

# THE MORPHOLOGY OF THE SWELLING PROCESS IN RAT LIVER MITOCHONDRIA

D. R. MYRON and J. L. CONNELLY

From the Department of Biochemistry, University of North Dakota, School of Medicine,  
Grand Forks, North Dakota 58201

## ABSTRACT

Through the use of combined spectrophotometric and electron microscope techniques, large amplitude swelling of rat liver mitochondria has been described as an ordered sequence of ultrastructural transitions. Prior to the actual swelling, mitochondria undergo two major conformational changes: condensed to twisted form and twisted to orthodox form. This sequence is independent of (a) the nature of swelling agents and (b) the time of onset of swelling. Agents that delay the onset of swelling act to increase the duration of the twisted conformation. Agents that prevent extensive swelling hold mitochondria in intermediate conformations. Gross swelling, immediately preceded by a decrease in electron opacity of the matrix, involves the rupture of the outer membrane and expansion of the inner compartment of the mitochondrion.

## INTRODUCTION

Swelling and contraction studies have comprised one major approach to the understanding of the complex bioenergetic machinery of the mitochondrion (1-3). More recently, the importance of the mitochondrial ultrastructure to energy-linked functions has become apparent (4-10). This latter development emphasized the potential value of morphological studies in the understanding of chemically induced volume changes.

Changes in the ultrastructure of mitochondria in response to a wide range of conditions have indicated that, in addition to the variation in metabolic states, such factors as ion transport or osmotic phenomena may influence the conformational status (11-16). Recently, conditions have been defined under which ultrastructural transformations of both orthodox<sup>1</sup> and condensed matrices are induced by osmotic variations (8).

<sup>1</sup> Although the terms "condensed," "twisted," and "orthodox" were originally devised in reference to the metabolic state—morphology relationship (5),

Many reports have described the gross changes in the ultrastructure of mitochondria swollen under a variety of conditions (17-23). Although the overall change in structure is well established, relatively little is known about the ultrastructural transformations which occur concomitant with swelling. This report discloses that the phosphate-induced swelling process can be described as a sequence of conformational changes, indicating that, morphologically, swelling involves more than a simple expansion of the mitochondrial membrane

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in this work these terms provide only a convenient descriptive title for the various morphological forms presented. These terms are applied in accordance with the following descriptions: condensed, dense matrix with pointed vacuoles; twisted, dense vesicular matrix; orthodox, low density, diffuse matrix with double-membrane cristae visible. The vacuoles noted in the bursting mitochondria appear to result from enlargements of the double-membrane cristae as the matrix emerges through the outer membrane.

system. Previous investigations have shown a direct influence of specific agents on the time of onset and extent of swelling (23-25). This work examines the responses of mitochondria, as reflected by changes in morphology, to the influence of a variety of environmental conditions, in an effort to characterize the specific effects of individual agents and conditions.

## MATERIALS AND METHODS

Liver mitochondria from young adult male Sprague-Dawley rats were prepared in 0.25 M sucrose as previously described (2), with an additional 10-min wash. Time of onset and extent of swelling were determined by measuring the change in optical density at 515 m $\mu$  of the mitochondrial suspension. Mitochondrial protein concentration ranged from 0.3 to 0.5 mg/ml throughout the swelling experiments. All determinations were conducted at room temperature, 25°C.

The ultrastructural status of mitochondria was determined on portions removed from each reaction mixture during the measurement of optical density change. Each portion (0.75 ml) of mitochondrial suspension was pipetted directly from the spectrophotometer tube into a small glass tube containing 0.25 ml of 10% glutaraldehyde buffered by 40 mM potassium phosphate (pH corresponding to that of the mitochondrial suspension). After mixing, the suspension was allowed to stand on ice for 10 min before centrifuging at 10,000 g for 8 min. The pellet was washed with a solution containing 0.14 M sucrose and 5 mM potassium phosphate (pH of the original suspension) prior to postfixation for 2 hr in 1% osmium tetroxide buffered by 10 mM potassium phosphate at pH 7.4. The small, flat pellet was dehydrated in ethanol and embedded in Epon 812 medium (26). The pellet (approximately 0.5 mm thick) was oriented in a position perpendicular to the cutting plane before hardening in a BEEM capsule. Silver-gold to light gold sections were mounted on copper grids and stained with lead citrate (27) and sodium uranyl acetate (28) before examination with a Philips EM-200 at 60 kv. The field of mitochondria was observed to be highly uniform throughout the cross-section of the pellet.

## RESULTS

### *The Sequence of Morphological Transitions Accompanying Swelling*

As demonstrated by the optical density curve in Fig. 1, mitochondria in 0.14 M sucrose in the presence of inorganic phosphate undergo a large amplitude swelling following a short time lag

(time of onset).<sup>2</sup> In order to determine the nature of the physical changes occurring during the swelling process, portions of the suspension were removed at selected times and fixed for electron microscopy. Although mitochondria, under these conditions, do not swell in complete synchrony, trends in ultrastructural transitions can be readily detected. Representative micrographs of mitochondria fixed at points *a* through *d* demonstrate that an ordered sequence of conformational changes of the mitochondrial inner compartment appears to take place prior to the bursting process. The initial change, which appears to be dependent on the presence of swelling agent, is the transformation of the matrix from a dense, compacted character (condensed conformation) to a dense, vesicular-appearing form (twisted).<sup>1</sup> Mitochondria suspended in sucrose in the absence of swelling agent will remain in the condensed conformation (Fig. 4 *a*). However, in the presence of phosphate (Fig. 1 *a*), twisted conformation appears within the 1st minute of incubation and is the principal form by 5 min. Concomitant with the rapid decrease in optical density is a second major transition of the inner compartment. As the matrix swells within the outer membrane, it becomes less dense, and the intracrystal space is diminished (Fig. 1 *d*). These mitochondria are similar in appearance to the conformation termed "orthodox" (5, 6, 29). Although this latter species is short-lived and is a component of a continually mixed population, it is consistently observed prior to the bursting and gross swelling illustrated in Figs. 1 *d* and *e*. The fact that this species is always observed between the twisted and swollen forms suggests that it is a sequential intermediate in the swelling process. Gross swelling is characterized by a rupture of the outer membrane followed by a protrusion of the intact inner compartment, and the loss of organization of the matrix. Note, in Fig. 1 *e*, that any organization of the inner membrane remaining in swollen mitochondria occurs only in regions in which the outer membrane is retained.

When phosphate is replaced by  $2 \times 10^{-5}$  M CaCl<sub>2</sub> in the suspending medium, the sequence of

<sup>2</sup> The transfer of mitochondria from sucrose suspension (250 mOsM) to the incubation medium (about 175 mOsM) results in a rapid osmotic volume change which is generally complete prior to the first optical density reading. Extent of swelling is measured from the plateau reached following this change.

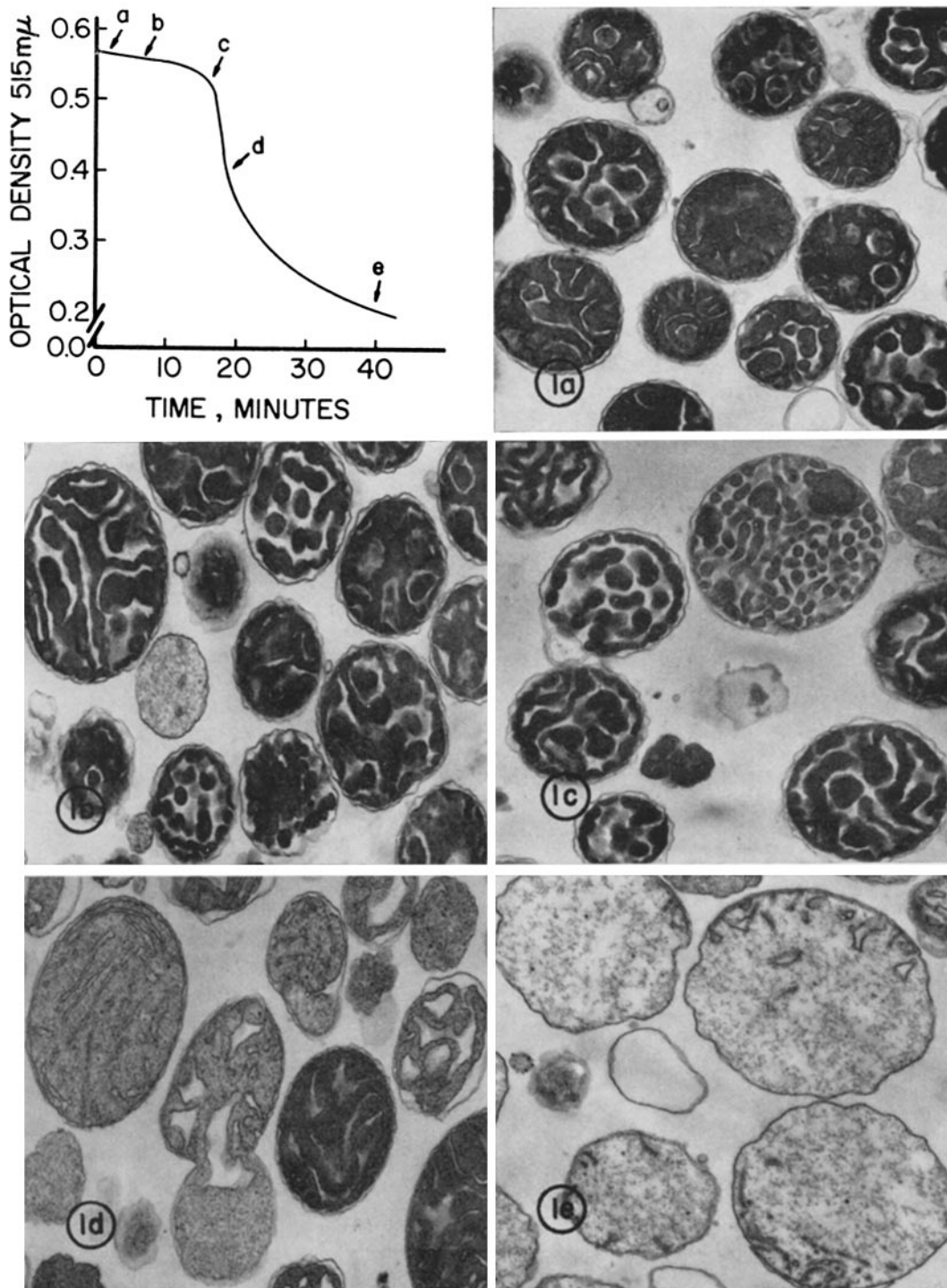


FIGURE 1 The sequence of morphological changes accompanying phosphate-induced swelling. The suspending medium (pH 7.4) contained 140 mM sucrose, 5 mM  $K^+PO_4$ , and 10 mM L-histidine. The ultrastructural statuses of mitochondria are as follows: (a) condensed, some twisted character; (b and c) twisted; (d) passing through the orthodox conformation prior to bursting and gross swelling, (e).  $\times 20,500$ .

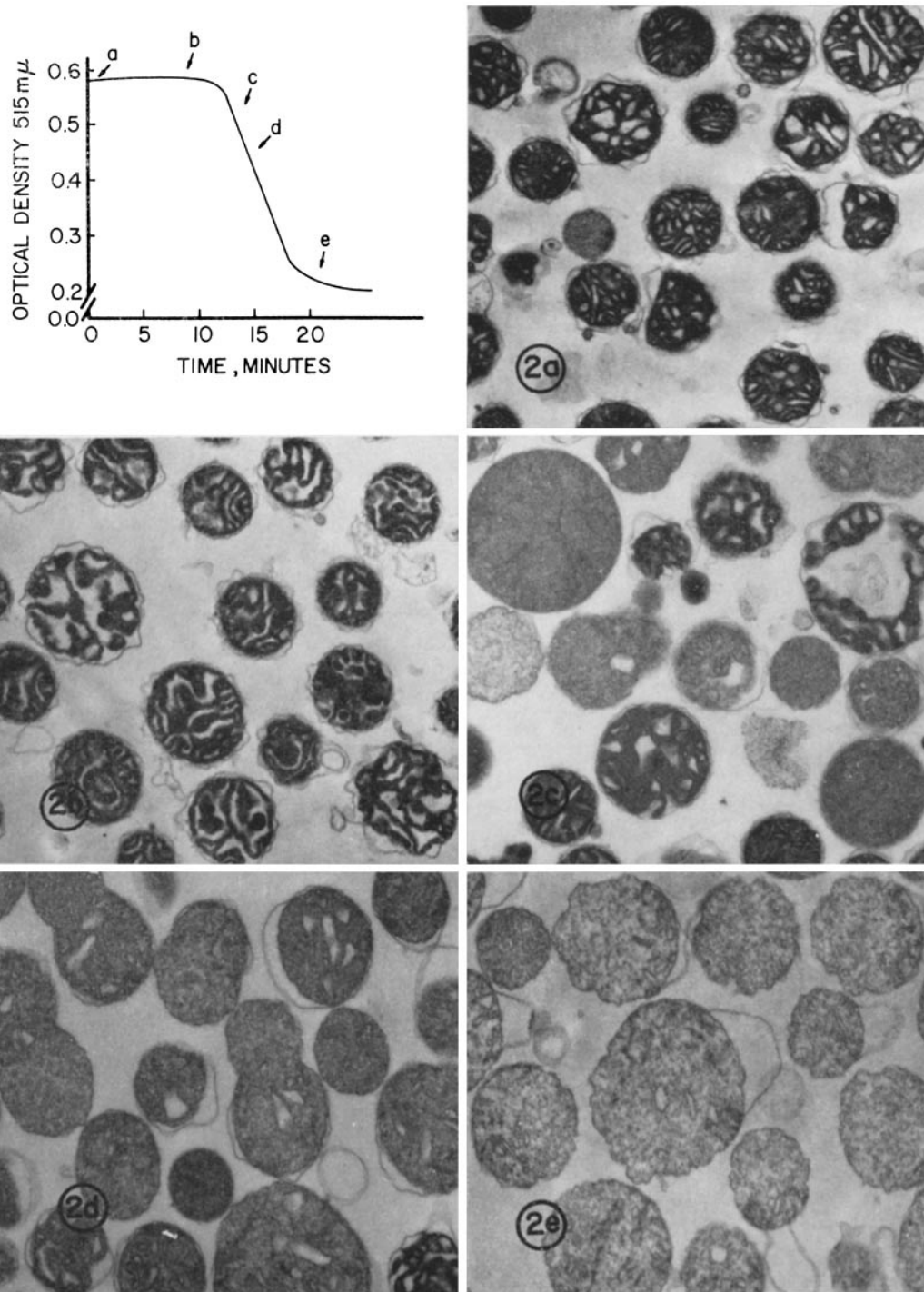


FIGURE 2 The sequence of morphological changes accompanying calcium-induced swelling. The medium (pH 7.4) contained 140 mM sucrose,  $2.0 \times 10^{-2}$  mM  $\text{CaCl}_2$ , and 10 mM L-histidine. The ultrastructural statuses of mitochondria are as follows: (a) initial condensed; (b) twisted; (c) appearance of some orthodox mitochondria; (d) and (e) bursting and swollen mitochondria.  $\times 12,500$ .

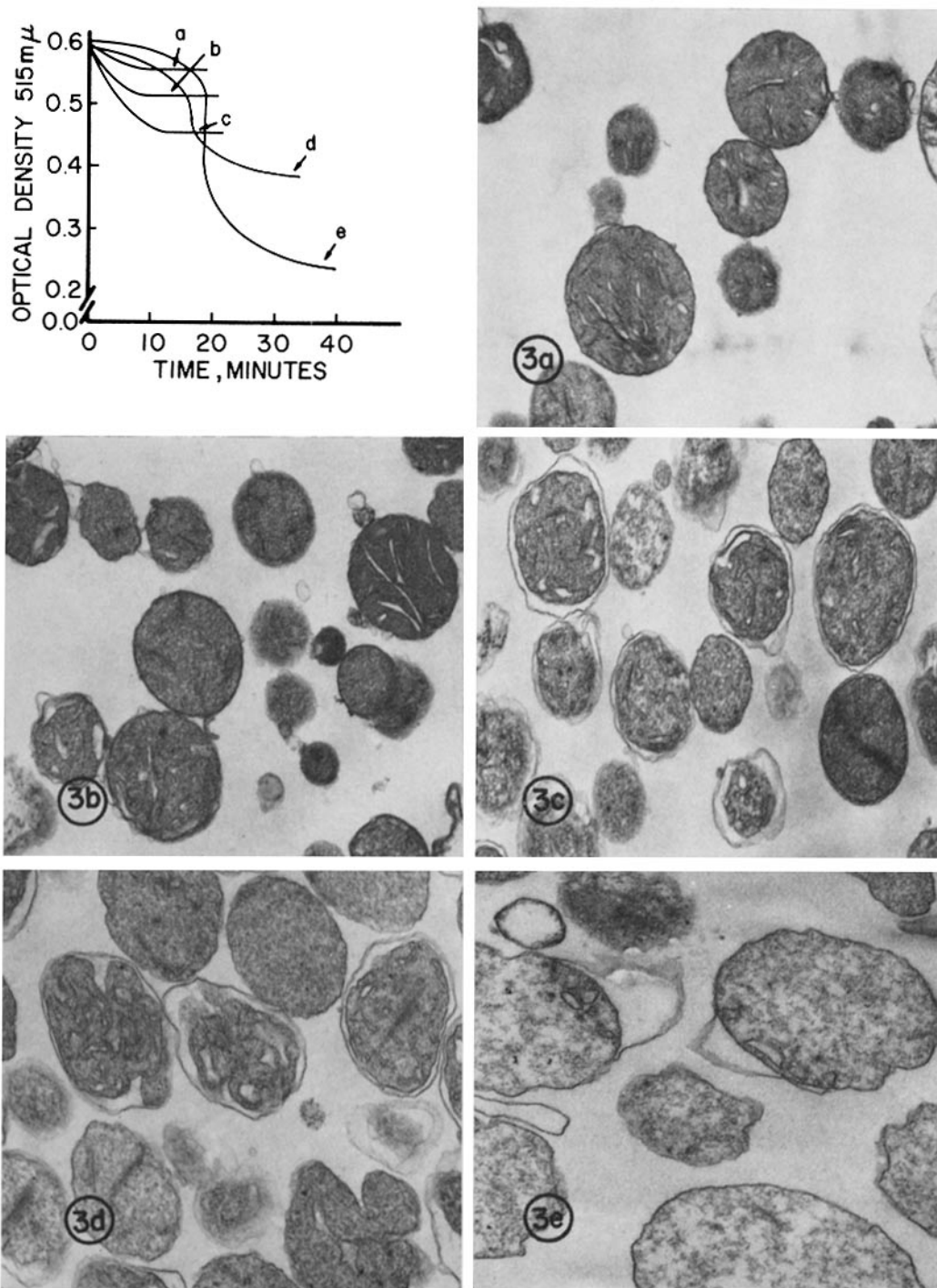


FIGURE 3 The morphology of change in extent of phosphate-induced swelling as influenced by pH. Suspending medium as in Fig. 1, except pH value. The ultrastructural statuses of mitochondria are as follows: (a) pH 6.2, orthodox; (b) pH 6.4, orthodox; (c) pH 6.6, intermediate appearance; (d) pH 7.0, some swelling; (e) pH 7.4, gross swelling.  $\times 20,500$ .

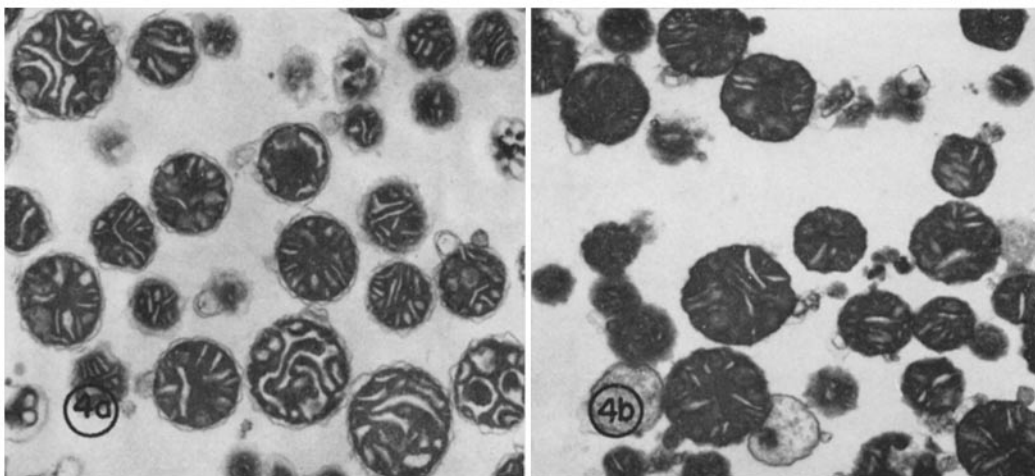


FIGURE 4 The influence of pH on mitochondria in the condensed conformation. Mitochondria were incubated for 10 min in a medium containing 140 mM sucrose and 10 mM L-histidine at (a) pH 7.4, and (b) pH 6.2. Note relative absence of intramembranal space at pH 6.2.  $\times 12,500$ .

conformational transitions of the inner compartment (Fig. 2 *a-e*) is essentially the same as that observed with phosphate. Similar results were obtained with oleate or thyroxine as the swelling agent. Thus, the swelling agent, in general, appears to be responsible for initiating the conditions which ultimately result in the series of conformational changes associated with the swelling of mitochondria. Furthermore, this latter response is independent of the nature of the swelling agent employed.

#### *The Influence of Change in Swelling Extent on the Morphology of Swelling*

Earlier studies have demonstrated that the extent of phosphate-induced swelling, as measured by optical density changes, is suppressed in a graded manner by decreasing the pH of the medium (2, 24). The morphology of mitochondria swollen to varied optical density changes, as limited by pH, is shown in Fig. 3.<sup>3</sup> In contrast to the transient-occurring conformation at pH 7.4, mitochondria exposed to the phosphate-containing medium at pH 6.2 (Figs. 3 *a* and *b*) remain in an intermediate conformation indefinitely and very

<sup>3</sup> In curves *a*, *b*, and *c*, the onset of swelling is immediate and the extents of swelling shown remain constant. See Fig. 1 of reference 2 and Fig. 3 of reference 24.

little bursting of the outer membrane is observed. However, as the pH is increased to 6.6, 7.0, and 7.4, the volume of the inner compartment increases with the coincident loss of internal organization and the bursting of the outer membrane. It appears that the swelling process, initiated by phosphate, is interrupted at various stages, depending upon the pH, such that a particular morphological form accumulates at each sustained optical density. It is suggestive of the possibility that each morphological form thus obtained corresponds to the form existing at the same optical density in the pH 7.4 swelling sequence.

At pH 6.2, the intramembranal space is undiscernible, suggesting that  $H^+$  exerts a direct influence on the nature of the binding between the inner and outer membranes. This phenomenon is independent of conformational status and the presence of swelling agent (Fig. 4).

An increase in the extent of phosphate-induced swelling at pH 6.2 may be effected by the addition of adenosine triphosphate (ATP) (Fig. 5) or oxidizable substrates (2, 24). A comparison of the structure of mitochondria in Fig. 4 *b* with that of mitochondria in Fig. 5 *b* indicates that the action of phosphate at pH 6.2 is to initiate a rapid transition from the condensed to orthodox conformation, which appears to proceed through an intermediate twisted form (Fig. 5 *a*). The addition of ATP to mitochondria in the orthodox form results in swelling and bursting (Fig. 5 *c-e*), which paral-

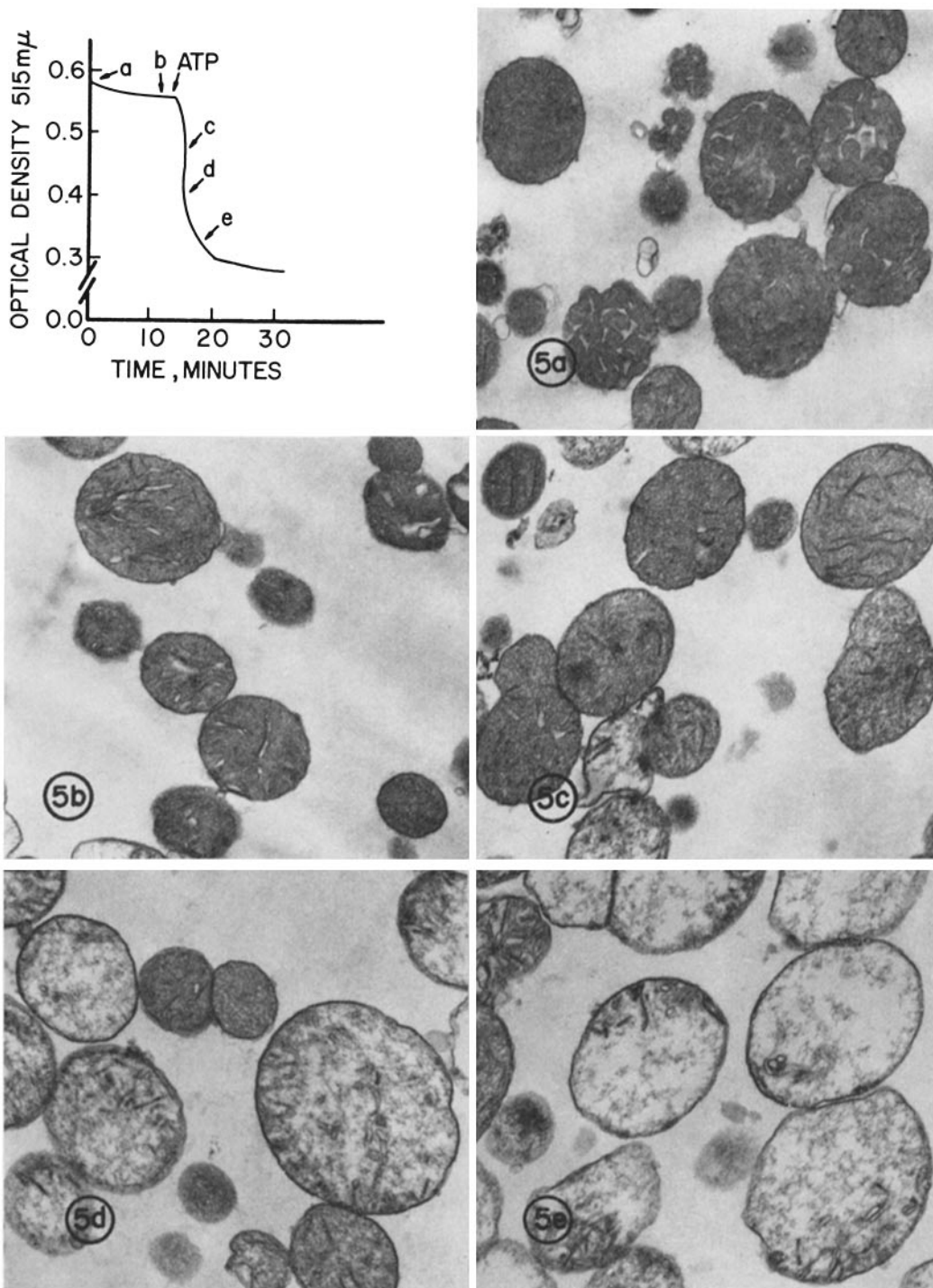


FIGURE 5 The ultrastructural expression of ATP-induced increase in extent of swelling. Medium as in Fig. 1, except pH 6.2. pH determined at 30 min = 6.2. The ultrastructural statuses of mitochondria are as follows: (a) indication of twisted conformation occurring between condensed (Fig. 4 b) and orthodox (b); (c) ATP-induced bursting, resulting in swelling (d) and (e).  $\times 20,500$ .



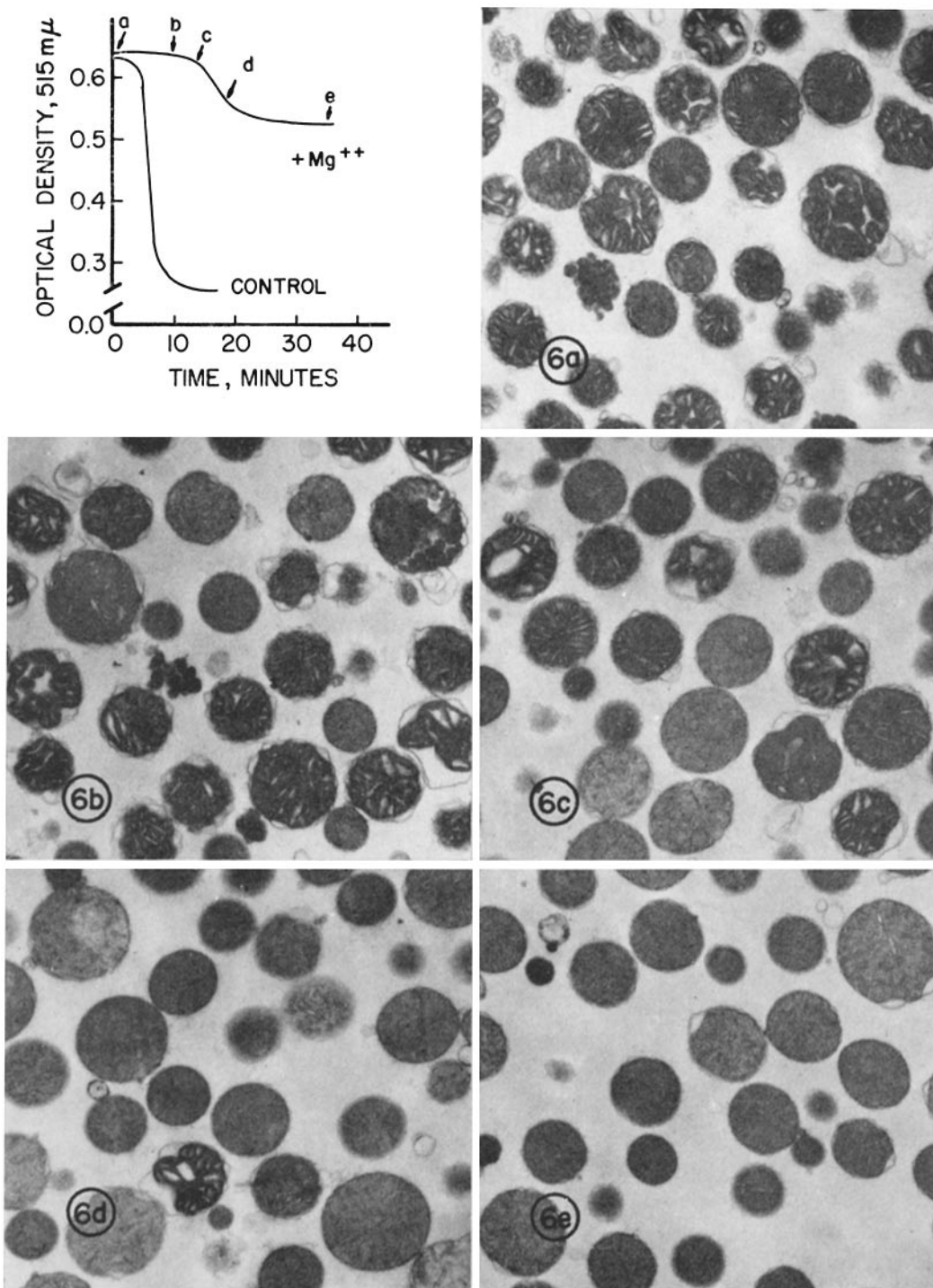
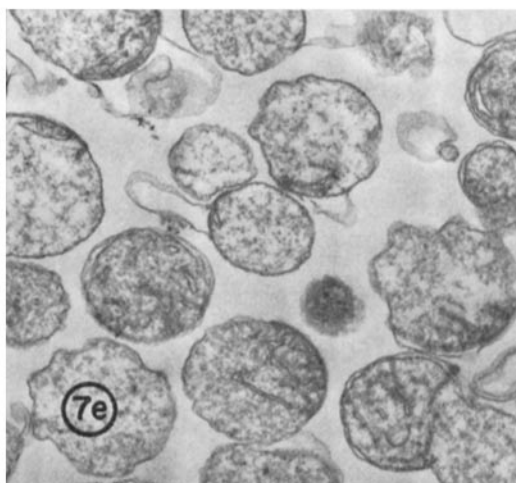
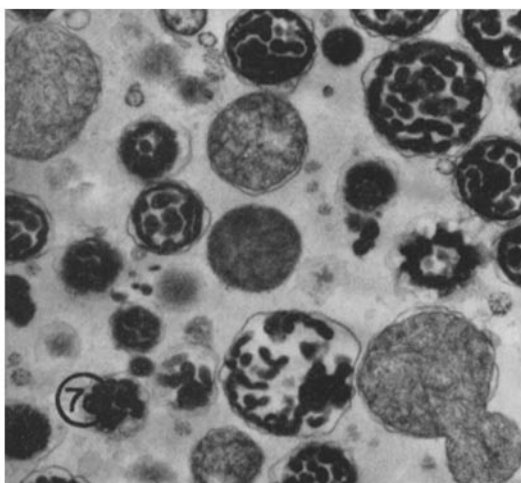
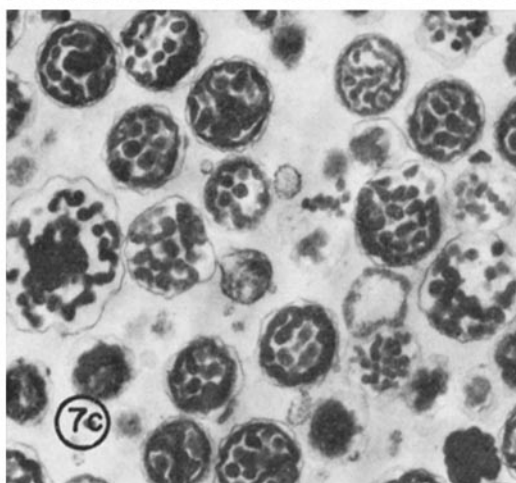
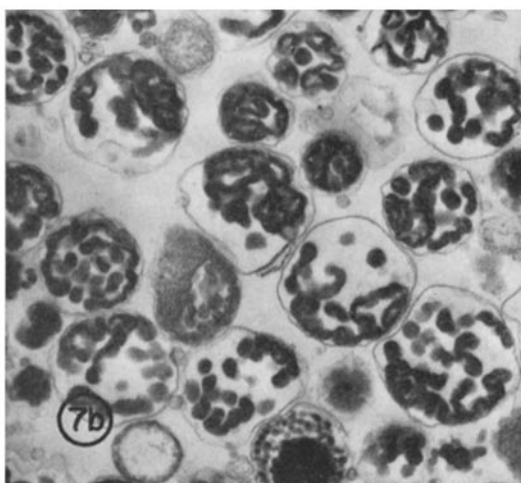
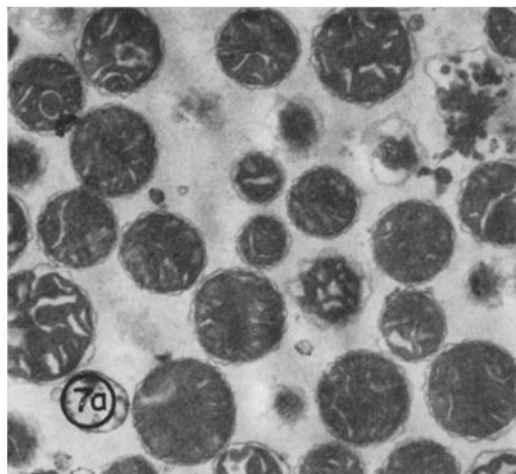
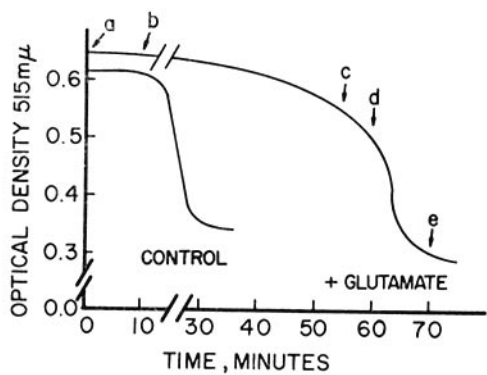


FIGURE 6 The morphology of  $Mg^{2+}$  inhibition of extent of swelling. Medium as in Fig. 1, except for added 5 mM of  $MgCl_2$ . The ultrastructural statuses of mitochondria are as follows: (a) predominantly condensed; (b, c) transition to orthodox; (d, e) predominantly orthodox.  $\times 12,500$ .





**FIGURE 7** The sequence of ultrastructural changes under conditions of delayed onset of swelling. Medium as in Fig. 1, plus 0.33 mM  $K^+$  glutamate, 0.2 mM ADP, and oligomycin, 1  $\mu$ g/ml. The ultrastructural statuses of mitochondria are as follows: (a) initial condensed; (b, c) highly twisted conformation; (d) appearance of orthodox and bursting forms; (e) swollen.  $\times 12,500$ .

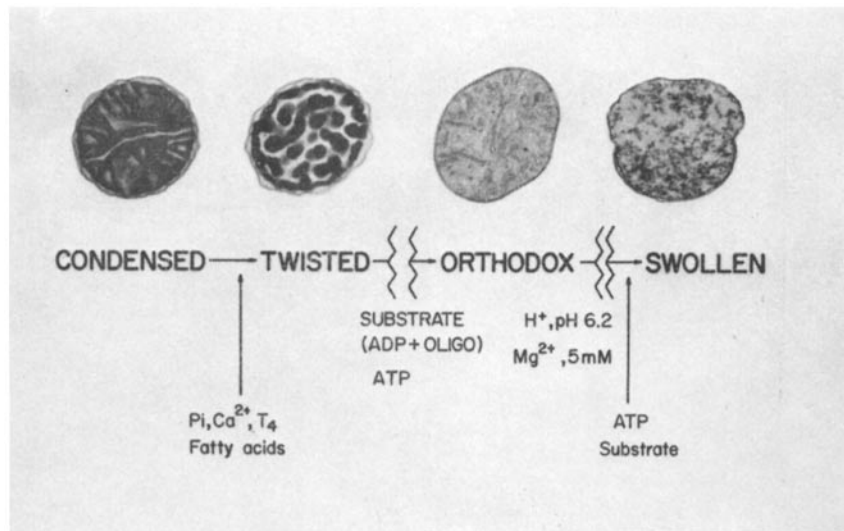


FIGURE 8 A generalized representation of the sequence of ultrastructural transitions occurring with large amplitude swelling of rat liver mitochondria. Approx.  $\times 11,000$ .

lets the morphological changes of the bursting process observed under pH 7.4 conditions. A similar pattern is obtained when ATP is replaced by respiratory substrates, such as succinate or glutamate. These experiments verify that the pH-intermembrane relationship noted above is independent of swelling status (extent).

In addition to being affected by hydrogen ion, the extent of swelling is also inversely related to the concentration of  $Mg^{2+}$  (3, 24). The protective influence of 5 mM  $MgCl_2$  in phosphate-induced swelling at pH 7.4 is shown in Fig. 6. Although the intermediate twisted conformation is not so pronounced, the over-all process results in a marked change from the condensed to the orthodox conformation. This transformation is identical to that described by Hackenbrock (5) for mitochondria incubated under state 1 conditions with 5 mM  $MgCl_2$ . It is apparent that  $Mg^{2+}$  somehow blocks the actual bursting process. Addition of ADP to these mitochondria does not regenerate the condensed form (8).

#### *Variation of Time of Onset of Swelling*

The time of onset of swelling is subject to regulation by a wide variety of agents including respiratory substrates, ATP and ADP (adonosediphosphate) (3, 25) which are intimately related to oxidative phosphorylation. The time of onset is

lengthened optimally in the presence of respiratory substrate, ADP, and oligomycin (25). The sequence of ultrastructural changes that occurs during swelling under the latter condition is shown in Fig. 7. Mitochondria quickly undergo a conformational transition from condensed to twisted form, and this form is maintained until the time of onset of swelling (Fig. 7 *d*). This finding was true regardless of the time of onset or the conditions (ADP plus substrate; substrate plus oligomycin; ATP; antimycin) used to vary the time of onset.

#### DISCUSSION

Of significance is the finding that the sequence of morphological transitions occurring during the large amplitude swelling is remarkably uniform regardless of the conditions under which swelling occurs. A general representation of this ordered sequence is given in Fig. 8. Although the mode of action of the swelling agents is unknown, all of them induce the same initial change in morphology. The degree of twisted character, prior to the onset of swelling, is condition-dependent. Mitochondria become most twisted under conditions that delay the onset of swelling (i.e. glutamate, ADP, and oligomycin, Fig. 7) and become least twisted in the presence of agents which inhibit extent of swelling (pH 6.2 or 5 mM  $MgCl_2$ ). Less twisted character was also observed when the

sucrose medium was replaced by potassium chloride.

The driving force behind the conformational transition from twisted to orthodox form is not readily apparent. Nevertheless, this transformation, resulting in a large increase in the volume of the inner compartment (matrix), is consistently observed as an intermediate step in the over-all process initiated by the swelling agent. Except under conditions in which the extent of swelling is limited, once the orthodox conformation evolves, a bursting of the outer membrane and gross swelling of the inner compartment continue to completion. It is suggested that ethylenediaminetetraacetate (EDTA), a known inhibitor of phosphate-induced swelling (2, 30), owes its effectiveness to an influence on the transition from twisted to orthodox form since in the presence of EDTA the condensed-to-twisted alteration proceeds unaffected.

It is noteworthy that cristae in swollen mitochondria are found only adjacent to segments of the inner membrane to which the outer membrane remains attached. A similar observation was reported with beef heart mitochondria (15). Moreover, the affinity between the inner and outer membranes is apparently increased under acidic conditions (Figs. 3 a, 4 b, and 5). These findings are consistent with the notion that there exist sites of binding between the outer and inner membranes of the mitochondrion (29), and, furthermore, that the degree of organization of the inner membrane may be influenced by association with the outer membrane.

Through a similar study with beef heart mitochondria under conditions of salt-induced swelling, a sequence of conformational changes was reported by Asai et al. (15). Although it is difficult to compare every characteristic of this sequence with every characteristic of the sequence for the rat liver, since minor variations may reflect a species difference, it is worth noting that a twisted inner membrane-matrix is observed as an intermediate in both cases.

This work has shown that the actual swelling process can, in effect, be suspended by agents which decrease the extent of swelling. The delay of mitochondrial swelling, which is accurately reflected by the lifetime of the twisted conformation, can be afforded by agents of a variety of classes including energy-linked metabolites (ATP, respiratory substrates), chelators (EDTA), respiratory inhibitors, and certain inorganic ions. Since, in

the presence of swelling agent, the twisted conformation is the only one whose lifetime can be varied, it follows that the mechanisms which maintain this conformation are the targets of the influence provided by known protective agents.

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#### REFERENCES

1. LEHNINGER, A. L. 1962. *Physiol. Revs.* **42**:467.
2. CONNELLY, J. L., and H. A. LARDY. 1964. *J. Biol. Chem.* **239**:3065.
3. CONNELLY, J. L., and H. A. LARDY. 1964. *Biochemistry.* **3**:1969.
4. WEINBACH, E. C., J. GARBUS, and H. G. SHEFFIELD. 1966. *Exp. Cell Res.* **46**:129.
5. HACKENBROCK, C. R. 1966. *J. Cell Biol.* **30**:269.
6. HACKENBROCK, C. R. 1968. *J. Cell Biol.* **37**:345.
7. DEAMER, D. W., K. UTSUMI, and L. PACKER. 1967. *Arch. Biochem. Biophys.* **121**:641.
8. HACKENBROCK, C. R., and A. I. CAPLAN. 1969. *J. Cell Biol.* **42**:221.
9. HARRIS, R. A., J. T. PENNISTON, J. ASAI, and D. E. GREEN. 1968. *Proc. Nat. Acad. Sci. U.S.A.* **59**:830.
10. GREEN, D. E., J. ASAI, R. A. HARRIS, and J. T. PENNISTON. 1968. *Arch. Biochem. Biophys.* **125**:684.
11. SORDAHL, L. A., Z. R. BLAILECK, G. H. KRAFT, and A. SCHWARTZ. 1969. *Arch. Biochem. Biophys.* **132**:404.
12. UTSUMI, K., and L. PACKER. 1967. *Arch. Biochem. Biophys.* **121**:633.
13. PACKER, L., J. M. WRIGGLESWORTH, P. A. G. FORTES, and B. C. PRESSMAN. 1968. *J. Cell Biol.* **39**:382.
14. HUNTER, G. R., Y. KAMISHIMA, and G. P. BRIERLEY. 1968. *J. Cell Biol.* **39**:64 a. (Abstr.)
15. ASAI, J., G. A. BLONDIN, W. J. VAIL, and D. E. GREEN. 1969. *Arch. Biochem. Biophys.* **132**:524.
16. WEBER, N. E., and P. V. BLAIR. 1969. *Biochem. Biophys. Res. Commun.* **36**:987.

17. LYNN, W. S., JR., S. FORTNEY, and R. H. BROWN. 1964. *J. Cell Biol.* **23**:9.
18. MALAMED, S. 1965. *Z. Zellforsch. Mikrosk. Anat.* **65**:10.
19. WLODOWER, P., D. F. PARSONS, G. R. WILLIAMS, and L. WOJTCZAK. 1966. *Biochim. Biophys. Acta.* **128**:34.
20. PARSONS, D. F., G. A. WILLIAMS, and B. CHANCE. 1966. *Ann. N. Y. Acad. Sci.* **137**:643.
21. CAPLAN, A. I., and J. W. GREENAWALT. 1966. *J. Cell Biol.* **31**:455.
22. MUNN, E. A., and P. V. BLAIR. 1967. *Z. Zellforsch. Mikrosk. Anat.* **80**:205.
23. BLAIR, P. V., and W. C. TAN. 1967. *Biochim. Biophys. Acta.* **143**:630.
24. CONNELLY, J. L., and C. H. HALLSTROM. 1966. *Biochemistry.* **5**:570.
25. CONNELLY, J. L., and C. H. HALLSTROM. 1967. *Biochemistry.* **6**:1567.
26. LUFT, J. H. 1961. *J. Biophys. Biochem. Cytol.* **9**:409.
27. VENABLE, J. H., and R. COGGESHALL. 1965. *J. Cell Biol.* **25**:407.
28. WATSON, M. L. 1958. *J. Biophys. Biochem. Cytol.* **4**:475.
29. HACKENBROCK, C. R. 1968. *Proc. Nat. Acad. Sci. U.S.A.* **61**:598.
30. SLATER, E. C., and K. W. CLELAND. 1952. *Nature (London).* **170**:118.