BBA 45961

EFFECT OF VALINOMYCIN ON MITOCHONDRIAL ULTRASTRUCTURE AND FUNCTION OF INTACT EHRLICH ASCITES TUMOR CELLS

EDWIN E. GORDON AND JAY BERNSTEIN

Department of Medicine, New York University Medical Center, New York, N. Y. 10016 and Departments of Pathology, Albert Einstein College of Medicine, Bronx, N. Y. 10461 and William Beaumont Hospital, Royal Oak, Mich. 48072 (U.S.A.)

(Received February 9th, 1970)

SUMMARY

Ultrastructural changes in the mitochondria of intact Ehrlich ascites tumor cells were observed after stimulation by valinomycin of the energy-dependent transport of K^+ into mitochondria. The mitochondria in cells taken directly from the animal displayed an orthodox configuration. After repeated washings of the cells, the mitochondria were converted to the 'condensed' or 'aggregated' state. The addition of valinomycin resulted in a transformation of mitochondria from the condensed to orthodox and markedly swollen forms. Alterations in cell size, O_2 uptake, and K^+ content accompanied the changes in mitochondrial morphology.

INTRODUCTION

Valinomycin has been shown to effect an energy-dependent mitochondrial K^+ transport in isolated mitochondria[1] and in intact ascites tumor cells[2]. The biochemical changes that result from valinomycin-stimulated K^+ transport in preparations of intact cells parallel the changes observed with isolated mitochondria, *i.e.* increased uptake of O_2 and extrusion of protons. With isolated mitochondria, the transport of K^+ has been associated with appreciable mitochondrial swelling as demonstrated by light-scattering techniques[3] and by electron microscopy[4]. In the present study transitional states of mitochondrial ultrastructure were observed in intact ascites tumor cells. Virtually all mitochondria assumed the condensed form after washing the cells repeatedly. Following initiation of valinomycin-induced mitochondrial K^+ transport, characteristic changes in mitochondrial ultrastructure were observed.

METHODS

Ehrlich ascites tumor cells of the hyperdiploid strain were harvested from the peritoneal cavity of white mice 6–8 days after inoculation. The cells were collected in ice-cold, K^+-free medium buffered with phosphate at pH 7.4. K^+-depletion was accomplished by washing the cells repeatedly over a period of 3 h in K^+-free medium as previously described[2]; the cells were finally suspended in medium to a cytocrit

of approx. 30 %. The concentration of ions in the medium used for collection, preparation and incubation of cells was: Na^+, 178; Mg^{2+}, 1.5; Cl^-, 154; SO_4^{2-}, 1.5; and PO_4^{3-}, 12 mM.

Incubations were carried out at room temperature in an air atmosphere with constant agitation; no exogenous substrate was added to the incubation mixture. Unless otherwise indicated, the volume of incubation was 3 ml, approx. 20 mg of cell protein were present, and the final concentrations of K^+ and valinomycin were 5.1 mM and 0.16 μM, respectively. At the end of the incubation, the cells were fixed for electron microscopy by adding to 1 vol. of sedimented cells 9 vol. of fixative. Cells were fixed in (a) 1 % osmium tetroxide buffered with Veronal to pH 7.4 and adjusted with sucrose to an osmolality of 320 mosM, or (b) 2.5 % glutaraldehyde buffered with *s*-collidine to pH 7.4 (364 mosM) and, after washing in buffer, osmicated in 1 % osmium tetroxide in Veronal buffer. Samples of fixed cells were dehydrated in graded alcohols and propylene oxide and were embedded in epoxy plastic (Epon 812). Sections were mounted on 200-mesh copper grids and stained with uranyl acetate and Reynolds' lead citrate[5].

Mitochondrial counts were performed in a single section of a cell block to avoid duplication. We obtained a sufficient density of cells in the pellet to carry out a count in the plane of one section. Electron microscopic examination was carried out through a binocular microscope at a screen magnification of approx. 4000 diameters. In the initial set, (Table I) 'aggregated' or 'condensed' mitochondria were counted in 100 cells (totaling 900–1000 mitochondria). A second count of 1000 consecutive mitochondria (Table II) included categories of 'orthodox' and markedly swollen. The former corresponds to mitochondrial configuration in cells fixed immedi-

TABLE I

PERCENTAGE OF CONDENSED MITOCHONDRIAL FORMS IN K^+-DEPLETED EHRLICH ASCITES TUMOR CELLS BEFORE AND AFTER TREATMENT WITH K^+ AND VALINOMYCIN

	Percentage			
	Expt. 1	*Expt. 2*	*Expt. 3*	*Average*
K^+-depleted	95	94	95	95
K^+-restored	92	96	97	95
Valinomycin	48	51	49	49
Valinomycin + K^+	7	6	5	6

TABLE II

CLASSIFICATION OF MITOCHONDRIAL ULSTRASTRUCTURE IN INTACT CELLS

1000 mitochondria counted.

	Condensed	*Orthodox*	*Markedly swollen*
K^+-depleted	929	51	20
K^+-restored	934	41	25
Valinomycin	471	147	382
Valinomycin + K^+	70	234	696

ately on removal from the animal and the latter refers to mitochondria with marked matrical swelling.

O_2 uptake by respiring cells at room temperature was detected with the Clark electrode and monitored continuously. Studies related to changes in cell volume were carried out with concentrated suspensions of cells in order to avoid the inaccuracies inherent in determining the cytocrit with dilute cell suspensions. Cell volume was calculated from the cytocrit and cell counts determined in a Neubauer chamber. The protein content was measured with a biuret technique[8]. The K^+ concentration of the cell was calculated from the cytocrit (uncorrected for trapped extracellular fluid) and the difference in K^+ content between the whole suspension and the incubation medium measured by flame photometry.

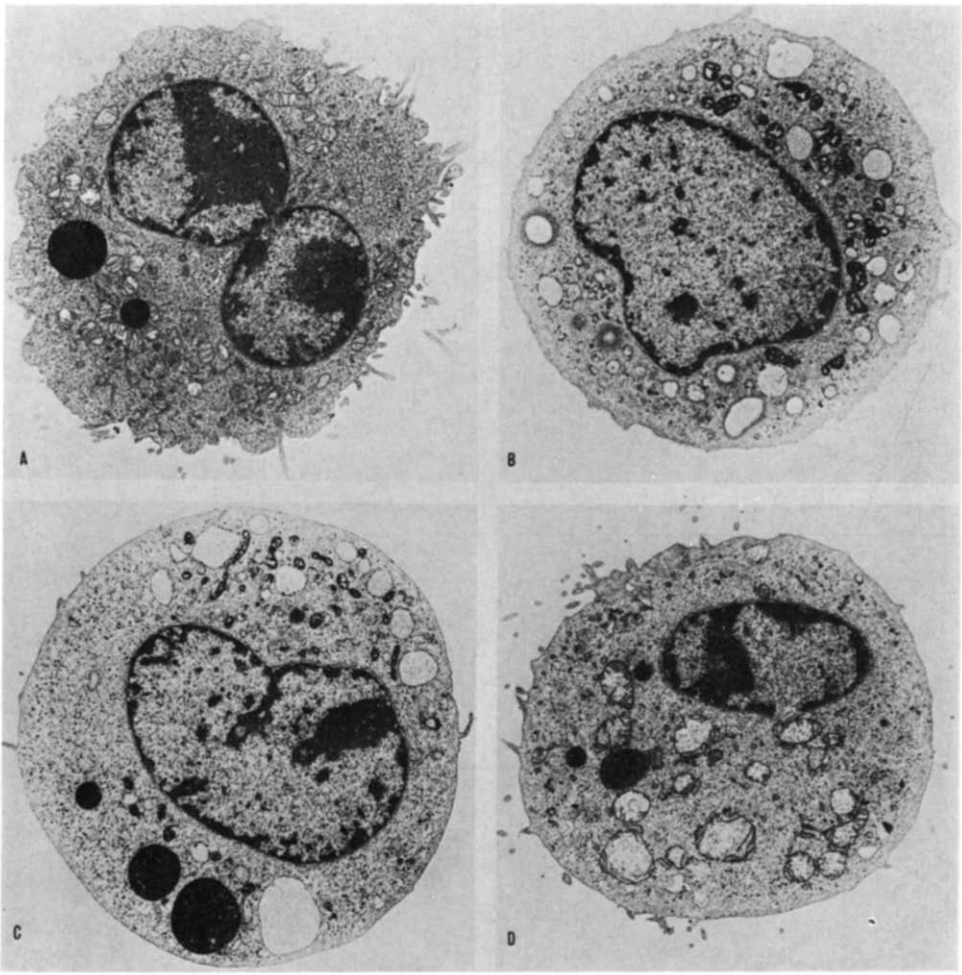

Fig. 1. Representative, intact ascites tumor cells: (A) immediate fixation, (B) K^+-depleted, (C) K^+-restored, (D) K^+ and valinomycin added. Mitochondria in cells fixed immediately after removal from animal display an orthodox configuration. After washing to deplete cells of K^+, the mitochondria are condensed and are not affected by restoring K^+ to the medium. The addition of valinomycin in the presence of K^+ leads to marked mitochondrial swelling in intact cells. Fixation in glutaraldehyde followed by osmium. × 3000.

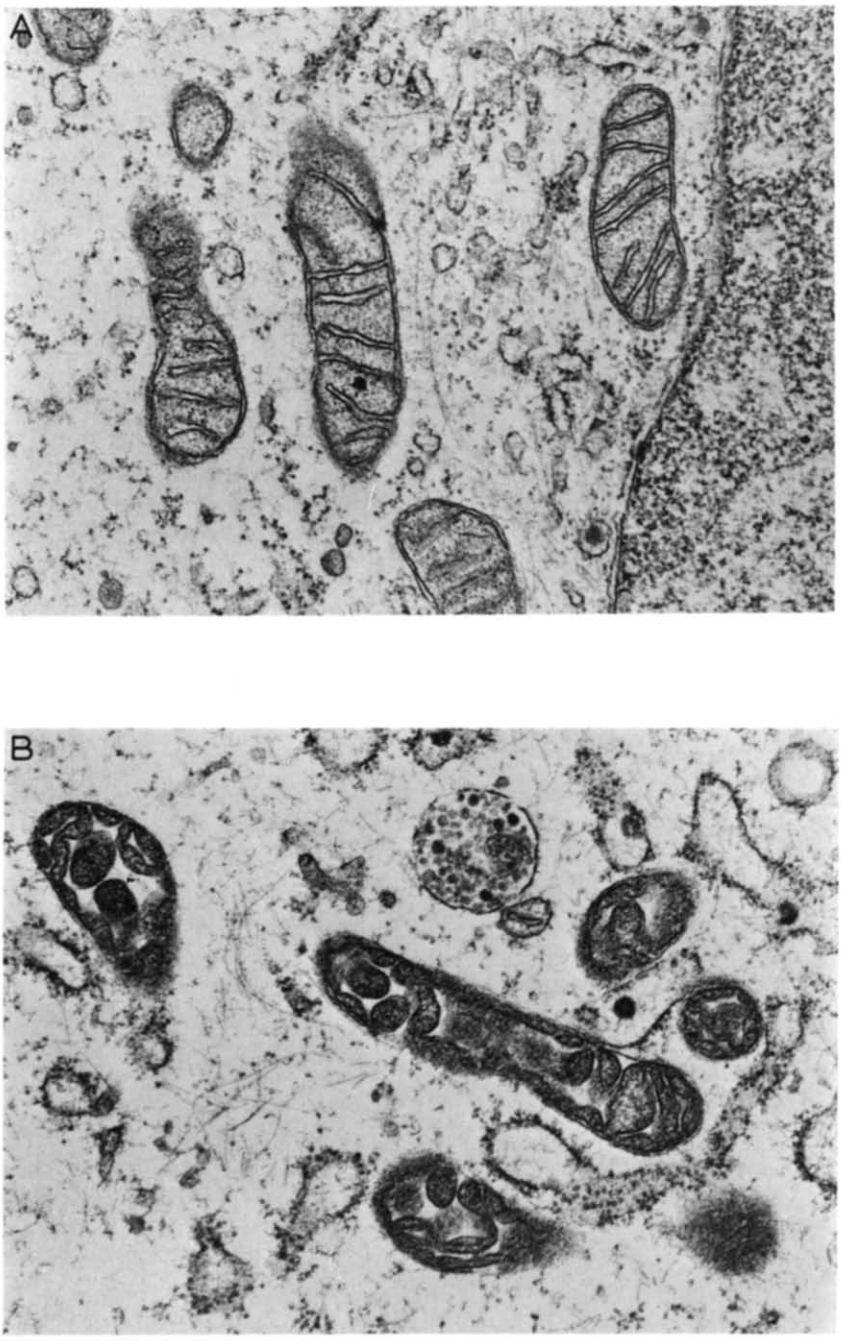

Fig. 2. A. Appearance of mitochondria, the orthodox configuration in ascites tumor cells fixed immediately. B. Condensed mitochondria in cells depleted of K^+ by repeated washing. The intercristal compartments are contracted into seemingly convoluted and twisted forms, and the matrix is electron-dense. The intracristal spaces are correspondingly enlarged. Fixation in osmium. × 40 000.

RESULTS

Ultrastructural changes

The characteristic ultrastructional features were observed in intact cells. The appearance of the plasma membranes and cytoplasmic constituents indicated that cellular integrity was preserved (Fig. 1). There was no difference in mitochondrial structure between cells fixed in osmium and those fixed initially in glutaraldehyde. The mitochondria of cells fixed directly upon removal from the animal displayed the characteristic features of the 'orthodox' form, *i.e.*, small size, a rounded or fusiform shape, and transverse, narrow cristae (Fig. 2A). In contrast, mitochondria in cells fixed after K^+ had been depleted by repeated washings in the absence of K^+ had a 'condensed'[6] or 'aggregated'[7] configuration (Fig. 2B). Other mitochondrial changes in K^+-depleted cells included marked swelling of the outer compartments and intracristal spaces. The matrix was condensed and extremely electron-dense, and the intercristal spaces containing the matrix were twisted into irregularly convoluted shapes. The mitochondria seemed to be slightly enlarged in comparison with those of the control cells. Matrical condensation was not uniform and was less pronounced in partially disrupted cells in which the plasma membrane was focally disrupted. Other alterations included peripheral cytoplasmic rarefaction and slight dilatation of cisterns of endoplasmic reticulum. In many of the cells the micro-

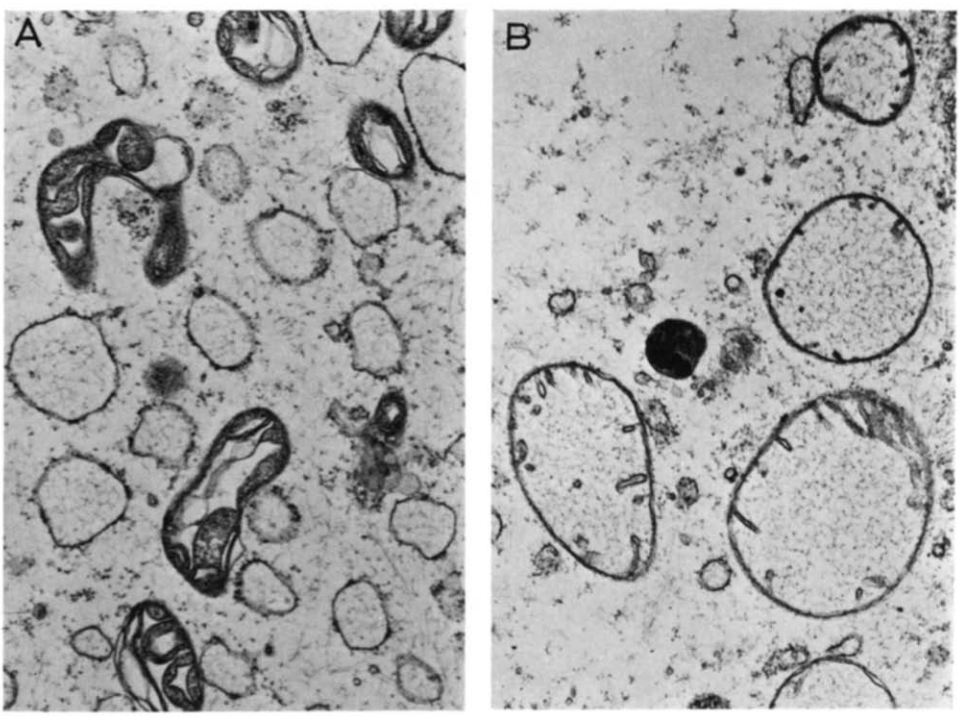

Fig. 3. Appearance of mitochondria in cells, to which (A) K^+ and (B) both K^+ and valinomycin were added. Restoring K^+ has no effect on mitochondrial ultrastructure. Adding valinomycin together with K^+ leads to marked swelling of almost all mitochondria. Fixation in osmium. × 18000.

villi were diminished in number and appeared to be blunted, suggesting stretching of the plasma membrane because of cellular swelling. Restoration of cell K^+ by incubation in a K^+-containing medium resulted in no further ultrastructural changes in the mitochondria (Fig. 3A).

The addition of valinomycin changed the morphology in approximately one-half of the mitochondria (Table I). The new alteration consisted of matrical swelling with either reversion to orthodox configuration or the development of a markedly swollen, ballooned appearance. The cristae in swollen mitochondria were narrow, tubular in shape and widely separated. There was also perhaps less cytoplasmic rarefaction and less dilation of the endoplasmic reticulum in cells with swollen mitochondria than in cells whose mitochondria retained an aggregated appearance. The addition of both valinomycin and K^+ to the incubation medium led to marked swelling of almost all mitochondria (Fig. 3B). In most of the swollen mitochondria the cristae were narrow and widely separated, often appearing to radiate from the periphery toward the center. The cristae were also shortened, suggesting a slight degree of unfolding of the inner membrane. The outer mitochondrial membrane remained intact. The outer compartments between the inner and outer membranes and the intracristal spaces appeared to be compressed. Swelling of other cytoplasmic structures was variable. Swelling of the endoplasmic reticulum was present in some cells and absent in others. Nuclear alterations after the addition of valinomycin and K^+ included an increased degree of fissuring, causing an appearance of cytoplasmic inclusions within nuclei and resulting in lobulated nuclei.

Mitochondrial types were counted in order to quantitate ultrastructural changes induced by valinomycin-stimulated mitochondrial K^+ transport. In three separate experiments approx. 1000 mitochondria in 100 cells were counted (Table I). The great majority, 95 % of mitochondria in K^+-depleted cells, were present in a condensed form. The percentages of condensed mitochondria were not altered by restoring K^+ to the medium. The addition of valinomycin resulted in swelling of approx. 50 % of the mitochondria, and the addition of both valinomycin and K^+ resulted in a transformation of almost all mitochondria to markedly swollen forms. A more detailed analysis of 1000 mitochondria was carried out in one experiment (Table II), confirming the previous percentages of condensed mitochondrial forms. The addition of valinomycin to condensed mitochondria of K^+-depleted cells resulted in a reversion to orthodox configuration of 10–15 % and swelling of 35–40 % of mitochondria. The combination of valinomycin and K^+ changed those figures further to 20 and almost 70 %, respectively.

Cell size and K^+ content

The impression of cytoplasmic rarefaction observed in the K^+-depleted cells was borne out by quantitative determination of cell volume (Table III). Depleting the cells of K^+ resulted in a movement of Na^+ and water into the cytoplasmic compartment[9]; the intracellular K^+ concentration was reduced to 3–4 mequiv/l of cells. Because of the absence of extracellular K^+, the coupled Na^+–K^+ pump of the plasma membrane is inoperative. When K^+ was restored to the medium, Na^+ and water were pumped out of the cells resulting in a significant ($P<0.001$) reduction of cell volume by about 10 %; K^+ was accumulated intracellularly against a concentration gradient. Addition of valinomycin to K^+-depleted cells once again increased

TABLE III

CELL VOLUME AND K^+ CONCENTRATION

The values represent the means ± S. E. with 5 different cell preparations. All incubations were carried out at 25° for 10 min. The volume of the incubation medium was 2 ml containing 15–25 % cells. Valinomycin was added to a final concentration of 1 μM, and K^+ to a final concentration of 7.7 mM.

	Cell vol. (μ^3)	*K^+* (*mequiv/l cells*)
K^+-depleted	1684 ± 43	3.9 ± 0.5
K^+-repleted	1545 ± 42	15.1 ± 1.7
Valinomycin	1885 ± 48	4.0 ± 0.6
Valinomycin + K^+	1919 ± 34	11.7 ± 0.6

cell volume; simultaneous addition of K^+ and valinomycin did not increase cell size beyond that seen after valinomycin alone. The ultrastructural studies suggest that the increase in cell volume in the presence of valinomycin was due in significant part to mitochondrial swelling.

O_2 uptake

Studies of O_2 uptake by the K^+-depleted cells and the K^+-repleted cells in the presence and absence of valinomycin were consistent with our previous findings[2]. As would be expected, the Q_{O_2} in the control state was somewhat lower in the present experiments due to the reduction in the temperature of incubation from 37 to 25°. A slight and inconstant increase in the rate of O_2 uptake was observed when either K^+ or valinomycin was present, but an appreciable increase in O_2 uptake required the addition of both K^+ and valinomycin (Fig. 4).

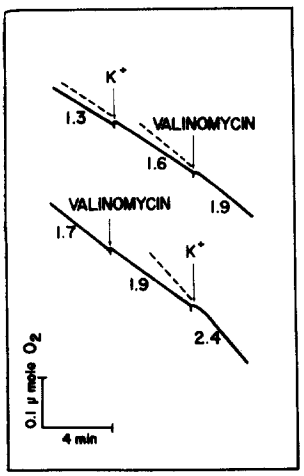

Fig. 4. Effect of K^+ and valinomycin on O_2 uptake of Ehrlich ascites tumor cells.

DISCUSSION

Ultrastructural alterations in the mitochondria of intact Ehrlich ascites tumor cells are similar to those described in isolated mitochondria. Cells washed in K^+-free medium contain mitochondria with dilated intracristal spaces and a dense, compact matrix, the so-called 'condensed' or 'aggregated' form. This appearance is strikingly different from the 'orthodox' configuration customarily seen in tissues or in ascites tumor cells fixed immediately. The condensed appearance is not, therefore, an artifact associated with the isolation of mitochondria, but can also be seen in intact cells. This mitochondrial configuration has been observed in intact cells of intestine[10] as well as in Ehrlich ascites tumor cells. It is possible that the process of depleting the cells of K^+ with attendant shifts of water and cations is responsible for the transition of the orthodox form (seen in fresh cells) to the condensed form. This interpretation is unlikely, however, since condensed forms are also observed in cells washed repeatedly in a K^+-containing medium, and after restoration of K^+ to K^+-depleted cells. The significance of the condensed configuration for mitochondrial function remains conjectural. Apparently the transition from one form to the other is accompanied by conformational changes of the inner membrane protein[11]. HACKENBROCK[12,13] has suggested that the condensed form corresponds to a 'low energy state' and the orthodox form to a 'high energy state'. He has further postulated that the transformation from a condensed to an orthodox form may occur as the result of an increase in the rate of electron transport through the respiratory chain (mechanochemical ultrastructural transformation) or through the energy-dependent accumulation of ions in the matrix (osmotic ultrastructural transformation). The valinomycin-induced mitochondrial changes in ascites cells in the presence of K^+ are consistent with the suggestion that the swelling is secondary to the active movement of K^+ into the matrix.

Approx. 50% of the mitochondria undergo an ultrastructural change from the condensed form to the orthodox and swollen forms after addition of valinomycin to K^+-depleted cells. It is likely that this submaximal effect follows from the mitochondrial transport of residual K^+ in the depleted cells, but some other mode of valinomycin action remains a possibility.

Despite significant contraction of the cell volume following a priming of the coupled Na^+–K^+ pump of the cell membrane with added K^+ (Table III), mitochondrial morphology remains unchanged (Fig. 3A and Table I). Initiation of mitochondrial K^+ transport by adding both K^+ and valinomycin results in the most dramatic morphological changes in essentially all mitochondria. The mitochondria become markedly swollen with what might be regarded as a reversion from condensed to a more orthodox form. The configuration is however, not normal, and the extreme degree of swelling results in widely separated, apparently compressed cristae. In these experiments there did not seem to be a reduction of cristae by flattening-out, as described by MALAMED[14] in osmotic swelling. Similar changes reversible upon addition of albumin, have been produced by the addition of pentachlorophenol to isolated rat liver mitochondria[15]. It is not known whether the marked swelling induced by valinomycin can be reversed in the intact cell. In addition to mitochondrial swelling, the entire cell volume seems to be markedly increased; this is corroborated by independent measurements of cell volume (Table III). These mitochon-

drial ultrastructural changes are presumably a consequence of valinomycin-induced K^+ transport into the mitochondria[2]. Functionally, there is an appreciable increase in O_2 uptake, a release of protons from the cell[2], and a multifold increase in the rate of glycolysis if exogenous glucose is added to the medium[16] upon stimulation of mitochondrial K^+ transport.

Although the transition of mitochondrial ultrastructure from the orthodox to the condensed form and then back through the orthodox and swollen forms represents an exaggerated series of events in the present experiments, it is clear that these transitions can take place in intact cells. The last phase probably represents the mitochondrial uptake of water as well as K^+ (ref. 17). However, such transitional mitochondrial forms can be shown to exhibit appreciable independence of gross changes in the water and ionic content of the extramitochondrial compartment, *i.e.* condensed mitochondria in the swollen K^+-depleted cells as well as in the contracted K^+-repleted cells. A more precise definition of the ultrastructural changes in functional terms must preceed the assessment of the biological significance of these observations.

ACKNOWLEDGEMENTS

This investigation was supported by U.S. Public Health Service Research Grants AM-12404 and AM-07840 from the National Institute of Arthritis and Metabolic Diseases and by a grant from the American Heart Association. E. E. G. is a Career Scientist of The Health Research Council of the City of New York under contract I-551.

The authors are indebted to Mr. Paul Delaney for invaluable assistance in performing electron microscopic procedures. The technical assistance of Maria de Hartog and Jane Hepple is gratefully acknowledged.

We are indebted to Dr. J. C. MacDonald of the Prairie Regional Laboratory, Saskatoon, Canada, for providing the valinomycin.

REFERENCES

1 C. MOORE AND B. C. PRESSMAN, *Biochem. Biophys. Res. Commun.*, 15 (1964) 562.
2 E. E. GORDON, K. NORDENBRAND AND L. ERNSTER, *Nature*, 213 (1967) 82.
3 B. C. PRESSMAN, *Proc. Natl. Acad. Sci. U. S.*, 53 (1965) 1076.
4 L. PACKER, J. M. WRIGGLESWORTH, P. A. G. FORTES AND B. C. PRESSMAN, *J. Cell Biol.*, 39 (1968) 382.
5 E. S. REYNOLDS, *J. Cell Biol.*, 17 (1963) 208.
6 C. R. HACKENBROCK, *J. Cell Biol.*, 30 (1966) 269.
7 D. E. GREEN, J. ASAI, R. A. HARRIS AND J. T. PENNISTON, *Arch. Biochem. Biophys.*, 125 (1968) 684.
8 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
9 M. MAIZELS, M. REMINGTON AND R. TRUSCOE, *J. Physiol.*, 140 (1958) 61.
10 D. K. JASPER AND J. R. BRONK, *J. Cell Biol.*, 38 (1968) 277.
11 J. M. WRIGGLESWORTH AND L. PACKER, *Arch. Biochem. Biophys.*, 128 (1968) 790.
12 C. R. HACKENBROCK, *Proc. Natl. Acad. Sci. U. S.*, 61 (1968) 598.
13 C. R. HACKENBROCK, *J. Cell Biol.*, 37 (1968) 345.
14 S. MALAMED, *Zellforsch. Mikroskop. Anat.*, 65 (1965) 10.
15 E. C. WEINBACH, H. SHEFFIELD AND J. GARBUS, *Proc. Natl. Acad. Sci. U. S.*, 50 (1963) 561.
16 E. E. GORDON AND M. DE HARTOG, *Biochim. Biophys. Acta*, 162 (1968) 220.
17 E. J. HARRIS AND K. VAN DAM, *Biochem. J.*, 106 (1968) 759.