THE ORGANIZATION OF RIBOSOMAL GRANULES WITHIN MITOCHONDRIAL STRUCTURES OF AEROBIC AND ANAEROBIC CELLS OF *SACCHAROMYCES CEREVISAE*

KENNETH WATSON. From the Department of Molecular Sciences, University of Warwick, Coventry CV4 7AL, England

INTRODUCTION

Difficulties in the fixation and embedding of yeast cells suitable for electron microscopy studies have been reported by many workers since the early observations on the presence of mitochondria in these cells (Agar and Douglas, 1957; Hagedorn, 1957; Hirano and Lindgren, 1961; Vitols et al., 1961; Yotsuyanagi, 1962). In particular, the reported absence of mitochondrial profiles in yeast cells grown anaerobically without lipid supplements (Polakis et al., 1964; Linnane, 1965; Wallace et al., 1968) has recently been shown to be due to inadequate fixation by potassium permanganate (Damsky et al., 1969; Plattner and Schatz, 1969; Watson et al., 1970, 1971).

The mitochondrial structures in the anaerobic lipid-depleted cell were observed by freeze etching (Plattner and Schatz, 1969) to be similar in morphology to those present in the aerobic and anaerobic lipid-supplemented cells. On the other hand, we have recently reported (Watson et al., 1970, 1971) that the mitochondrial precursors in the lipid-depleted cell are quite distinct, both morphologically and biochemically, to mitochondrial structures in the aerobic and anaerobic lipid-supplemented cells. Damsky et al. (1969) have also reported the presence of primitive mitochondrial structures, similar to those isolated by Watson et al. (1970, 1971), in partially disrupted anaerobic lipid-depleted cells.

A further difference was the nondetectability of mitochondrial ribosomal RNA species (Forrester et al., 1971) and an active mitochondrial protein synthesizing system (Watson et al., 1971) in the lipid-depleted anaerobic cell, both of which are present in the case of the lipid-supplemented anaerobic cell (Watson et al., 1971; Forrester et al., 1971). The experiments on the lipid-depleted

anaerobic cell are, however, complicated by the difficulties experienced in the isolation of relatively intact mitochondrial structures from such cells without a previous prefixation with glutaraldehyde (Watson et al., 1970, 1971).

While the present communication attempts to resolve some of these difficulties, the primary objective of this paper is to report electron microscopy procedures which allow a description of the organization of ribosomal-like granules within mitochondrial structures of both aerobic and anaerobic yeast cells. The aldehyde fixation procedure described, unlike potassium permanganate (Luft, 1956), preserves the nucleic acids of the cell and allowed a preferential and an intense staining of ribonucleoproteins after brief staining with lead citrate. By comparison, the cell membranes were seen in high negative contrast.

METHODS

Growth of Cells

ANAEROBIC: Cells were grown anaerobically, essentially as previously described (Watson et al., 1970). Lipid-depleted cells were grown on galactose (4%) yeast extract-salts medium and lipid-supplemented cells on the same medium supplemented with Tween 80 (5 g/liter) and ergosterol (20 mg/liter). In some experiments, cells were grown anaerobically with glucose (10%) as carbon source.

AEROBIC: Aerobic growth cells were grown on yeast extract-salts medium with glucose (5%) or ethanol (1%) as carbon source to give, respectively, catabolite repressed and maximally derepressed cells. The cells were grown in 2-liter fluted conical flasks, containing 700 ml media, and shaken on a New Brunswick gyrotory shaker at 250 rpm. (New Brunswick Scientific Co., Inc., New Brunswick, N.J.). A few drops of tributyl citrate were used as an antifoaming agent. The temperature was 30°C.

Electron Microscopy

Anaerobic cells, grown to late log phase, were cooled by immersion of the culture flasks in crushed ice. A stream of oxygen-free nitrogen gas was passed through the flasks for 10–15 min before collection of the cells. The cells were washed once with ice-cold 0.3 M sucrose and 50 mM sodium cacodylate buffer (pH 7.2) and then fixed by the addition of 10% glutaraldehyde and 10% acrolein to give final concentrations of 5 and 2%, respectively. Cells were routinely fixed in an ice bath for 4–6 hr under a constant stream of oxygen-free nitrogen. Aerobically grown cells were similarly treated except for the omission of the nitrogen. In some experiments, one-fifth volume of 25% glutaraldehyde and 10% acrolein were added directly into the cooled anaerobic culture flasks under a constant stream of oxygen-free nitrogen.

After fixation, the cells were washed with 0.3 M sucrose and 50 mM cacodylate buffer (pH 7.2) and rapidly dehydrated through a graded acetone series. The dehydrated pellets were treated with a 1:1 mixture of propylene oxide and Epon-Araldite 1:1 resin mixture and left for 1–2 hr after which the mixture was replaced with Epon-Araldite 1:1 mixture and left overnight. The Epon-Araldite mixture was replaced the next day and the sample left for a further 24 hr. The pellets were finally placed in Beem capsules (LKB Instruments, Inc., Stockholm) and the samples polymerized for a minimum period of 48 hr at 60°C. The use of the Epon-Araldite mixture (Dr. S. Ito, personal communication) resulted in a marked improvement in the sectioning properties of the yeast cells as compared to cells embedded in Araldite alone.

Sections were routinely stained for 5 min with a 2% aqueous solution of uranyl acetate, washed in CO_2-free water, and further stained for 10–30 sec in 0.2% lead citrate. The stained sections were washed in 0.01 N NaOH, twice in water, and then allowed to air-dry. The uranyl acetate staining procedure was omitted in the case of samples preferentially stained with lead citrate only. Grids were examined in a Hitachi HU-11A or HU-125 electron microscope at an accelerating voltage of 75 kv.

Materials

Glutaraldehyde, approximately 25%, was obtained from Koch-Light Laboratories Ltd. (Colnbrook, England). The stock solutions gave absorption bands at 280 and 235 nm. Purification by distillation resulted in the disappearance of the 235 nm band, the latter peak presumably due to impurities in the glutaraldehyde (Anderson, 1967). The concentration of glutaraldehyde was determined by titration (Anderson, 1967) and solutions were stored as 25 and 10% aqueous solutions at 4°C. The solutions were further treated with solid $BaCO_3$ just before use.

Acrolein was obtained from BDH Chemicals Ltd. (Poole, England) and purified by distillation. 0.1% hydroquinone was added as stabilizer and the acrolein stored as a 10% aqueous solution at 4°C. Lead citrate and uranyl acetate were Analar quality (BDH Chemicals Ltd.).

RESULTS

Aerobic Cells

Fig. 1 shows electron micrographs of mitochondria in cells grown on 1% ethanol so as to give catabolite derepressed cells. In Fig. 1 *a* the

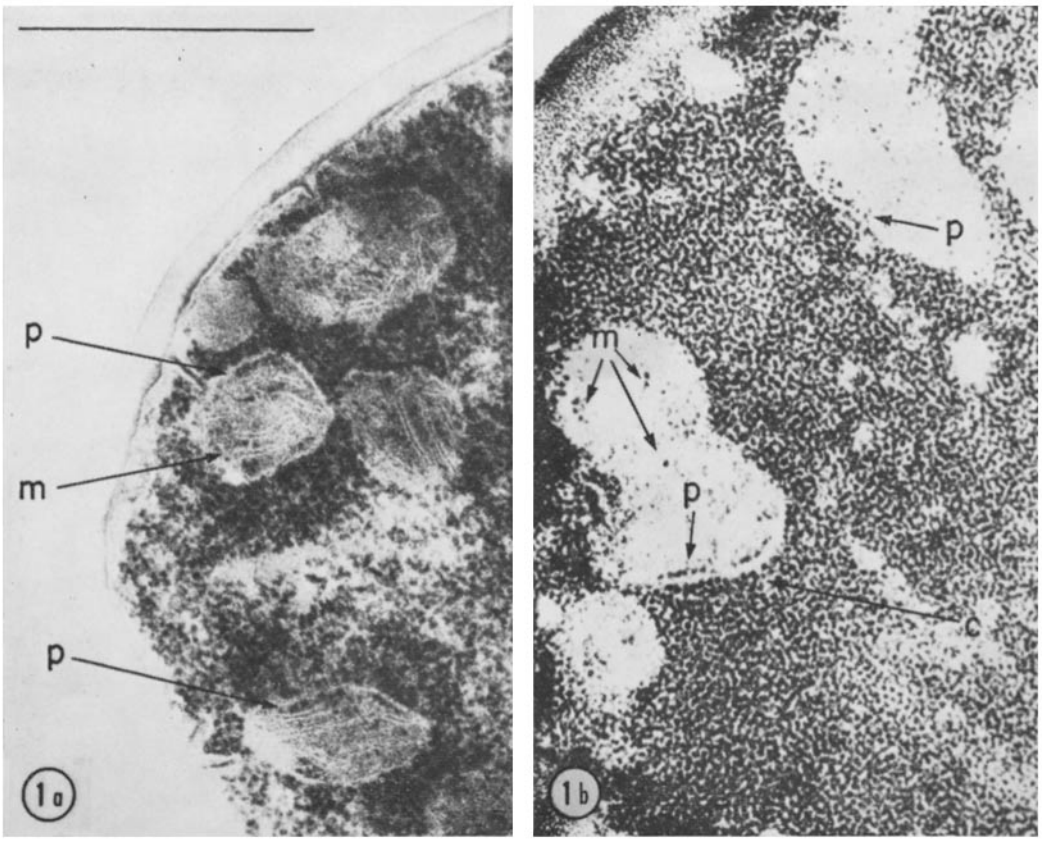

FIGURE 1 Electron micrographs of yeast cells grown on 1% ethanol as substrate. Fixation with glutaraldehyde-acrolein. (*a*) Uranyl acetate and lead citrate staining. Densely staining ribosomal granules are seen in the cytoplasm and also within the mitochondrial structures. *m*, ribosomal-like granules in the mitochondrial matrix close to cristae; *p*, ribosomal-like granules aligning the periphery of the inner mitochondrial membrane. The bar corresponds to 0.5 μ. (*b*) Lead citrate staining. The mitochondrial membranes are not visible but densely staining ribosomal granules are seen in the cytoplasm and within the mitochondria. *m*, ribosomal granules in the mitochondrial matrix; *p*, ribosomal granules aligning the periphery of the inner mitochondrial membrane; *c*, cytoplasmic ribosomal granules close to the outer mitochondrial membrane. The bar corresponds to 0.5 μ.

thin sections were stained with uranyl acetate followed by lead citrate while in Fig. 1 *b* the sections were stained with lead citrate only. The double staining procedure visualized the mitochondrial membranes in negative contrast while by comparison no mitochondrial membranes were visible after section staining with lead citrate only. On the other hand, both staining procedures resulted in an intense staining of ribosomal granules in the cell cytoplasm and also within the mitochondrial structures. The ribosomal-like granules within the mitochondria were observed along the periphery of the inner membrane (Fig. 1 *a* and *b*) as well as in the matrix areas (Fig. 1 *b*) and in some cases close to the cristae membranes (Fig. 1 *a*).

The arrangement of the mitochondrial granules along the periphery of the inner membrane was particularly evident when the cells, after aldehyde fixation, were briefly stained with lead citrate only (Fig. 1 *b*) as compared to the normal procedure of uranyl acetate followed by lead citrate (Fig. 1 *a*). Lead staining has been reported to result in an intense staining of ribonucleoprotein particles (Watson, 1958; Huxley and Zubay, 1961) and it has been further observed that after alde-

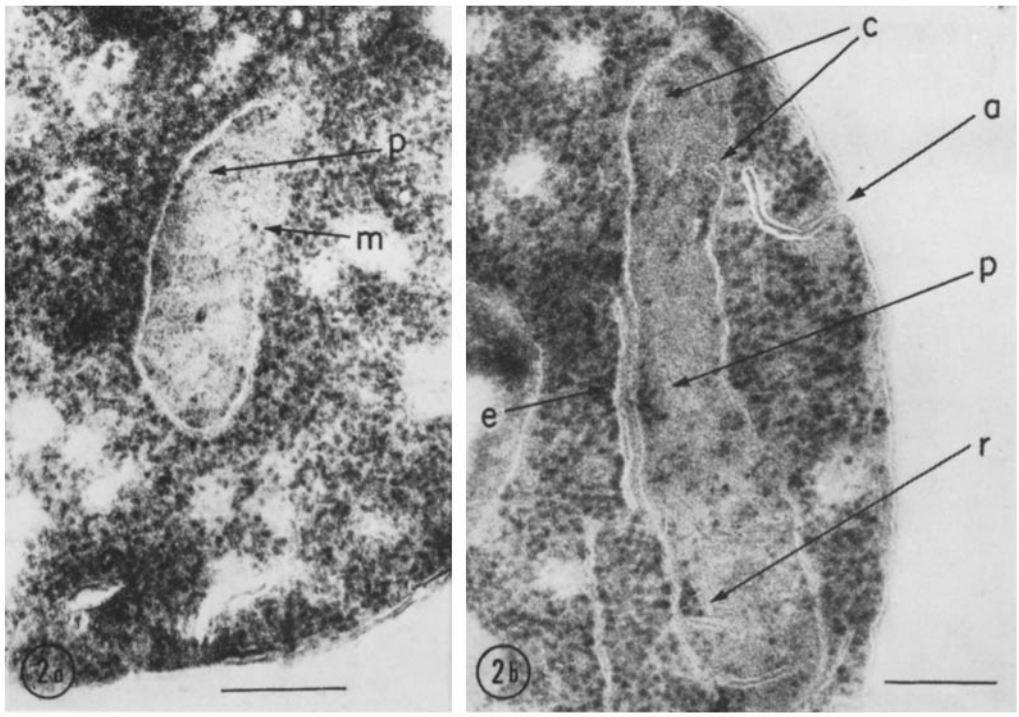

FIGURE 2 Electron micrographs of mitochondrial structures in anaerobically grown cells. Fixation with glutaraldehyde-acrolein and staining with uranyl acetate and lead citrate. Densely staining ribosomal granules are observed in the cytoplasm and within the mitochondrial structures. (*a*) Lipid-depleted anaerobic cell grown on 10% glucose. The cells contained 8% of the total fatty acid as unsaturated fatty acid. We have adopted as a criterion of the degree of anaerobiosis of the lipid-depleted cell the percentage unsaturated fatty acid of the total fatty acid of the cell. We have found this percentage to be consistently between 5–10% in the case of cells grown on glucose and between 10–15% in the case of cells grown on galactose. *m*, ribosomal-like granules within the mitochondrial matrix; *p*, ribosomal-like granules aligning the periphery of the mitochondrion. The bar represents 0.2 μ. (*b*) Lipid-supplemented anaerobic cell grown on 10% glucose plus Tween 80 and ergosterol. *a*, invagination of the plasma membrane; *e*, endoplasmic reticulum close to the mitochondrion; *c*, cristae membranes; *r*, ribosomal granules close to the short cristae; *p*, ribosomal granules along the inner mitochondrial membrane. The bar represents 0.2 μ.

hyde fixation, without postosmication, ribonucleoprotein-containing structures are preferentially stained (Marinozzi and Gautier, 1962; Marinozzi, 1963; Andre and Marinozzi, 1965; Daems and Persijn, 1963; Bernard, 1969). As shown in Fig. 1 *b*, densely staining intramitochondrial granules were clearly seen arranged along the periphery of the mitochondria as well as within the matrix areas. Under the conditions of staining employed, that is, lead staining only, the membranes were not visible. However, the ribosomal-like granules within the mitochondria were clearly separated from the surrounding cytoplasmic granules by a distinct broad band, presumably the outer and inner mitochondrial membranes. The surrounding, slightly larger, cytoplasmic granules were seen apparently associated with or possibly even attached to the outer mitochondrial membrane (Fig. 1 *b*).

Anaerobic Cells

Electron micrographs of mitochondrial structures in cells grown anaerobically in the absence and presence of lipid supplements are shown in Figs. 2 *a* and *b*, respectively. The aldehyde fixation procedure followed by staining with uranyl and lead salts unequivocally showed the presence of mitochondrial structures in both types of anaer-

obic cell. Although membrane elements reminiscent of cristae were clearly visible in the mitochondrial structure of the lipid-supplemented cell (Fig. 2 *b*), the inner membrane was not extensively invaginated and the cristae were short and stunted by comparison with mitochondria in the aerobic cell (cf. Fig. 1 *a*). In the case of the mitochondrial structure in the anadrobic lipid-depleted cell, there was no obvious folded inner membrane (Fig. 2 *a*). Densely staining ribosomal-like granules were observed within mitochondrial structures of both lipid-depleted and lipid-supplemented anaerobic cells. The granules were again predominantly localized along the periphery of the inner mitochondrial membrane and less frequently within the matrix areas. A few granules were observed in some cases close to the short cristae membranes of mitochondria in the lipid-supplemented cell (Fig. 2 *b*).

DISCUSSION

The glutaraldehyde-acrolein procedure described in this paper was effective as a fixative for intact yeast cells grown aerobically or anaerobically with or without lipids. Mitochondrial structures were clearly identified in anaerobic yeast cells, the lipid-depleted structures showing very little internal membrane organization and the lipid-supplemented structures showing a few short cristae. These observations were consistent with our recent reports on the morphology and biochemical properties of the isolated mitochondrial profiles (Watson et al., 1970, 1971). The present double-aldehyde fixation technique clearly distinguishes the morphology of the mitochondrial structures in the aerobic, the lipid-supplemented anaerobic, and the lipid-depleted anaerobic cell and has, therefore, some advantage over freeze-etching studies which show close morphological similarities (Plattner and Schatz, 1969).

The membranes of the yeast cell, in particular the mitochondrial membranes, were not visible after aldehyde fixation and lead staining (Fig. 1 *b*). Daems and Persijn (1963) have earlier reported a similar observation using mouse liver tissues and they attributed the lack of contrast as due to the requirement of reduced osmium for the attachment of lead to the polar groups of the membrane phospholipids. However, the present studies have shown that if lead staining was preceeded by uranyl acetate, then the membranes of the cell, including the mitochondrial membranes, were well contrasted. Uranyl salts have been reported to have a stabilizing effect on DNA (Ryter and Kellenberger, 1958) and to have a staining affinity for DNA-containing structures (Stoeckenius, 1961; Zobel and Beer, 1961; Huxley and Zubay, 1961). More recently it has been shown that uranyl salts also have a stabilizing effect on bacterial membranes (Silva et al., 1971) and malarial parasite membranes (Terzakis, 1968). Lead salts, on the other hand, have a strong affinity for ribosomes and ribonucleoprotein-containing structures, particularly after aldehyde fixation (Marinozzi, 1963; Daems and Persijn, 1963; Bernard, 1969). Further studies on the mode of action of lead and uranyl salts in relation to different fixation, dehydration, and embedding procedures may be expected to yield useful morphological information.

A few densely staining ribosomal granules were observed within mitochondrial structures of the lipid-depleted anaerobic cell (Fig. 2 *a*). We have previously reported the absence of significant amino acid incorporation activity (Watson et al., 1971) and mitochondrial ribosomal RNA species (Forrester et al., 1971) in the isolated mitochondrial structures. The apparent discrepancy between the in vitro and the present in vivo observations may be due to the extreme fragility of these lipid-depleted mitochondrial precursors which cannot be isolated intact without a previous stabilization with glutaraldehyde (Watson et al., 1970, 1971). The latter prefixation, however, strongly inhibits amino acid incorporation activity (Watson et al., 1971) and RNA determination (I. T. Forrester, personal communication). Despite these difficulties, we suggest that if the lipid-depleted anaerobic cell does have mitochondrial ribosomal RNA species, the amount would be much less than that in the aerobic cell.

The presence of ribosomal-like granules in the mitochondrial structures of the lipid-supplemented anaerobic cell was not unexpected since these structures have been shown to have active mitochondrial protein synthesizing both in vitro (Watson et al., 1971) and in vivo (Schatz and Saltzgaber, 1969) as well as unique mitochondrial ribosomal RNA species (Forrester et al., 1971).

The arrangement of the densely staining mitochondrial granules was of some interest. Previous descriptions of these ribosomal-like granules have been somewhat arbitrary and they have been generally described as situated close to the cristae membranes and also within the matrix areas (Swift, 1965; Andre and Marinozzi, 1965; Swift

et al., 1968). The localization of the ribosomal-like granules close to the cristae membranes and within the matrix areas have been confirmed in the present studies on yeast cells. Furthermore, we particularly emphasize that the ribosomal granules were characteristically observed aligning the periphery of the inner mitochondrial membrane in aerobic as well as anaerobic cells (Figs. 1 and 2). Vignais et al. (1969) have recently reported that mitochondria isolated from *Candida* show the presence of ribosomal-like granules close to the periphery of the inner membrane. The significance of the peripheral arrangement of the mitochondrial granules is not clear. It is possible that the localization of the ribosomal granules whether cristal, matrix, or peripheral, as well as the number of granules, may vary with the physiological state of the cell. It is interesting to note that Curgy (1970), using radioautography with tritiated uridine, has recently reported that newly synthesized mitochondrial ribosomal RNA is preferentially localized in the peripheral areas of mitochondria in rat liver. It would be tempting to speculate that the peripheral arrangement of the mitochondrial ribosomal granules would be of some significance in potential mitochondrial-cytoplasmic interactions.

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REFERENCES

AGAR, D. H., and H. C. DOUGLAS. 1957. *J. Bacteriol.* **73**:365.

ANDERSON, P. J. 1967. *J. Histochem. Cytochem.* **15**:652.

ANDRE, J., and V. MARINOZZI. 1965. *J. Microsc. (Paris).* **4**:615.

BERNARD, W. 1969. *J. Ultrastruct. Res.* **27**:250.

CURGY, J. -J. 1970. *Exp. Cell Res.* **62**:359.

DAEMS, W. T., and J. P. PERSIJN. 1963. *J. R. Microsc. Soc.* **81**:199.

DAMSKY, C., W. H. NELSON, and A. CLAUDE. 1969. *J. Cell Biol.* **43**:174.

FORRESTER, I. T., K. WATSON, and A. W. Linnane. 1971. *Biochem. Biophys. Res. Commun.* **43**:409.

HAGEDORN, H. 1957. *Naturwiss. Rundsch.* **44**:642.

HIRANO, T., and C. C. LINDGREN. 1961. *J. Ultrastruct. Res.* **5**:321.

HUXLEY, H. E., and G. ZUBAY. 1961. *J. Biochem. Biophys. Cytol.* **11**:729.

LINNANE, A. W. 1965. *In* Oxidases and Related Redox Systems. T. E. King, H. S. Mason, and M. Morrison, editors. John Wiley & Sons, Inc., New York. **2**:1102.

LUFT, J. 1956. *J. Biochem. Biophys. Cytol.* **2**:799.

MARINOZZI, V. 1963. *J. R. Microsc. Soc.* **81**:141.

MARINOZZI, V., and A. GAUTIER. 1962. *J. Ultrastruct. Res.* **7**:436.

PLATTNER, H., and G. SCHATZ. 1969. *Biochemistry.* **8**:335.

POLAKIS, E. S., W. BARTLEY, and G. A. MEEK. 1964. *Biochem. J.* **90**:369.

RYTER, A., and E. KELLENBERGER. 1958. *Naturforscher.* **13b**:597.

SCHATZ, G., and H. SALTZGABER. 1969. *Biochem. Biophys. Res. Commun.* **37**:996.

SILVA, M. T., J. M. SANTOS, J. V. MELO, and F. C. GUERRA. 1971. *Biochim. Biophys. Acta.* **233**:513.

STOECKENIUS, W. 1961. *J. Biochem. Biophys. Cytol.* **11**:297.

SWIFT, H. 1965. *Am. Nat.* **99**:201.

SWIFT, H., M. RABINOWITZ, and G. GETZ. 1968. *In* Biochemical Aspects of the Biogenesis of Mitochondria. E. C. Slater, J. M. Tager, S. Papa, and E. Quagliariello, editors. Adriatica Editrice, Bari, Italy. 3.

TERZAKIS, J. A. 1968. *J. Ultrastruct. Res.* **22**:168.

VIGNAIS, P. V., J. HUET, and J. ANDRE. 1969. *FEBS. (Fed. Eur. Biochem. Soc.) Lett.* **3**:177.

VITOLS, E., R. J. NORTH, and A. W. LINNANE. 1961. *J. Biochem. Biophys. Cytol.* **9**:689.

WALLACE, P. G., M. HUANG, and A. W. LINNANE. 1968. *J. Cell Biol.* **37**:207.

WATSON, K., J. HASLAM, and A. W. LINNANE. 1970. *J. Cell Biol.* **46**:88.

WATSON, K., J. HASLAM, B. J. VEITCH, and A. W. LINNANE. 1971. *In* Autonomy and Biogenesis of Mitochondria and Chloroplasts. N. K. Boardman. R. M. Smillie, and A. W. Linnane, editors. North Holland Publishing Co., Amsterdam. 161.

WATSON, M. L. 1958. *J. Biochem. Biophys. Cytol.* **4**:475.

YOTSUYANAGI, Y. 1962. *J. Ultrastruct. Res.* **7**:121.

ZOBEL, C. R., and M. BEER. 1961. *J. Biochem. Biophys. Cytol.* **10**:335.