final tonicities of the suspending solutions before fixation was initiated were: (a) 255 mosM; (b) 135 mosM; (c) 95 mosM; and (d) 55 mosM. Fixed mitochondria were sedimented in 400 ul polyethylene microcentrifuge tubes in a Model 152 Microfuge (Beckman Instruments, Inc.), dehydrated in graded ethanol, and embedded in Dow Epoxy Resin according to a procedure modified slightly from that of Lockwood (7). Thin sections were stained with uranyl acetate and lead citrate (8). x 10,000.

osmotically-induced swelling states according to a procedure found suitable for preserving the inner membranes. While suspended at 255 mosM tonicity (Fig. 1a), the mitochondria have a highly contracted appearance similar to that of the "non-energized" mitochondria of Green et al. (3). Decreasing the ambient tonicity to levels as low as about 95 mosM (Fig. 1c) results in swelling of the inner compartment primarily at the expense of the outer compartment. At 135 mosM (Fig. 1b) the degree of inner compartment swelling is similar to that of the "energized" mitochondria of Green et al. (3). Decreasing the ambient tonicity to levels beyond about 95 mosM results in rupture of the outer membrane and expansion of the inner compartment through the break (Fig. 1d).

Companion studies showed that mitochondrial optical density [measured with a Coleman Model 6A spectrophotometer and corrected in accordance with the findings of Tedeschi and Harris (9,10)] decreases with swelling as follows: 255 mosM, 100%; 135 mosM, 88%; 95 mosM, 83%; and 55 mosM, 67%. Companion studies also showed that the changes in mitochondrial ultrastructure and optical density occurring in media of about 95 mosM tonicity and above are completely reversible. Mitochondria "reversed" from the 55 mosM swelling state (Fig. 1d) have a normal contracted appearance except for a ruptured outer membrane and a slightly distorted inner compartment configuration on the side on which the break in the outer membrane occurred during swelling.

Figs. 2 and 3 summarize the results of the experiments designed to simulate energy-dependent induction of the "energized-twisted" conformational state. Fig. 2a shows the form swollen mitochondria often take when exposed to glutaraldehyde at 30° C, and Figs. 2b, 2c, 2d, and 3b show variations of the "energized-twisted" state swollen mitochondria assume when induced to contract at the same time (Figs. 2b and 2c) or shortly after (Figs. 2d and 3b) they are exposed to glutaraldehyde at 30° C. That transformation to the "energized-twisted" state occurs during glutaraldehyde fixation and not during osmium fixation is indicated by the observation that the inner membranes tend to re-

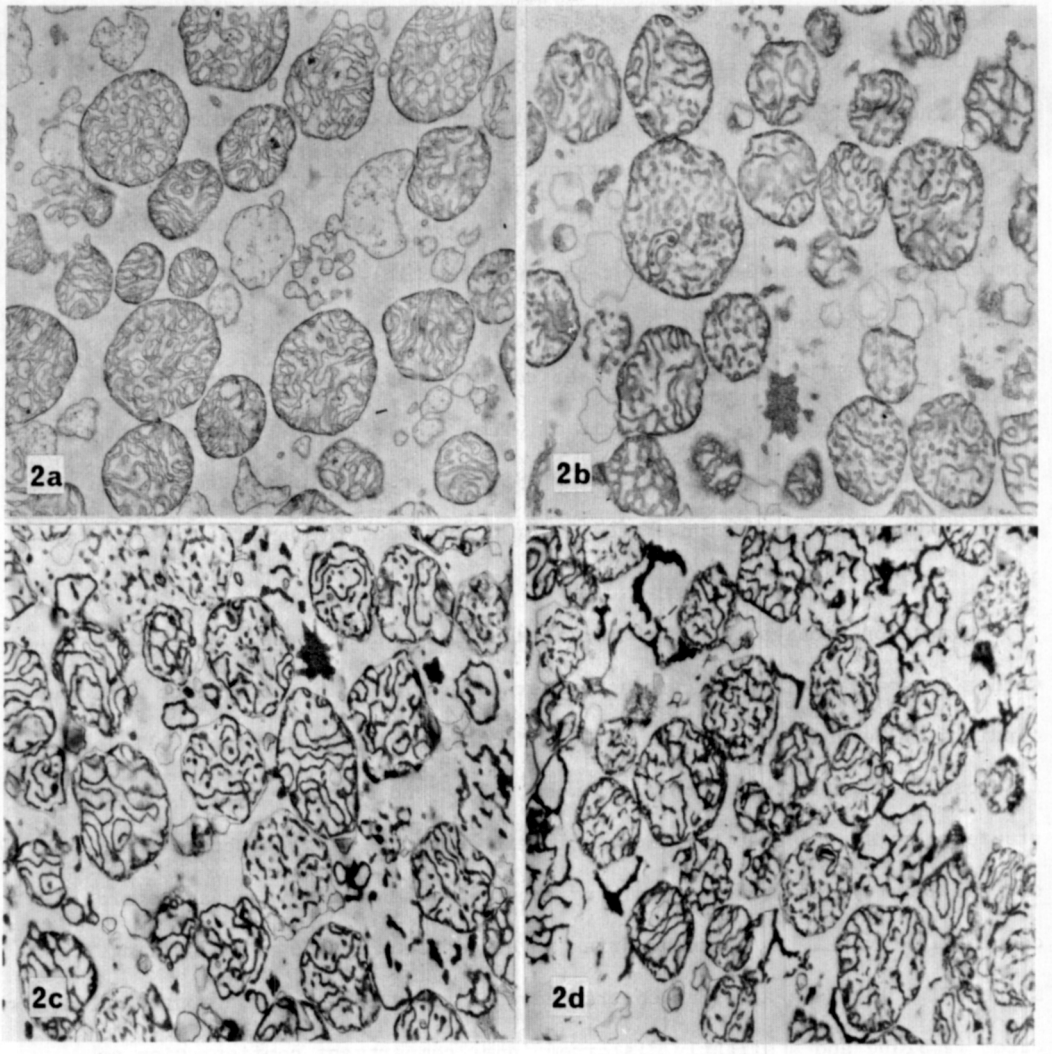

Figure 2. Passive induction of the "energized-twisted" conformational state. Mitochondria were suspended initially at 1 mg protein/ml in 35 mM sucrose - 5 mM K-HEPES, pH 7.6 - 2 uM rotenone (Final tonicity = 50 mosM). Mitochondria of Figs. 2a through 2c were incubated for 5 min at 30° C and then diluted gradually to 0.5 mg protein/ml over 0.5 to 1 min with solutions (30° C) containing 5 mM K-HEPES, pH 7.6, 4% glutaraldehyde, and sufficient sucrose to take the final tonicity (disregarding the glutaraldehyde) to: (a) 50 mosM; (b) 140 mosM; and (c) 260 mosM. Mitochondria of Fig. 2d were incubated for 5 min at 30° C and then exposed to 0.6% glutaraldehyde (added to the suspension as 15% solution) for 1 min prior to diluting the suspension and taking the final tonicity to 260 mosM as described above. While additions were being made, the suspensions were stirred vigorously to minimize sudden exposure of the mitochondria to high concentrations of sucrose. Subsequent to dilution, the mitochondria were (1) maintained at 30° C for 15 min, (2) cooled to 0° C, and (3) fixed with osmium either by addition of 4% OsO_4 to the suspension (Figs. 2a and 2b) as described in Fig. 1 or by exposure of sedimented mitochondria to 2% OsO_4 - 50 mM K-cacodylate, pH 7.5 - 250 mM sucrose for 1 hr at 0° C (Figs. 2c and 2d). Sedimentation, dehydration, and embedding were achieved as described in Fig. 1. x 10,000.

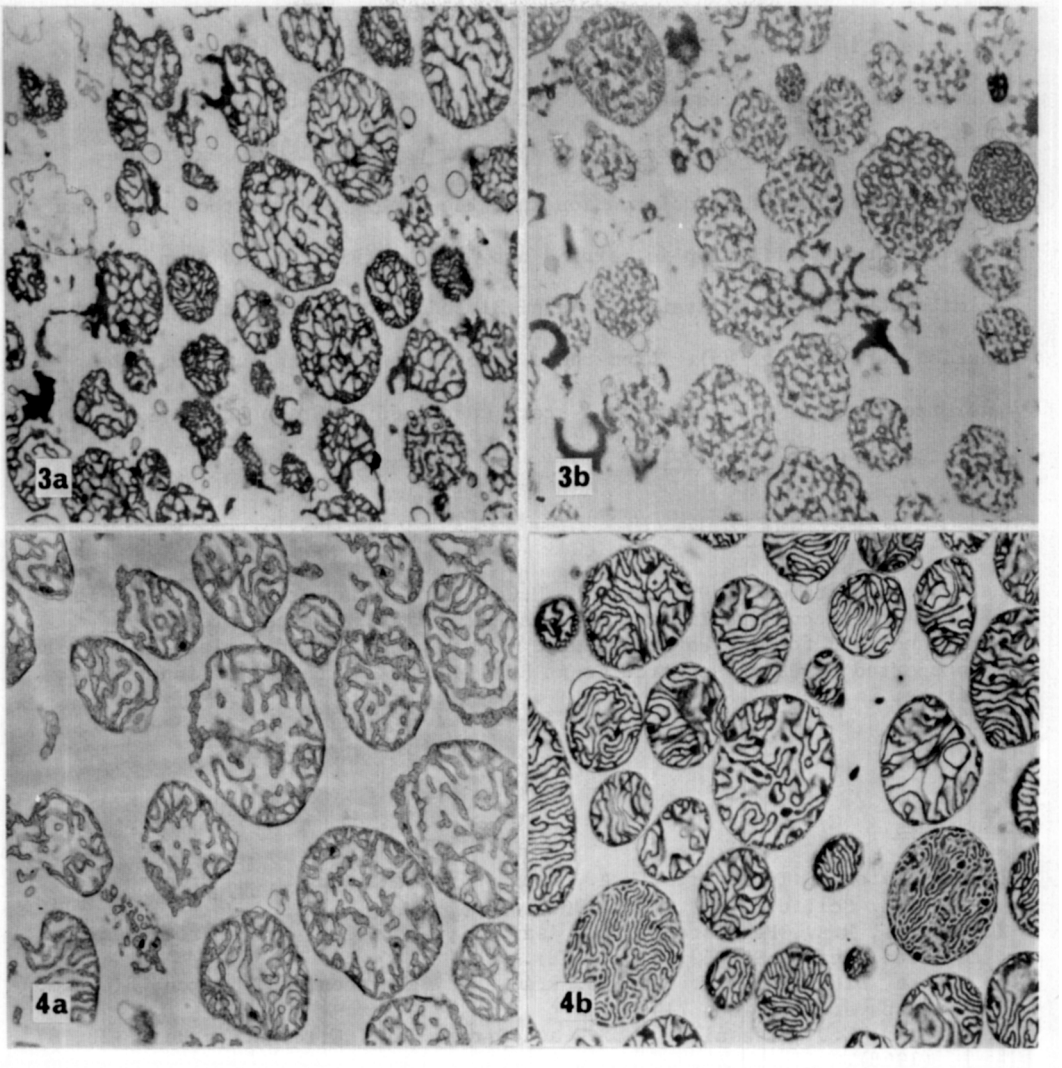

Figure 3. Effect of temperature of glutaraldehyde fixation on disruption of the inner mitochondrial membrane. Mitochondria were suspended initially at 1 mg protein/ml in 50 mM sucrose - 5 mM K-HEPES, pH 7.6 (final tonicity = 65 mosM) and preincubated, exposed to 0.6% glutaraldehyde, and induced to contract (260 mosM final tonicity) as described for the mitochondria of Fig. 2d except that the mitochondria of Fig. 3a were maintained at 0° C. Osmium fixation, sedimentation, dehydration, and embedding were carried out as described in Fig. 1. x 10,000.

Figure 4. Disruption and distortion of the inner compartments of mitochondria fixed initially for 1 hr at 0° C with 1% glutaraldehyde and secondarily with osmium by exposure of sedimented mitochondria to OsO_4 vapors for 1 hr at 0° C. Prior to fixation, the mitochondria were suspended at 0.5 mg protein/ml and preincubated for 5 min at 0° C in solutions containing 5 mM K-barbital, pH 7.7 and sufficient sucrose to give final tonicities of: (a) 50 mosM and (b) 250 mosM. Glutaraldehyde was added to the suspensions as 25% solution. Sedimented mitochondria were exposed to osmium vapors (from 4% OsO_4) in a moist chamber. Sedimentation, dehydration, and embedding were achieved as described in Fig. 1. x 10,000.

main intact in mitochondria exposed to glutaraldehyde and then induced to contract while maintained at 0° C (Fig. 3a). However, when mitochondria are exposed to glutaraldehyde for relatively long periods (1 hr), extensive changes suggestive of transformation into the "energized-twisted" state can occur even though fixation is carried out at 0° C (Fig. 4) and even though the mitochondria are maintained in a highly contracted state throughout the fixation period (Fig. 4b). From these observations we conclude that the "energized-twisted" conformational state described by Green et al. (1,2,3) may be an artifact of fixation.

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References.

1. Penniston, J.T., Harris, R.A., Asai, J., and Green, D.E. Proc. Natl. Acad. Sci. U.S. 59, 624 (1968).
2. Harris, R.A., Penniston, J.T., Asai, J., and Green, D.E. Proc. Natl. Acad. Sci. U.S. 59, 830 (1968).
3. Green, D.E., Asai, J., Harris, R.A., and Penniston, J.T. Arch. Biochem. Biophys. 125, 684 (1968).
4. Stoner, C.D., Ressallat, M.M., and Sirak, H.D. Circulation Res. 23, 87 (1968).
5. Hatefi, Y., Jurtshuk, P., and Haavik, A.G. Arch. Biochem. Biophys. 94, 148 (1961).
6. Anderson, P.J. J. Histochem. Cytochem. 15, 652 (1967).
7. Lockwood, W.R. Anat. Rec. 150, 129 (1965).
8. Reynolds, E.S. J. Cell Biol. 17, 208 (1963).
9. Tedeschi, H. and Harris, D.L. Arch Biochem. Biophys. 58, 52 (1955).
10. Tedeschi, H. and Harris, D.L. Biochim. Biophys. Acta 28, 392 (1958).