

## ON THE "ENERGIZED-TWISTED" CONFIGURATION OF ISOLATED BEEF HEART MITOCHONDRIA

GEORGE R. HUNTER and GERALD P. BRIERLEY. From the Department of Physiological Chemistry College of Medicine, Ohio State University, Columbus, Ohio 43210

At least two distinct morphological alterations as a function of metabolic activity of isolated beef heart mitochondria can be detected by electron microscopy. The first is an osmotic swelling and expansion of the matrix which can result from either the energy-linked or the passive uptake of solutes such as  $K^+$  acetate or phosphate (1, 2). The matrix expansion seen under these conditions closely resembles that seen when the mitochondria are swollen in hypotonic media (3). The expanded matrix network can be recontracted by osmotic pressure of sucrose or KCl (3) or, under some conditions, by energy-linked ion extrusion (2). A similar reversible expansion of the matrix of liver mitochondria has been reported by Hackenbrock (4-6). Green and coworkers (7-12) have noted that the matrix of heart mitochondria expands slightly during respiration in the absence of  $P_i$  and have designated this form the "energized" configuration.

The second type of alteration is a striking rearrangement of the matrix network into a mass of twisted tubules as first reported by Green and coworkers (7-9) and designated by them as the "energized-twisted" configuration. These morphological alterations have provided the basis for a model of energy transduction involving major conformational shifts in the membrane components (12) and an elaborate topological analysis of mitochondrial morphology (10). However, the relationship of this twisted configuration to energy transduction is by no means clear. For example, similar transitions are not seen in coupled heart mitochondria prepared by alternative procedures

(13), or in skeletal muscle mitochondria (14), or liver preparations (4-6). Butler and Judah (15) found no change in conformation of mitochondria with respiration state when buffered potassium permanganate was used as a fixative. In addition it has been reported that addition of ADP in the absence of  $P_i$  induces the highly condensed "non-energized" configuration which remains unchanged when substrates and  $P_i$  are introduced (16). Stoner and Sirak (17) have suggested that the energized-twisted form is an artifact which results from glutaraldehyde-induced rupture of the inner membranes of swollen mitochondria and simultaneous contraction as a result of sucrose in the incubation mixture. Unfortunately the conditions used for the passive induction of the energized-twisted configuration (17) were quite different from those of Green and coworkers (7-9), and the possibility of a fixation artifact seems to have been discarded by the latter group (see reference 11, for example). In the present communication we present evidence that extensive changes in light scattering occur following fixation of heart mitochondria under the conditions necessary for induction of the energized-twisted form. These light scattering changes are those predicted by the Stoner and Sirak (17) model and strongly support their interpretation that the energized-twisted form is an artifact of fixation.

### METHODS

Beef heart mitochondria were prepared with Nagarse (Enzyme Development Corporation, New York) and ethyleneglycol bis( $\beta$ -aminoethylether) $N,N',N',N'$ -

tetraacetic acid (EGTA) as previously described (18). Oxygen consumption, pH, and absorbance at 546  $m\mu$  were recorded simultaneously by using a Clark electrode, a combination pH electrode, and a circular glass cuvette equipped with a stirrer and water jacket and mounted on an Eppendorf photometer (Brinkman Instruments Inc., Westbury, N. Y.) (19). Mitochondria were fixed for electron microscopy by the addition of 8% neutralized glutaraldehyde (Polysciences, Inc., Warrington, Pa.) to a final volume of 0.2 or 0.6% as indicated. The mitochondria were centrifuged out of the glutaraldehyde suspension after 30 min at 0°C, washed once, and postfixed in buffered 1% osmium tetroxide. Mitochondria fixed in this way were washed three times in phosphate buffer and then distilled water, dehydrated in a graded ethanol series, embedded in Dow Epoxy Resin (Dow Chemical Co., Midland, Mich.), thin sectioned, and stained with uranyl acetate and lead citrate (see reference 2 for details). Sections were examined in a JEM-7 electron microscope. A number of low power fields of mitochondria fixed in different metabolic states were chosen at random and the number of mitochondria in each of the various configurational forms described by Green et al. (see reference 9, Figs. 1-3) was evaluated. Percentages reported are based on counts of 200-400 individual mitochondria in each case. Mitochondria were designated energized-twisted (ET) if the matrix was broken up into a series of twisted tubules rather than being the usual highly compressed network (nonenergized). A slight expansion of the intact network was designated as energized (E). If the matrix network had expanded to more than twice its volume in the highly condensed (nonenergized) form, the particles were designated as swollen (see reference 2, Fig. 3 A). From 3 to 7% of the particles in our fields could not be placed in any of these categories and were left unclassified.

## RESULTS AND DISCUSSION

The study shown in Fig. 1 establishes that isolated beef heart mitochondria assume the energized-twisted configuration when they respire in the presence of phosphate or arsenate, but in the absence of adenosine diphosphate (ADP). Fig. 2 *a* shows the appearance of beef heart mitochondria fixed while respiring but in the absence of  $P_i$ . These particles show a more expanded matrix than uncoupled (nonenergized) mitochondria, but the matrix can be further expanded by either hypotonic swelling (3) or by respiration-dependent ion accumulation (Fig. 2 *b*). Addition of phosphate results in conversion of more than 85% of the mitochondria to the energized-twisted form (Fig.

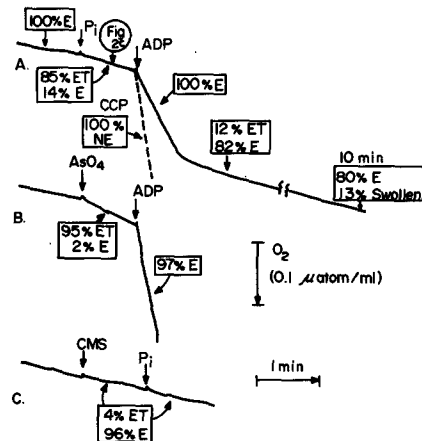
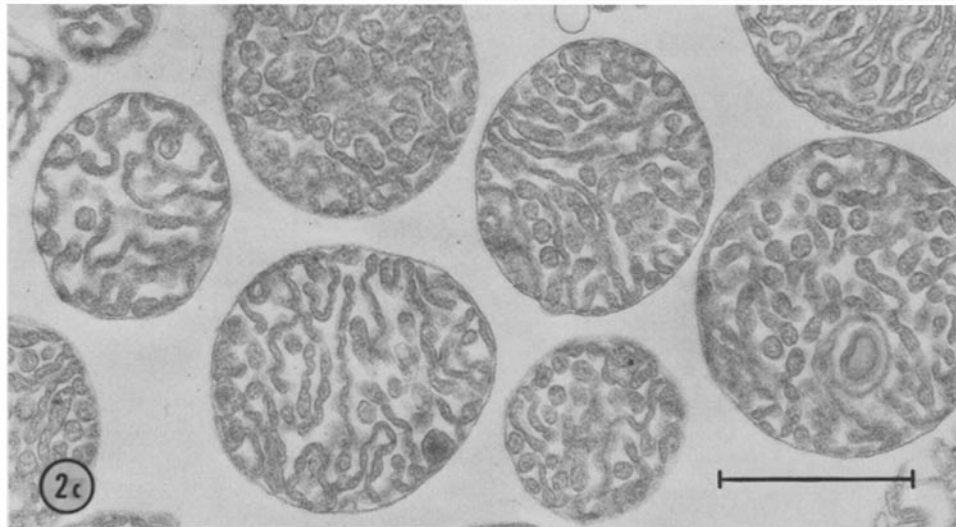
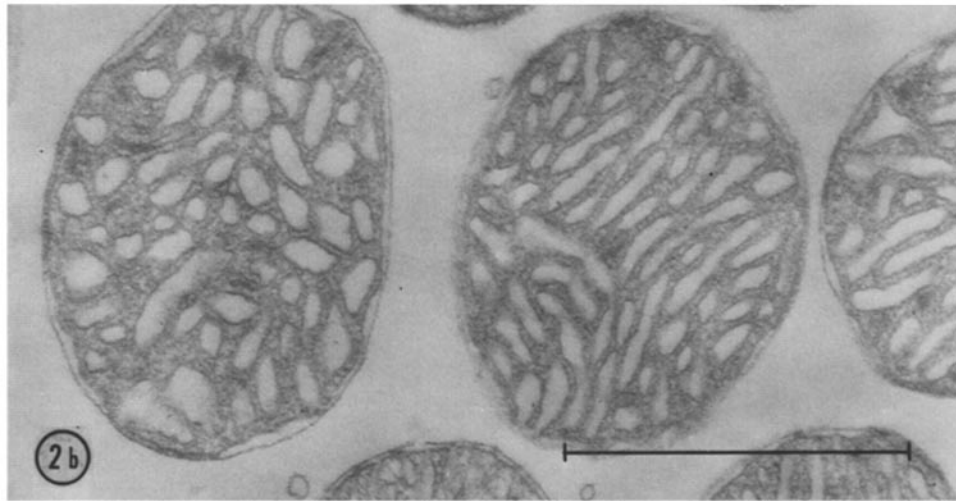
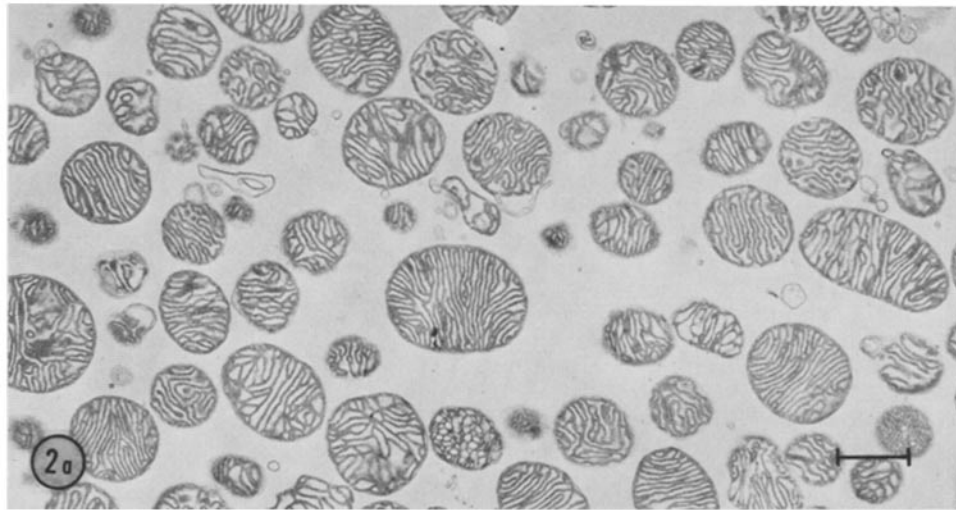


FIGURE 1 Conditions for the generation of the energized-twisted configuration. Beef heart mitochondria (5 mg of protein) were incubated at 25°C in 8 ml of sucrose (0.25 M) containing  $K^+$  succinate (2 mM, pH 7), and respiration was monitored with a Clark electrode. Where indicated, the following additions were made:  $K^+$  phosphate (10 mM),  $K^+$  arsenate (10 mM), adenosine diphosphate (ADP, 0.1 mM), *m*-chlorocarbonyl cyanide phenylhydrazine (CCP,  $5 \times 10^{-7}$  M), *p*-chloromercuriphenyl sulfonate (CMS, 100  $\mu$ M). At each of the points indicated the reaction was stopped by the addition of glutaraldehyde, and the mitochondria were examined in the electron microscope as described in the text. The percentage of mitochondria in each of the morphological categories defined is tabulated at the point of fixation. ET, energized-twisted; NE, nonenergized; E, energized (see Green et al. [9]).

2 *c*). Addition of ADP to establish State 3 respiration (20) discharges the energized-twisted condition, as does the addition of an uncoupler (Fig. 1). When State 4 respiration resumes following the ADP pulse, a portion of the mitochondria (12%) revert to the twisted form (Fig. 1). Prolonged incubation in State 4 results in the appearance of a greater number of mitochondria with swollen matrix and disappearance of the twisted form. These results confirm the reports of Green and coworkers (7-12) as to the conditions necessary to establish the energized-twisted configuration. It is of interest to note, however, that these are also the conditions which support the spontaneous accumulation of monovalent cations and phosphate (21-24). In this regard Fig. 1 C shows that the generation of the energized-twisted form is blocked by the presence of *p*-chloromercuriphenyl sulfonate (CMS), a reagent which does not interfere with State 4 succinate respiration under these



**FIGURE 2 a** Typical low power field of beef heart mitochondria in the energized configuration. This field was obtained at the point on the respiration trace shown in Fig. 1 and indicated by "100% E." Bar equals  $1 \mu$ .  $\times 8700$ .

**FIGURE 2 b** Expanded matrix form of isolated beef heart mitochondria obtained under conditions of respiration-dependent ion uptake (corresponding to Fig. 3 D). Bar equals  $1 \mu$ .  $\times 45,000$ .

**FIGURE 2 c** Energized-twisted configuration field obtained at the point indicated on Fig. 1 A. Bar indicates  $1 \mu$ .  $\times 25,000$ .

conditions, but which blocks the mitochondrial phosphate transporter system (25–27). This result suggests that the accumulation of  $P_i$  is essential for the change in morphology. The conversion to the energized-twisted form is not specific for  $K^+$ , however, since substitution of choline salts of  $P_i$  and succinate has little effect on the relative proportions in the twisted configuration (83% ET, 5% E, and 7% swollen were obtained with the choline salts under the conditions of Fig. 1 A following the addition of phosphate). Heart mitochondria accumulate choline under similar conditions (21).

It is now clear that isolated heart mitochondria are capable of accumulating large amounts of phosphate and acetate salts in the absence of added ionophores by a respiration-dependent reaction, and that this uptake of salts results in osmotic swelling (21–24). In a medium containing sucrose the osmotic pressure of the nonpenetrating sucrose strongly opposes this respiration-dependent osmotic swelling so that the net phosphate accumulation and osmotic expansion of the matrix are limited. However, the swelling is of sufficient magnitude to be clearly visible by conventional absorbance or light scattering measurements (Fig. 3 A). Interruption of the supply of energy by addition of an uncoupler or an inhibitor of respiration results in rapid contraction due to excess sucrose osmotic pressure in the absence of a driving force to maintain the ion gradient established by respiration. Ionophores, such as valinomycin, further accelerate the contraction (Fig. 3 A). The light scattering results reported by Harris et al. (28) show this phenomenon clearly, but have been interpreted as evidence for the conformational changes which result in the establishment of the energized-twisted configuration.

Addition of glutaraldehyde (0.2%) to contracted mitochondria (Fig. 3 A) results in little further absorbancy change and the particles are fixed rapidly (in less than 1 min) by the criterion that they are not lysed by Triton X-100. In contrast, if glutaraldehyde is added to mitochondria swollen by spontaneous phosphate accumulation (Fig. 3 C), a slow contraction results which ceases only after about 10 min and at an absorbancy value identical with that of glutaraldehyde-treated contracted mitochondria (compare Figs. 3 A and 3 C). Mitochondria fixed under these conditions show the energized-twisted form. Unfixed mitochondria are rapidly lysed by Triton,

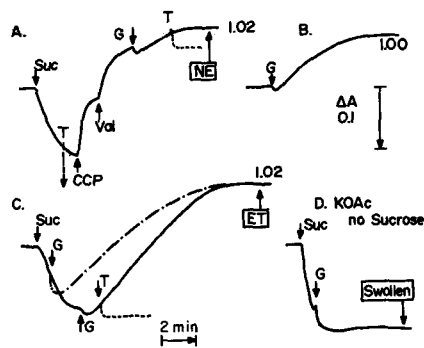


FIGURE 3 Absorbance changes, following fixation with glutaraldehyde, of heart mitochondria swollen by phosphate uptake. Rotenone-treated mitochondria (5 mg of protein) were incubated at 25°C in 8 ml of sucrose (0.25 M) containing  $K^+$  phosphate (10 mM, pH 7) and swelling was initiated by the addition of  $K^+$  succinate (2 mM). Absorbance at 546 m $\mu$  was recorded with an Eppendorf photometer. Where indicated, glutaraldehyde (G) was added to a final concentration of 0.2% and Triton X-100 (T) to a final concentration of 0.3%; CCP,  $5 \times 10^{-7}$  M; valinomycin (Val),  $2 \times 10^{-7}$  M. In Fig. 3 D the sucrose-phosphate medium was replaced by a medium of  $K^+$  acetate (120 mM, pH 7). Electron microscopy of the fixed mitochondria of Fig. 3 C shows the energized-twisted configuration (Fig. 2 c), Fig. 3 A, nonenergized, and Fig. 3 D, swollen matrix (Fig. 2 b).

but, as shown in Fig. 3 C, even the swollen mitochondria are rapidly fixed by glutaraldehyde by the criterion of the response to Triton. Higher concentrations of glutaraldehyde (0.6%) cause even more rapid contraction to the same final absorbancy value. Mitochondria which are allowed to swell less extensively contract less on addition of glutaraldehyde and reach the identical final absorbancy reading (Fig. 3 C). Mitochondria which have not been swollen by these procedures show little contraction with glutaraldehyde fixation (Fig. 3 B). Where swelling occurs with no sucrose in the medium (Fig. 3 D), there is no swelling or contraction on fixation with glutaraldehyde and micrographs show an intact swollen matrix network (see reference 2, Fig. 7). This result also establishes convincingly that the apparent contraction does not result from glutaraldehyde-induced aggregation of the mitochondrial suspension. These studies indicate that glutaraldehyde rapidly fixes a suspension of mitochondria in such a way as to prevent lysis by a detergent but has not fixed the membrane to a degree which prevents an osmotic contraction due to sucrose in the

medium. The absorbancy changes appear to provide direct evidence in support of the suggestion of Stoner and Sirak (17) that the energized-twisted configuration is an artifact which results from glutaraldehyde cross-linking combined with sucrose osmotic contraction of an expanded matrix. In the presence of lower concentrations of sucrose (100 mM), the contraction following glutaraldehyde addition is much slower and less extensive than that shown in Fig. 3 C. This may explain the fact that Deamer et al. (29) were able to fix the matrix expanded and the contracted states of liver mitochondria by direct addition of glutaraldehyde with little subsequent swelling or contraction. These studies and others (2, 21-24, 29-31) have firmly established the close relationship between matrix expansion and ion accumulation. It should be noted that fixation of isolated mitochondria with glutaraldehyde in sucrose media does not result in contraction if osmotic equilibrium is established before the addition of the fixative (see reference 3 and reference 30, Fig. 6).

#### CONCLUSIONS

The present study confirms that isolated beef heart mitochondria assume the energized-twisted configuration almost quantitatively under the conditions specified by Green and coworkers. These identical conditions, however, promote the spontaneous accumulation of phosphate salts by these mitochondria, and the resulting osmotic swelling can be followed by changes in absorbancy of the suspension. Addition of glutaraldehyde to mitochondria swollen under these conditions results in rapid fixation against detergent lysis but fails to prevent a slow osmotic contraction due to the osmotic pressure of sucrose in the suspending medium. It therefore appears quite likely that the mitochondria as finally visualized in the electron microscope are not in the same configuration as at the time of addition of the fixative. These considerations make it incumbent upon authors, who emphasize the importance of morphological transitions to energy coupling in isolated mitochondria, to establish that the observed transitions are not the result of secondary changes following fixation. Similar secondary changes may be possible *in situ* and whenever fixation is attempted on systems not at osmotic equilibrium.

We thank Dr. D. G. McConnell of the Institute for Research in Vision, Ohio State University for the

use of his electron microscope facilities, and Doctor C. D. Stoner and Doctor R. A. Dilley for helpful discussions. A verbal report of this study was made in November of 1969 (32).

These studies were supported in part by a Grant-in-Aid from the American Heart Association, and by United States Public Health Service Grant HE09364.

Received for publication 4 December 1970, and in revised form 12 January 1971.

#### REFERENCES

- HUNTER, G. R., and G. P. BRIERLEY. 1969. *Biochim. Biophys. Acta.* **180**:68.
- HUNTER, G. R., Y. KAMISHIMA, and G. P. BRIERLEY. 1969. *Biochim. Biophys. Acta.* **180**:81.
- STONER, C. D., and H. SIRAK. 1969. *J. Cell Biol.* **43**:521.
- HACKENBROCK, C. R. 1966. *J. Cell Biol.* **30**:269.
- HACKENBROCK, C. R. 1968. *J. Cell Biol.* **37**:345.
- HACKENBROCK, C. R. 1968. *Proc. Nat. Acad. Sci. U. S. A.* **61**:598.
- PENNISTON, J. T., R. A. HARRIS, J. ASAI, and D. E. GREEN. 1968. *Proc. Nat. Acad. Sci. U. S. A.* **59**:624.
- HARRIS, R. A., J. T. PENNISTON, J. ASAI, and D. E. GREEN. 1968. *Proc. Nat. Acad. Sci. U. S. A.* **59**:830.
- GREEN, D. E., J. ASAI, R. A. HARRIS, and J. T. PENNISTON. 1968. *Arch. Biochem. Biophys.* **125**:684.
- KORMAN, E. F., A. D. F. ADDINK, T. WAKABAYASKI, and D. E. GREEN. 1970. *Bioenergetics.* **1**:9.
- WILLIAMS, C. A., W. J. VAIL, R. A. HARRIS, M. CALDWELL, D. E. GREEN, and E. VALDIVIA. 1970. *Bioenergetics.* **1**:147.
- GREEN, D. E., and R. A. HARRIS. 1969. *FEBS Letters.* **5**:241.
- SORDAHL, L. A., Z. R. BLAIBLOCK, G. H. KRAFT, and A. SCHWARTZ. 1969. *Arch. Biochem. Biophys.* **132**:404.
- KUNER, J. M., and R. E. BEYER. 1970. *J. Membrane Biol.* **2**:71.
- BUTLER, W. H., and J. D. JUDAH. 1970. *J. Cell Biol.* **44**:278.
- WEBER, N. E., and P. V. BLAIR. 1969. *Biochem. Biophys. Res. Commun.* **36**:987.
- STONER, C. D., and H. SIRAK. 1969. *Biochem. Biophys. Res. Commun.* **35**:59.
- SETTLEMIRE, C. T., G. R. HUNTER, and G. P. BRIERLEY. 1968. *Biochim. Biophys. Acta.* **162**:487.
- BRIERLEY, G. P., M. JURKOWITZ, K. M. SCOTT, and A. J. MEROLA. 1970. *J. Biol. Chem.* **245**:5404.
- CHANCE, B., and G. R. WILLIAMS. 1955. *J. Biol. Chem.* **242**:318.

21. BRIERLEY, G. P., C. T. SETTLEMIRE, and V. A. KNIGHT. 1968. *Arch. Biochem. Biophys.* 126:276.
22. IZZARD, S., and H. TEDESCHI. 1970. *Proc. Nat. Acad. Sci. U. S. A.* 67:702.
23. ANAGNOSTI, E., and H. TEDESCHI. 1970. *J. Cell Biol.* 47:520.
24. BLONDIN, G. A., and D. E. GREEN. 1969. *Arch. Biochem. Biophys.* 132:509.
25. TYLER, D. D. 1969. *Biochem. J.* 111:665.
26. FONYO, A., and S. J. BESSMAN. 1968. *Biochem. Med.* 2:145.
27. SCOTT, K. M., V. A. KNIGHT, C. T. SETTLEMIRE, and G. P. BRIERLEY. 1970. *Biochemistry.* 9:714.
28. HARRIS, R. A., M. A. ASBELL, J. ASAI, W. W. JOLLY, and D. E. GREEN. 1969. *Arch. Biochem. Biophys.* 132:545.
29. DEAMER, D. W., K. UTSUMI, and L. PACKER. 1967. *Arch. Biochem. Biophys.* 121:641.
30. PACKER, L., J. M. WIGGLESWORTH, P. A. G. FORTES, and B. C. PRESSMAN. 1968. *J. Cell Biol.* 39:382.
31. PFAFF, E., M. KLINGENBERG, E. RITT, and W. VOGELL. 1968. *Eur. J. Biochem.* 5:222.
32. HUNTER, G. R., and G. P. BRIERLEY. 1969. *J. Cell Biol.* 43(2, Pt. 2):56a. (Abstr.)