*CIRCULARITY OF YEAST MITOCHONDRIAL DNA**

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There no longer is much question that mitochondria contain the necessary components of a genetic apparatus which is at least partially independent of nuclear genes.[1, 2] DNA-dependent RNA polymerase[2] and DNA polymerase[3] activities of mitochondria may be responsible for the synthesis of presumptive messenger RNA[4] and the conservation of the DNA moiety,[5] respectively. The duplex DNA molecules[6] may occur as open or supercoiled cyclic filaments,[7] may have two free ends,[8–10] or may exist in all these conformations.[11, 12] There is increasing evidence of mutable nonchromosomal factors in yeast,[13] as well as of Mendelian genes which govern various mitochondrial traits.[14] Mounolou *et al.*[15] showed that mitochondrial DNA of an isogenic series of wild-type and petite yeast had different buoyant densities in CsCl gradients. Also, we[12] reported that cyclic molecules predominated in wild-type mitochondrial DNA, whereas filaments with two free ends typified the mitochondrial DNA of an isogenic vegetative petite strain of haploid yeast. There was a heterodisperse length distribution for these molecules from both strains.[12]

The data from the present study of diploid isogenic wild-type and vegetative petite strains extend and confirm the earlier observations of haploids. Additional evidence was collected to evaluate the effects upon filament length and conformation of several variables introduced during DNA purification and in spreading of DNA or mitochondria during the Kleinschmidt[16] monolayering procedure.

Materials and Methods.—The diploid isogenic strains iso-N (wild-type) and DP-28 (vegetative petite) of *Saccharomyces cerevisiae* have been described relative to origin, culture, and phenotype.[17, 18] Mitochondria were isolated from spheroplast lysates[12, 17] of 16-hr or 24-hr mid-stationary phase liquid cultures[19] by centrifugation for 20 min at 10,000 *g*, and then were purified further in continuous or discontinuous four-step sucrose gradients ranging from 1.0 to 2.5 *M* in concentration. Mitochondria either were centrifuged for 20 min at 50,000 rpm in the SW50 rotor of the Spinco model L ultracentrifuge or for 2.5 hr at 13,000 rpm in the SCR swinging-bucket rotor of the Lourdes model A-2 Beta-fuge. All operations were performed at 0–4°C.

Isolation of mitochondrial DNA: Intact organelles recovered from sucrose gradients were treated with deoxyribonuclease-I and pronase,[12] and proteins were extracted after denaturation by phenol or chloroform–*iso*amyl alcohol.[20] The emulsions were shaken at various speeds depending upon the experiment, and the aqueous phase was removed and treated with pancreatic and T_1 ribonucleases prior to final deproteinization. The DNA contained in the aqueous phase was dialyzed exhaustively against several changes of saline–sodium citrate,[20] and its concentration was determined by absorption at 260 mμ. In some cases, whole-cell DNA was obtained from spheroplast lysates with the same methods as just described for mitochondrial DNA.

CsCl density-gradient centrifugation: Analytical sedimentation was carried out according to the method of Meselson *et al.*[21] in a Spinco model E ultracentrifuge at 25°C. DNA from the *Bacillus subtilis* phage PBS1 served as the density marker (density 1.723

gm/cm^3). A Joyce-Loebl recording microdensitometer was used to scan the ultraviolet photographs. The densities of the DNA samples were expressed relative to the density of *Escherichia coli* DNA,[11] taken as 1.710 gm/cm^3.

Monolayering DNA: Freshly isolated intact mitochondria were suspended in ice-cold ammonium acetate–0.01% cytochrome *c* and applied to an ice-cold distilled water hypophase, essentially as described earlier.[12] The concentration of ammonium acetate used to suspend the mitochondria varied from 2.0 to 7.0 *M* depending upon the experimental design. Purified mitochondrial DNA was diluted to a concentration of 1–4 μg/ml in 2 *M* ammonium acetate–0.01% cytochrome *c* and monolayered in the same manner as were whole mitochondria. In some cases, 10 μg/ml deoxyribonuclease-I and 5 mM $MgCl_2$ were added to DNA mixtures just before spreading.[12]

Platinum shadowing and electron microscopy: Samples were picked up on collodion-coated, carbon-stabilized nickel grids and then were dehydrated for 10–20 sec in absolute ethanol. The grids were drained dry and then were shadowed with Pt/Ir (80:20) at an angle of 6–7° in a Cenco evaporator at a vacuum of less than 10^{-4} mm Hg. The grids rotated at 12 rpm for 4–8 min at about 7 cm from the source. The grids were scanned and photographed at initial plate magnifications of 6700 and 9500 times with an RCA-3G electron microscope operated at 50 kv.

Results.—Except for the deoxyribonuclease controls, circular filaments occurred in all mitochondrial DNA preparations (Fig. 1). But their frequency

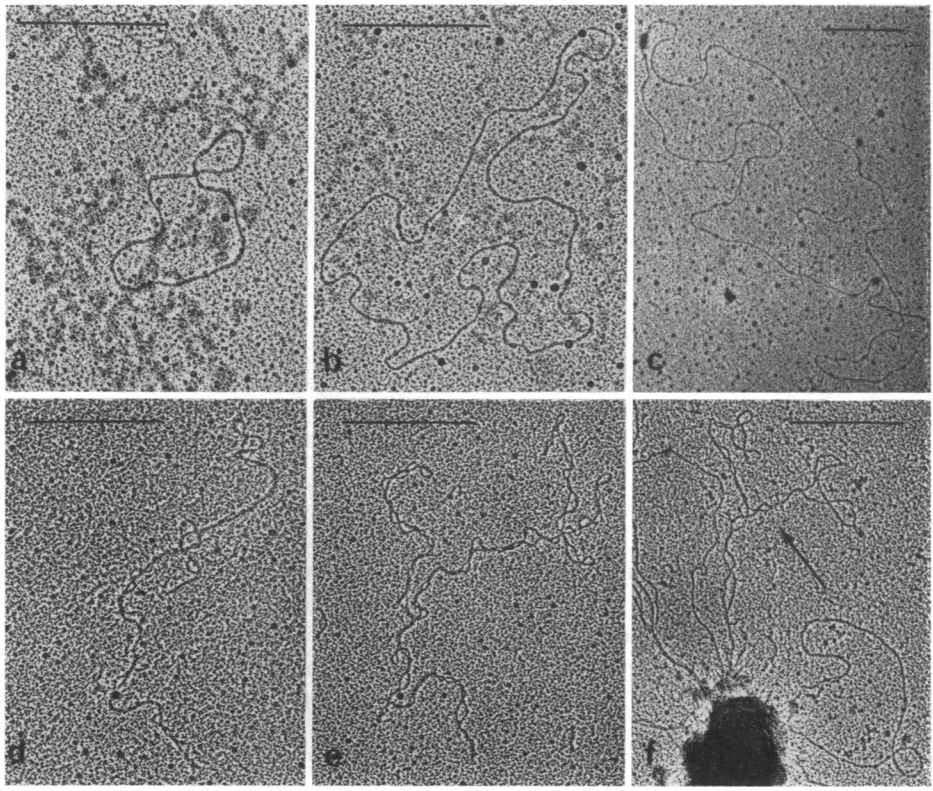

FIG. 1.—Circular DNA filaments from wild-type mitochondria. Open circles measure (*a*) 2.2 μ, (*b*) 5.4 μ, and (*c*) 9.0 μ. Supercoiled circles from purified DNA measure (*d*) 4.9 μ and (*e*) 8.6 μ; (*f*) from an osmotically ruptured mitochondrion the molecule (at *arrow*) measured 5.0 μ. The bar represents 0.5 μ.

(Fig. 2) varied depending upon (*a*) what strain was used, (*b*) at what speed the emulsion was shaken during deproteinization to purify the DNA, (*c*) whether phenol or chloroform was the denaturing agent for deproteinizing the crude samples, (*d*) what degree of osmotic shock occurred during spreading of intact mitochondria onto the hypophase in monolayering, and (*e*) whether we used purified mitochondrial DNA or obtained samples from osmotically ruptured mitochondria on the hypophase.

Osmotically ruptured mitochondria from wild-type iso-N yielded the highest frequency of circular filaments (Fig. 2), but even this frequency varied depending

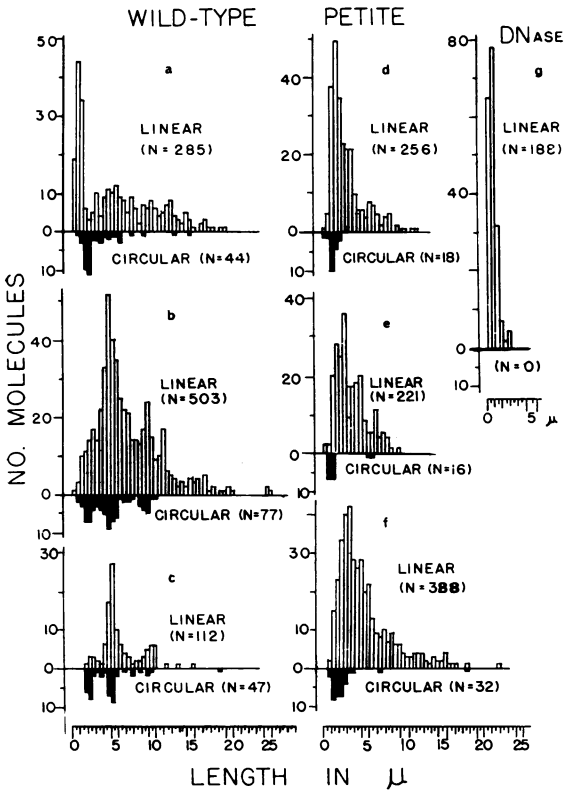

FIG. 2.—Length distribution histograms showing frequencies of linear (two free ends) and circular (no free ends) mitochondrial DNA molecules from wild-type strains: (*a*) phenol-extracted DNA, (*b*) chloroform-extracted DNA, and (*c*) osmotically lysed mitochondrial DNA; and from petite DNA extruded from mitochondria lysed while suspended in ammonium acetate concentrations of (*d*) 6.0 *M*, (*e*) 4.0 and 5.0 *M*, and (*f*) 3.00–3.75 *M*. Filaments from samples in contact with DNase for 3–5 min during monolayering are shown in (*g*).

upon the violence of osmotic lysis as a function of the concentration of ammonium acetate used to dilute the mitochondria for monolayering. With 5 *M* ammonium acetate, 50 per cent of the filaments measured were circular, whereas the frequency was only 8 per cent when 7 *M* ammonium acetate was used. Purified wild-type mitochondrial DNA contained 11–15 per cent cyclic filaments, with the highest frequency occurring in samples shaken gently with chloroform.

The contour lengths of circular filaments from wild type generally varied from 0.9 to 10.5 μ, with modes occurring at 1.5–2.5, 4.5–5.5 and 9.0–10.0 μ (Fig. 2), suggesting a multiple-length distribution.[11, 22] Phenol-extracted wild-type mitochondrial DNA yielded few circles larger than 2.5 μ, and only a single clear mode occurred at 1.5–2.5 μ (Fig. 2).

DNA molecules with two free ends in wild-type preparations varied in length from 0.5 to 25.6 μ, with modes at 4.5–5.5, 9.0–10.0, and 11.0–12.0 μ (Fig. 2). There was an excess of short linear fragments in phenol-extracted mitochondrial DNA as compared with chloroform-extracted DNA, even when the latter was shaken vigorously. Except for the fragments, the remaining linear filament groups were essentially the same for both types of purified mitochondrial DNA.

A few supercoiled cyclic molecules[23] were detected in lysed-mitochondrial and in purified DNA samples (Fig. 1). The lengths of these supercoils were consistent with the lengths found for relaxed (non supercoiled) circles.

Filaments extruded from lysed wild-type mitochondria during monolayering occurred principally as closed loops (Fig. 3), although some strands with free ends were observed. Of 165 such extruded DNA filaments which could be

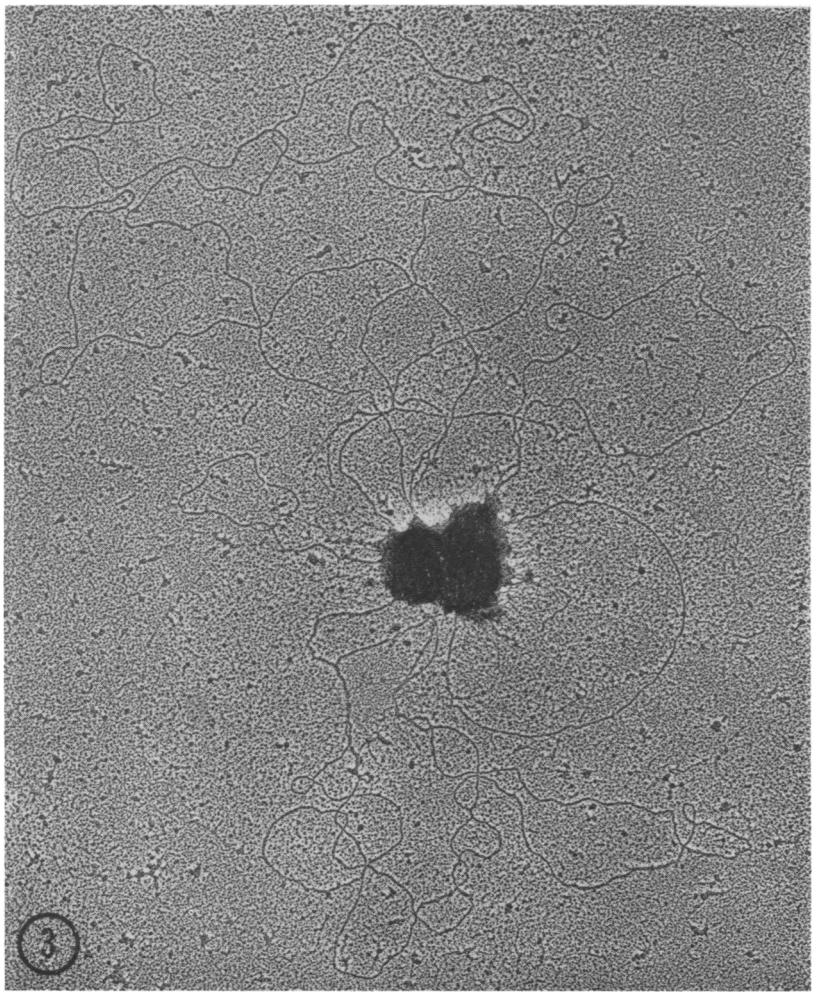

FIG. 3.—DNA filaments extruded upon lysis of an intact wild-type mitochondrion during monolayer formation. Total length, 48.5 μ. Magnification $\times$29,000.

measured, 114 (69%) had no visible free ends. The total length of DNA filaments per mitochondrion was measured from 14 mitochondria which were fixed in the protein monolayer while lysing.[16] From 40.0 to 55.8 μ of DNA could be measured from a single mitochondrial ghost of iso-N, representing a total of six to eight loops and strands still attached to the organelle membrane.

Comparison of mitochondrial DNA extruded from osmotically lysed organelles of wild-type and petite strains revealed several differences: (*a*) the maximum circle frequency for petites was 7.6 per cent, which did not change appreciably with variation in ammonium acetate concentration, whereas wild-type preparations contained a minimum of 8 per cent and a maximum of 50 per cent circles (Fig. 2); (*b*) only the 1.5–2.5-μ class of circular filaments occurred in petites, whereas circles measuring 4.5–5.5 μ in contour length were the principal size class in wild-type samples; (*c*) petite DNA filaments with two free ends presented a unimodal distribution which was skewed considerably toward the shorter lengths centering at about 2.0–3.5 μ, whereas wild-type molecules were distributed across a broad range with more than one obvious mode; and (*d*) filaments extruded from petite mitochondria ruptured during monolayering generally occurred as strands with obvious free ends, in contrast with the predominance of closed loops in wild-type preparations (Fig. 3).

Buoyant density determinations of purified mitochondrial and whole-cell DNA samples revealed peak density values of 1.700 for nuclear DNA of both strains, 1.684 for wild-type mitochondrial DNA, and 1.681 for petite mitochondrial DNA.

Discussion.—These data confirm the occurrence in yeast mitochondria of DNA filaments of both circular and linear conformations in diploids as well as the haploids described earlier.[12] The two sets of data agreed in showing that cyclic filaments occurred in substantially higher frequencies in the wild-type than in the vegetative petite strains. In wild-type strains, cyclic filaments were of various lengths with a predominant mode at 4.5–5.5 μ, whereas petites contained few circular molecules and these principally measured 1.5–2.5 μ in contour length. In lysed-mitochondrial preparations of wild type, up to 50 per cent of the DNA filaments lying close to, but not attached to, membranes were circular, whereas petites yielded a maximum of 8 per cent circles in similar preparations.

A large part of the problem in resolving the conflicting reports on yeast mitochondrial DNA[8, 10–12] resides in the fragility of these molecules in yeast as compared with mitochondrial DNA in animal species.[7, 9, 24] In the present study we found that circle frequency was reduced from 50 to 8 per cent when iso-N mitochondria were diluted for monolayering in 7 *M* rather than 5 *M* ammonium acetate, and that petite mitochondrial DNA circle frequency could be increased to 8 per cent from 6 per cent if organelles were suspended in 3 *M* rather than 4–6 *M* ammonium acetate. Since the salt concentration only influences physical rupture of the mitochondria during monolayering, it seems unlikely that nuclease or ligase variations[11] induced the observed differences in circle frequency. Breakage of DNA filaments also must have occurred during purification of mitochondrial DNA since the organelles were lysed by detergent action[20] at the start of the procedure and then were shaken in phenol or chloroform to effect deproteiniza-

tion. Since mitochondrial DNA may be membrane-bound,[9, 24, 25] organelle lysis may lead to breaks in the molecules at the point of attachment to the membrane. Also, if DNA replication occurs at the membrane,[26] the replication point might be a weaker site at which breakage could occur preferentially. The similarity of length distribution and modal peaks for linear and circular molecules in iso-N provide some support for this idea of preferential breakage. Also, since DNA extruded from osmotically lysed mitochondria represents a temporal state close to *in vivo*, it is significant that up to 50 per cent of these filaments were circular, whereas only 15 per cent circles were observed as a maximum in purified iso-N mitochondrial DNA.

Although mitochondrial lysis was effected by detergent action for all preparations, there were many fragments of length 1–2 μ in phenol-extracted DNA and not in chloroform-extracted samples. More significantly, there was a substantial reduction in frequency of circular filaments longer than 2.5 μ. Phenol extraction has been reported[27] to remove selectively the A + T-rich satellite DNA while not affecting the G + C satellite. Fragmentation also may occur during one or more of the preparative steps required to process yeast mitochondrial DNA during CsCl density-gradient centrifugation; Sinclair *et al.*[8] and Shapiro *et al.*[11] found few linear filaments longer than 5 μ. Both groups also reported that few circular filaments occurred in the phenol-extracted mitochondrial DNA recovered from CsCl gradients.

We did not observe nuclear DNA contamination in the mitochondrial DNA samples subjected to analytical sedimentation in CsCl; however, minor levels of contamination may have been obscured in our samples. Therefore, a more sensitive assessment of contamination was made by direct observations or specimen grids which contained unlysed mitochondria suspended in 2 *M* ammonium acetate for monolayering. Such grids never had DNA filaments, whereas mitochondria from the same isolations which were suspended in lysing concentrations of the salt always showed DNA filaments among the remnants of mitochondrial membranes. If present, contaminating nuclear DNA filaments or nucleoprotein fibers[12] should have been observed among the many grids scanned for both strains. Although this serves as a monitor for contamination only for whole mitochondrial preparations, the comparable length distributions for purified and lysed-mitochondrial DNA of iso-N indicate that contamination probably was lacking in the purified DNA samples, too. Indeed, Borst *et al.*[28] demonstrated that deoxyribonuclease treatment effectively removed nuclear contamination from mitochondrial suspensions without affecting the DNA within the intact organelle.

Although we earlier estimated that at least 10 μ of DNA filament might occur in a single haploid yeast mitochondrion containing 3×10^{-17} gm of DNA,[12] more direct estimates now can be made of the minimum DNA content per wild-type mitochondrion because iso-N organelle lysis can be controlled so that extruded DNA filaments remain attached or in the vicinity of the mitochondrial membranes. From 14 such mitochondrial figures, a single ruptured organelle contained a total filament length of 40–56 μ. Such photographs do not permit us to determine whether this total represents one, or more than one, molecule per mitochondrion. But, since osmotically lysed circular mitochondrial DNA oc-

curred in modal length groups of 2, 5, and 10 μ, it seems unlikely that one large DNA filament fragmented at preferential sites in every case. To accept such an interpretation we would have to invoke the coincident events of preferential fragmentation followed by hydrogen-bonding of cohesive ends.[11] Since the DNA released during mitochondrial lysis is adsorbed to the protein film almost immediately with little chance for gross molecular alterations,[16] it seems most unlikely that circular mitochondrial DNA is the consequence of "sticky ends"[11] in any substantial portion of the population. Instead, the existence of more than one DNA molecule per mitochondrion is suggested by the occurrence of more than one mode for lengths of both circular and linear filaments, as well as the direct observations of six to eight loops extruded from a single mitochondrion. The occurrence of more than one DNA molecule per mitochondrion has been proposed for yeast on the basis of genetic studies,[13] and for protozoa and mammals on the basis of molecular studies.[9, 29]

The fragility of the membranes and DNA filaments of petite mitochondria rendered it difficult to compare those DNA molecules with the wild type. Fragmentation may have been responsible for up to 94 per cent linear filaments, as well as the virtual absence of circles measuring more than 3 μ. A reasonable interpretation would be that the smallest circles are the same for the two strains, but that either few larger circles occur in petites or these molecules differ physically or chemically from wild-type circular DNA. It is possible that alterations, perhaps of a deletion nature, have produced molecules richer in A + T and therefore more susceptible to scissions and molecule fragmentation. This interpretation is supported by the buoyant density determinations which showed a higher A + T content for petite than for wild-type mitochondrial DNA. Such a situation also could explain the rarity of petite DNA filaments measuring more than 8 μ, especially when mitochondria were suspended in ammonium acetate of higher ionic strengths, and for the lack of a comparable length distribution pattern in the two isogenic strains.

As we reported for haploid yeast,[12] there seems to be a correspondence between distinctive organelle phenotypes and organelle DNA in diploids, too. The complexity of the length distributions and the sensitivity to external influences prevent firm statements at present concerning the differences between petite and wild-type mitochondrial DNA. At the least, there are differences in frequency of circular filaments and in the fragility of these molecules in the two strains. Although it is still unknown whether differences in mitochondrial DNA lead to specific phenotype differences, it is significant that distinctive mitochondrial DNA populations occur in wild-type and petite mitochondria of both haploids and diploids. Although this correlation may be fortuitous, there may be a more direct relationship between the particular features of mitochondrial DNA filaments and the particular phenotypic traits of the organelles in which these are contained.

Summary.—Purified and osmotically lysed mitochondrial DNA occurred in both circular and linear conformations in wild-type and vegetative petite diploid strains. Frequencies of circular filaments varied in the wild type in that purified DNA yielded a maximum of 15 per cent circles, whereas lysed mitochondria contained up to 50 per cent circular filaments. A maximum of 8 per cent circles

was found for petite lysed-mitochondrial DNA. Multiple-length distributions characterized wild-type DNA samples, whereas petite molecules exhibited a unimodal length distribution, centering at the shortest lengths. A total of 40–56 μ of DNA per mitochondrion was measured from single lysed organelles of wild type, presumably representing from six to eight individual molecules. Fragmentation as a function of mechanical shear was considered a likely explanation for the variations observed in samples prepared under diverse conditions for purification and for monolayering of DNA.

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[1] Barnett, W. E., and D. H. Brown, these PROCEEDINGS, **57**, 452 (1967); Barnett, W. E., J. L. Epler, and D. H. Brown, these PROCEEDINGS, **57**, 1775 (1967); Rifkin, M. R., D. D. Wood, and D. J. L. Luck, these PROCEEDINGS, **58**, 1025 (1967).

[2] Reich, E., and D. J. L. Luck, these PROCEEDINGS, **55**, 1600 (1966).

[3] Wintersberger, E., *Biochem. Biophys. Res. Commun.*, **25**, 1 (1966).

[4] Suyama, Y., and J. Eyer, *J. Biol. Chem.*, **243**, 320 (1968).

[5] Stone, G. E., and O. L. Miller, Jr., *J. Exptl. Zool.*, **159**, 33 (1965).

[6] Rabinowitz, M., J. Sinclair, L. DeSalle, R. Haselkorn, and H. H. Swift, these PROCEEDINGS, **53**, 1126 (1965).

[7] Pikó, L., A. Tyler, and J. Vinograd, *Biol. Bull.*, **132**, 68 (1967).

[8] Sinclair, J. H., B. J. Stevens, P. Sanghavi, and M. Rabinowitz, *Science*, **156**, 1234 (1967).

[9] Suyama, Y., and K. Miura, these PROCEEDINGS, **60**, 235 (1968).

[10] Guérineau, M., C. Grandchamp, Y. Yotsuyanagi, and P. P. Slonimski, *Compt. Rend.*, **266**, 1884 (1968).

[11] Shapiro, L., L. I. Grossman, J. Marmur, and A. Kleinschmidt, *J. Mol. Biol.*, **33**, 907 (1968).

[12] Avers, C. J., these PROCEEDINGS, **58**, 620 (1967).

[13] Linnane, A. W., G. W. Saunders, E. B. Gingold, and H. B. Lukins, these PROCEEDINGS, **59**, 903 (1968); Thomas, D. Y., and D. Wilkie, *Biochem. Biophys. Res. Commun.*, **30**, 368 (1968).

[14] Sherman, F., and P. P. Slonimski, *Biochim. Biophys. Acta*, **90**, 1 (1964).

[15] Mounolou, J. C., H. Jakob, and P. P. Slonimski, *Biochem. Biophys. Res. Commun.*, **24**, 218 (1966).

[16] Kleinschmidt, A. K., in *Molecular Genetics*, ed. J. H. Taylor (New York: Academic Press, 1967), vol. 2, p. 47.

[17] Avers, C. J., *J. Bacteriol.*, **94**, 1225 (1967).

[18] Avers, C. J., M. W. Rancourt, and F. H. Lin, these PROCEEDINGS, **54**, 527 (1965).

[19] Avers, C. J., F. H. Lin, and C. R. Pfeffer, *J. Histochem. Cytochem.*, **13**, 344 (1965).

[20] Marmur, J., *J. Mol. Biol.*, **3**, 208 (1961).

[21] Meselson, M., F. W. Stahl, and J. Vinograd, these PROCEEDINGS, **43**, 681 (1957).

[22] Rush, M. G., A. K. Kleinschmidt, W. Hellman, and R. C. Warner, these PROCEEDINGS, **58**, 1676 (1967).

[23] Bode, V. C., and L. A. MacHattie, *J. Mol. Biol.*, **32**, 673 (1968).

[24] Nass, M. M. K., in *Organizational Biosynthesis*, ed. H. J. Vogel, J. O. Lampen, and V. Bryson (New York: Academic Press, 1967), p. 503.

[25] Yotsuyanagi, Y., and C. Guerrier, *Compt. Rend.*, **260**, 2344 (1965).

[26] Chai, N. C., and K. G. Lark, *J. Bacteriol.*, **94**, 415 (1967); Ryter, A., *Bacteriol. Rev.*, **32**, 39 (1968).

[27] Skinner, D. M., and L. L. Triplett, *Biochem. Biophys. Res. Commun.*, **28**, 892 (1967).

[28] Borst, P., G. J. C. M. Ruttenberg, and A. M. Kroon, *Biochim. Biophys. Acta*, **149**, 140 (1967).

[29] Nass, M. M. K., these PROCEEDINGS, **56**, 1215 (1966); Clayton, D. A., and J. Vinograd, *Nature*, **216**, 652 (1967); Parsons, J. A., and R. C. Rustad, *J. Cell Biol.*, **37**, 683 (1968).