Fleischer, S., and Fleischer, B. (1967), *Methods Enzymol. 10*, 406.
Folch, J., Lees, M., and Sloane Stanley, G. H. (1957), *J. Biol. Chem. 226*, 497.
Houtsmuller, U. M. T., and van Deenen, L. L. M. (1965), *Biochim. Biophys. Acta 106*, 564.
Jollow, D., Kellerman, G. M., and Linnane, A. W. (1968), *J. Cell Biol. 37*, 221.
Kagawa, Y., and Racker, E. (1966), *J. Biol. Chem. 241*, 2467.
Kováč, L., Šubik, J., Russ, G., and Kollár, K. (1967), *Biochim. Biophys. Acta 144*, 94.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Marinetti, G. V., Erbland, J., and Kochen, J. (1957), *Federation Proc. 16*, 837.
Morrison, W. R., and Smith, L. M. (1964), *J. Lipid Res. 5*, 600.
Parker, F., and Peterson, N. F. (1965), *J. Lipid Res. 6*, 455.
Schatz, G. (1965), *Biochim. Biophys. Acta 96*, 342.
Schatz, G., Penefsky, H. S., and Racker, E. (1967), *J. Biol. Chem. 242*, 2552.
Serlupi-Crescenzi, G., and Barcellona, S. (1966), *Ann. Ist Super. Sanita 2*, 431.
Skidmore, W. D., and Entenman, C. (1962), *J. Lipid Res. 3*, 471.
Skipski, V. P., Peterson, R. F., and Barclay, M. (1964), *Biochem. J. 90*, 374.
Stadtman, T. C. (1957), *Methods Enzymol. 3*, 392.
Wagner, H., Hörhammer, L., and Wolff, P. (1961), *Biochem. Z. 334*, 175.

Promitochondria of Anaerobically Grown Yeast. III. Morphology*

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ABSTRACT: Electron micrographs of frozen-etched, anaerobically grown *Saccharomyces cerevisiae* cells reveal the presence of mitochondria-like structures. These nonrespiring "promitochondria" resemble aerobic yeast mitochondria with respect to their characteristic cristae, their double-layered envelope, and their brittle outer membrane which exhibits slits of approximately 100 × 1000 Å. Promitochondria were found regardless of whether the cells had been grown in the presence or the absence of Tween 80 and ergosterol. These observations support the view that anaerobically grown *S. cerevisiae* still contains mitochondrial structures.

The cytology of aerobically grown yeast cells has been extensively investigated and appears fairly well established (Agar and Douglas, 1957; Hagedorn, 1957; Hashimoto *et al.*, 1959; Hirano and Lindegren, 1961; Vitols *et al.*, 1961; Marquardt, 1962; Yotsuyanagi, 1962). In contrast, the structural organization of the anaerobically grown cells has received considerable less attention and is still under dispute. One of the main uncertainties centers around the question of whether the anaerobic cells still contain mitochondrial organelles. In one of the earliest papers on this subject, Linnane *et al.* (1962) reported that anaerobically grown *Torulopsis utilis* cells were devoid of mitochondria and instead contained a pronounced reticular or myelinlike membrane system. These membranes were interpreted as mitochondrial precursors. Absence of mitochondria was subsequently also claimed for anaerobically grown *Saccharomyces cerevisiae* (Wallace and Linnane, 1964; Polakis *et al.*, 1964). In these cells, however, the cytoplasm appeared to be essentially empty and it was therefore proposed that respiratory adaptation of *S. cerevisiae* involved *de novo* formation of the mitochondrial structures (Wallace and Linnane, 1964).

On the other hand, electron micrographs of anaerobic *S. cerevisiae* by Morpurgo *et al.* (1964) suggested that mitochondria were still present, provided the cells had been grown in a medium enriched with Tween 80 and ergosterol. The isolation of mitochondria-like subcellular particles from these anaerobic, lipid-supplemented cells (Schatz, 1965) further supported this view. Wallace *et al.* (1968) have therefore recently modified their original opinion concerning the effect of anaerobic growth on the mitochondrial organelles. They now admit that anaerobic yeast cells are not necessarily devoid of mitochondria but they still maintain that anaerobic growth in the absence of added lipids induced a complete loss of the mitochondrial structures.

The morphological study reported here was prompted

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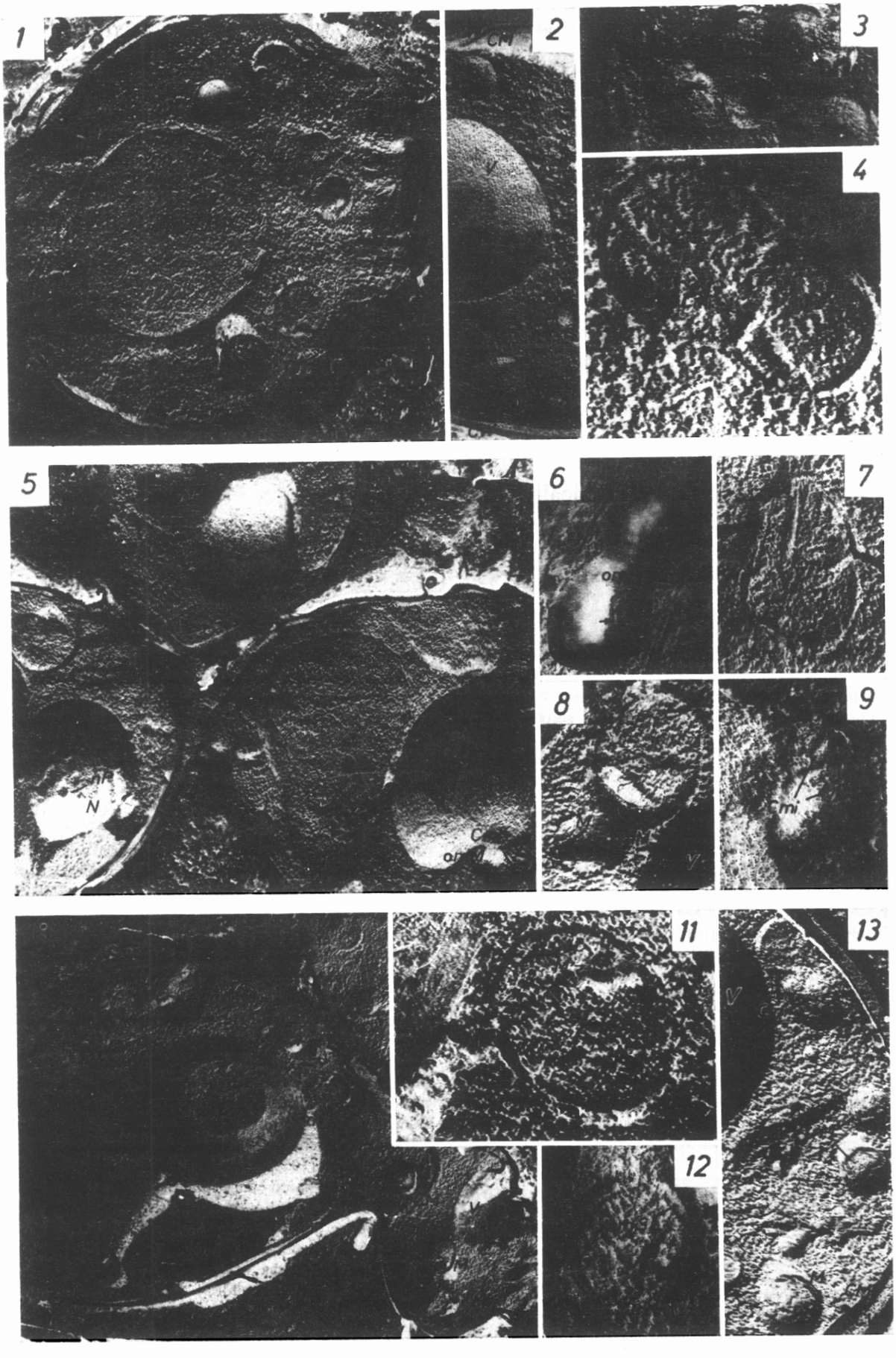
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by biochemical experiments which indicated that mitochondria-like particles are present in anaerobic *S. cerevisiae* regardless of the lipid composition of the growth medium (Criddle and Schatz, 1969). It seemed thus mandatory to reinvestigate the cytology of the anaerobic yeast cells in the hope of detecting intracellular structures which, upon homogenization, could give rise to the "promitochondrial" particles. Since the chemical composition of anaerobic yeast cells differs significantly from that of the aerobic ones (Kováč *et al.*, 1967; Jollow *et al.*, 1968; Paltauf and Schatz, 1969), we decided to abandon the conventional chemical fixatives and to rely instead on "physical" fixation by the freeze-etching procedure of Moor *et al.* (1961). As will be shown below, this method satisfactorily reveals the structural features of anaerobic *S. cerevisiae* and affords an identification of promitochondria even in the lipid-deficient anaerobic yeast cells.

Materials and Methods

The wild-type *S. cerevisiae* strain D 273–10 B (Sherman, 1965) was grown and harvested exactly as described in the first paper of this series (Criddle and Schatz, 1969) except that the concentration of glucose in the growth medium was lowered to 0.8%. In order to facilitate comparison, both the aerobic and anaerobic cultures were poisoned with cycloheximide and iodoacetate prior to harvesting. The isolated yeast cells were suspended in 10 mM Tris-SO_4 (pH 7.4)–50 μg/ml of cycloheximide so that each milliliter of the suspension contained 500–700 mg (wet weight) of cells. Single drops of the suspensions were placed on scratched copper plates which were immediately immersed into liquid Freon 12 cooled with liquid nitrogen. In an attempt to minimize artifacts, pretreatment of the cells with an antifreeze agent was omitted even though this entailed a somewhat rougher ice background relief. Until being processed further, the quick-frozen samples were stored in liquid nitrogen. They were then cut in the freeze-etching device (Model BA 360 M) manufactured by Balzers AG. (Switzerland) and successively shadowed with platinum and carbon. The shadowing angles were 45 and 90°, respectively. The shadowed preparations were examined at 80 kV in a Siemens Elmiskop I electron microscope.

Results

Aerobically Grown Cells. Like chemically fixed and embedded specimens of aerobic yeast cells (*cf.*, *e.g.*, Agar and Douglas, 1957; Vitols *et al.*, 1961; Yotsuyanagi, 1962), freeze-etched samples of these cells reveal numerous mitochondria with diameters between 0.2 and 0.8 μ (Figures 1–4). The organelles are generally situated close to the periphery of the cell. Their identity may often be inferred from the characteristic cristae mitochondriales that are exposed in the cross-sectioned structures (Figure 4). More frequently, however, the mitochondria have remained more or less intact and present themselves only in surface view. Even in these cases, identification is nearly always possible because of the characteristic surface structure, in particular the unique 100 × 1000 Å slits of the mitochondrial outer

FIGURES 1–4: Aerobically grown *S. cerevisiae* cells.

FIGURE 1: Basic organization (only partially represented). Visible are the cell wall, the cell membrane, the nucleus, several lipid droplets with typical myelin sheets, and some mitochondria. The large perinuclear vacuole is not in the plane of section. Magnification 32,500 ×.

FIGURE 2: A large perinuclear vacuole studded with particles. Note that the vacuolar envelope appears essentially intact and shows no sign of splintering. Magnification 32,500 ×.

FIGURE 3: Surface view of mitochondria with partly splintered outer membrane. Note the difference to the intact membrane surface of the vacuole. Magnification 40,000 ×.

FIGURE 4: Cross-sectioned mitochondrion exhibiting several distinct cristae. Magnification 82,000 ×.

FIGURES 5–9: Anaerobic cells grown in the presence of Tween 80 and ergosterol.

FIGURE 5: Basic organization (only partially represented). The electron micrograph shows nuclei with distinct pores in their envelope, cytoplasmic membranes, a lipid droplet, and large perinuclear vacuoles. In the lower right-hand corner, a promitochondrion is seen in surface view. Magnification 23,000 ×.

FIGURE 6: Surface relief of a promitochondrion. The arrow points at a slit in the promitochondrial outer membrane. Magnification 60,000 ×.

FIGURE 7: Cross-sectioned promitochondrion. Whereas the double envelope is only poorly resolved, the cristae are clearly visible. Magnification 50,000 ×.

FIGURE 8: A promitochondrion, seen partly in cross-section and partly in surface relief. The latter reveals two distinct surrounding membranes spaced 150–200 Å apart. Magnification 50,000 ×.

FIGURE 9: Surface relief of a promitochondrion. The partial removal of the outer membrane affords an "external" view of the infoldings of the inner membrane. Magnification 50,000 ×.

FIGURES 10–13: Anaerobic cells grown in the absence of Tween 80 and ergosterol.

FIGURE 10: Basic organization. Note the remarkable structural organization of the cell. The electron micrograph shows a nucleus and an adjacent large vacuole, several smaller vascuoles, lipid droplets, a Golgi apparatus, a cell membrane, a cell wall, and several promitochondria which present themselves either as cross sections or partly in surface relief. Magnification 23,000 ×.

FIGURES 11 and 12: Promitochondria surrounded by double membranes spaced approximately 200–300 Å apart. The sections are virtually devoid of cristae. Magnification 110,000 × (Figure 11) and 80,000 × (Figure 12).

FIGURE 13: Surface reliefs of promitochondria. The parallel contours probably represent an external view of the cristae. These are also evident in the partially cross-sectioned specimen in the upper right-hand corner. Magnification 35,000 ×. Abbreviations are: CM = cell membrane; Cmi = cristae mitochondriales; cyM = cytoplasmic membrane; CW = cell wall; GA = Golgi apparatus; Gly = glycogen; L = lipid droplet; M = mitochondrion or promitochondrion; N = nucleus; nM = nuclear membrane; nP = nuclear pore; omM = outer mitochondrial (or promitochondrial) membrane; omS = outer mitochondrial (or promitochondrial) space; pS = perinuclear space; and V = vacuole.

membrane (*cf.* Moor and Mühlethaler, 1963). The outer membrane also splinters easily and thereby partially uncovers the inner one (Figure 3).

In contrast, the numerous intracellular lipid droplets appear homogeneous in cross sections and are surrounded by a single, smooth envelope. Their surface relief exhibits several myelinlike layers, each of them 40–50 Å thick. The vacuoles, which may also be in the same size range as the mitochondria, possess yet another type of surface; they are covered by a single membrane which is studded with globular units approximately 150 Å in diameter.

In addition, our electron micrographs of the aerobic cells reveal most of the structures that have already been found in earlier studies of frozen-etched aerobic yeast cells (Moor and Mühlethaler, 1963; Moor, 1966a). These structures include: (1) a nucleus surrounded by a porous double envelope; (2) a nuclear "crystalloid;" (3) a Golgi apparatus; (4) poorly developed cytoplasmic membranes ("endoplasmic reticulum"); (5) homogeneous "metachromatin" granules; (6) ribosomes; (7) glycogen rosettes; (8) a cell membrane which exhibits occasional rodlike buildings as well as unidentified aggregates from which fine fibers extend to the adjacent (9) cell wall.

Cells Grown Anaerobically in the Presence of Tween 80 and Ergosterol. The electron micrographs of these nonrespiring cells (Figures 5–9) show most of the morphological details typical of the corresponding aerobic cells. Again, cross-sectioned structures with clearly identifiable cristae can be discerned (Figure 7). In those instances in which the mitochondrial outer membrane has merely splintered, the cristae can be seen from the "outside" and appear as several parallel contours on the mitochondrial inner membrane (Figure 9). As in the aerobic cells, some mitochondrial reliefs exhibit the characteristic slits in the mitochondrial outer membrane (Figure 6).

Cells Grown Anaerobically in the Absence of Tween 80 and Ergosterol. In contrast to chemically fixed and embedded specimens of these cells (Wallace and Linnane, 1964; Morpurgo *et al.*, 1964; Wallace *et al.*, 1968), frozen-etched preparations reveal numerous clearly defined intracellular structures (Figures 10–13). Indeed, the cytology of the anaerobic, lipid-deficient cells appears to be quite similar to that of the aerobic ones. Of particular interest in the present context are the mitochondrial structures which can again be identified by their double envelope (Figure 12), their brittle outer membrane (Figure 13), and their characteristic inner membrane contours (Figure 13) which, after partial cross sectioning, can be recognized as cristae mitochondriales (Figure 13). Lipid droplets and vacuoles exhibit the same morphological features as in aerobic yeast cells and can thus in most cases be distinguished from the mitochondrial structures.

Discussion

The present morphological findings with frozen-etched *S. cerevisiae* indicate that the anaerobically grown cells still contain mitochondria-like structures. These nonrespiring "promitochondria" resemble aerobic yeast mitochondria with respect to the following morphological properties: (1) their size (diameter 0.2–0.8 μ); (2) a double-layered envelope; (3) an unusually brittle outer membrane exhibiting characteristic 100 × 1000-Å slits; and (4) typical cristae mitochondriales that are visible after cross sectioning or after partial removal of the outer membrane. Taken together, these features strongly suggest a mitochondrial nature and permit a rather unambiguous distinction from other intracellular structures such as lipid droplets or vacuoles. It is therefore reasonable to conclude that the promitochondria described here represent the *in vivo* equivalent of the mitochondria-like particles which can be isolated from homogenates of anaerobic yeast cells (Schatz, 1965; Criddle and Schatz, 1969; Paltauf and Schatz, 1969).

Our interpretation of the present electron micrographs assumes that freeze-etching reveals the *surface* of the intracellular membranes (*cf.* Moor and Mühlethaler, 1963; Mühlethaler *et al.*, 1965; Moor, 1966b, 1967). This view was recently challenged by Branton and Park who claimed that the fracturing plane in frozen-etched chloroplasts and myelin sheets passes along the *inner* side of the respective membranes (Branton and Park, 1967; Branton, 1966, 1967). However, the mitochondrial fracturing reliefs observed by us as well as by other authors in various cell types (Moor and Mühlethaler, 1963; Branton and Moor, 1964; Moor *et al.*, 1964; Plattner, 1968) are difficult to reconcile with this concept. In particular, the pictures showing the splintering of entire segments of the mitochondrial outer membrane with concomitant exposure of the invaginated inner one favor the original suggestion (Moor and Mühlethaler, 1963) that freeze-etching provides a surface view of biological membranes.

The present electron micrographs of the aerobic yeast cells resemble those obtained after chemical fixation (Agar and Douglas, 1957; Vitols *et al.*, 1961; Yotsuyanagi, 1962) and thus confirm the validity of the freeze-etching method. In addition, however, our investigations indicate that "physical" fixation by freeze-etching may be far superior to chemical methods if cells in different physiological states and, hence, of different chemical composition are compared. Thus, according to previous studies with other *S. cerevisiae* strains, chemical fixation of the anaerobic, lipid-deficient cells merely reveals an essentially empty cytoplasm and faint outlines of the nucleus, the cell membrane, the cell wall, and a sparse endoplasmic reticulum (Wallace and Linnane, 1964; Morpurgo *et al.*, 1964; Wallace *et al.*, 1968). In contrast, the freeze-etching procedure uncovers a wealth of clearly defined cytoplasmic structures and suggests that the cytology of these anaerobic cells is basically the same as that of the aerobic ones. The reasons for these discrepancies are not clear at present. Although it is tempting to speculate that they are related to the extremely low unsaturation of the promitochondrial lipids (Paltauf and Schatz, 1969), the present uncertainties about the interaction between chemical fixatives and biological membranes *in situ* preclude a definitive answer to this interesting question.

The main point emerging from this study is the fact that anaerobic yeast cells contain promitochondrial

structures even after growth in a lipid-deficient culture medium. It should be stressed that this conclusion rests not merely on morphological evidence, but is also borne out by the biochemical data summarized in the first paper of this series (Criddle and Schatz, 1969). Indeed, even if one were to negate the validity of the present electron micrographs, one would still have to explain the observation that the lipid-deficient anaerobic cells contain subcellar particles equipped with oligomycin-sensitive mitochondrial adenosine triphosphatase (F_1). The combined evidence leaves thus little doubt that at least our *S. cerevisiae* strain never completely loses its mitochondrial organelles.

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References

Agar, D. H., and Douglas, H. C. (1957), *J. Bacteriol. 73*, 365.

Branton, D. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 1048.

Branton, D. (1967), *Exptl. Cell Res. 45*, 703.

Branton, D., and Moor, H. (1964), *J. Ultrastruct. Res. 11*, 401.

Branton, D., and Park, R. B. (1967), *J. Ultrastruct. Res. 19*, 283.

Criddle, R. S., and Schatz, G. (1969), *Biochemistry 8*, 322 (this issue; paper I).

Hagedorn, H. (1957), *Naturwissenschaften 44*, 641.

Hashimoto, T., Conti, S. F., and Naylor, H. B. (1959), *J. Bacteriol. 77*, 344.

Hirano, T., and Lindegren, C. C. (1961), *J. Ultrastruct. Res. 5*, 321.

Jollow, D., Kellerman, G. M., and Linnane, A. W. (1968), *J. Cell Biol. 37*, 221.

Kováč, L., Šubik, J., Russ, G., and Kollár, K. (1967), *Biochim. Biophys. Acta 144*, 94.

Linnane, A. W., Vitols, E., and Nowland, P. G. (1962), *J. Cell Biol. 13*, 345.

Marquardt, H. (1962), *Z. Naturforsch. 17b*, 42.

Moor, H. (1966a), *J. Cell Biol. 29*, 153.

Moor, H. (1966b), *Balzers High-Vacuum Rept. 9* (DN 1002), 1.

Moor, H. (1967), *13th Meeting German Soc. Electron Microscopy, Marburg-Lahn*, Abstract A1.

Moor, H., and Mühlethaler, K. (1963), *J. Cell Biol. 17*, 609.

Moor, H., Mühlethaler, K., Waldner, H., and Frey-Wyssling, A. (1961), *J. Biophys. Biochem. Cytol. 10*, 1.

Moor, H., Ruska, C., and Ruska, H. (1964), *Z. Zellforsch. 62*, 581.

Morpurgo, G., Serlupi-Crescenzi, G., Tecce, G., Valente, F., and Venetacci, D. (1964), *Nature 201*, 897.

Mühlethaler, K., Moor, H., and Szarkowski, J. W. (1965), *Planta 67*, 305.

Paltauf, F., and Schatz, G. (1969), *Biochemistry 8*, 335 (this issue; paper II).

Plattner, H. (1968), *Ber. Naturwissenschaften-Med. Verein (Innsbruck)* (in press).

Polakis, E. S., Bartley, W., and Meek, G. A. (1964), *Biochem. J. 90*, 369.

Schatz, G. (1965), *Biochim. Biophys. Acta 96*, 342.

Sherman, F. (1965), Mecanismes de Regulation des Activites Cellulaires chez les Microorganismes, Paris, The Centre National du Recherche Scientifique, p 465.

Vitols, E., North, R. J., and Linnane, A. W. (1961), *J. Biophys. Biochem. Cytol. 9*, 689.

Wallace, P. G., Huang, M., and Linnane, A. W. (1968), *J. Cell Biol. 37*, 207.

Wallace, P. G., and Linnane, A. W. (1964), *Nature 201*, 1191.

Yotsuyanagi, Y. (1962), *J. Ultrastruct. Res. 7*, 121.