MITOCHONDRIAL DNA FROM SHEEP HEART

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It has been established unequivocally in recent years that mitochondria contain DNA.[1] A genetic function for this DNA is indirectly suggested by two lines of evidence. Firstly, in yeast and molds, cytoplasmic mutations exist that influence structure and function of the mitochondria. The cytoplasmic determinant can be transmitted by purified mitochondria and, since the mitochondrial DNA of some of these mutants was found[2, 3] to differ markedly in buoyant density from the wild-type mitochondrial DNA, it was suggested[2–4] that the cytoplasmic determinant and mitochondrial DNA are identical. Secondly, experiments with actinomycin and acriflavin indicate that RNA and protein synthesis in isolated mitochondria is dependent on intramitochondrial DNA. This suggests that mitochondrial DNA contains genetic messages that can be transcribed and translated by the mitochondrial protein-synthesizing system (for review see ref. 5).

Surveys of DNA base composition have shown that the base composition of the DNA of related organisms is usually very similar.[6] If mitochondrial DNA has genetic significance, one would expect it to fit this pattern. It has indeed been demonstrated that the buoyant density in CsCl of mitochondrial DNA from different plants is the same (1.706 gm/cm^3),[7] and that there is only a small variation in the case of birds (1.707–1.711 gm/cm^3)[5, 8, 9] and mammals (1.701–1.704 gm/cm^3).[5, 7–15]

A striking exception to this rule, however, was reported independently by Kalf and Grece[16] and Corneo *et al.*[13] They found that mitochondrial DNA from sheep heart[13, 16] and brain[13] had a buoyant density in CsCl of 1.714, which corresponded to the density of a minor satellite component present in the DNA from homogenates of sheep tissues, the major component of which banded at 1.704 gm/cm.[3] The large difference in density of mitochondrial DNA of sheep (1.714) and other mammals (1.701–1.704) was unexpected, and the identical density of mitochondrial DNA and satellite DNA in sheep was also rather surprising since in ox tissues a similar satellite (which also bands at 1.714 gm/cm^3) is a nuclear component while mitochondrial DNA bands at 1.703 gm/cm^3 (refs. 5, 9, 12, 13).

These marked differences between the mitochondrial DNA of two closely related animals cast some doubt on its functional significance. We have therefore reinvestigated the buoyant density of mitochondrial DNA from sheep heart using the criteria established earlier[5, 17–19] to identify the DNA in mitochondrial preparations as mitochondrial DNA.

Materials and Methods.—Mitochondria: Fresh sheep hearts were obtained from the Amsterdam slaughterhouse. Mitochondria were prepared in 0.25 *M* sucrose, 0.01 *M* sodium EDTA, pH 7.5, as described for ox heart by Crane *et al.*[20]

Nuclei: Nuclei were prepared from a small piece of sheep liver by the procedure of Widnell and Tata,[21] slightly modified[19] to exclude completely contamination by mitochondria.

Isolation of DNA: DNA was isolated by a modification[19] of the method of Marmur.[22] The alcohol precipitations were replaced by dialysis against 0.15 *M* NaCl, 0.015 *M* sodium citrate,

pH 7.0, and the isopropanol precipitation by chromatography on a methylated albumin-on-kieselguhr column.[23]

Gradient centrifugation: Equilibrium density gradient centrifugation in CsCl (E. Merck, AG, Germany, quality 2038) was performed in a Beckman-Spinco model E ultracentrifuge according to the procedure of Schildkraut *et al.*[24] with the minor modifications described elsewhere.[9] The UV-absorption photographs were traced with a Beckman-Spinco RB Analytrol densitometer. Densities were calculated in relation to DNA from *Micrococcus lysodeikticus* (ρ = 1.731). Linear gradients of 20–5% sucrose in 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.0, were made in three centrifuge tubes simultaneously from the same stock solutions with the aid of a simple mixing device. The DNA preparations (0.9 ml) were layered on top of the gradients. Centrifugation was for 16 hr at 0° and 22,000 rpm in the SW25 rotor of a Spinco model L preparative ultracentrifuge. One-ml fractions were collected after puncturing the bottom of the tube.

Denaturation and renaturation: Thermal denaturation and renaturation was followed by heating DNA solutions in a thermostated cuvette and measuring the change in absorbancy at 260 mμ in a Zeiss PMQ II spectrophotometer.[25] The temperature was measured by means of a calibrated thermistor directly immersed in the DNA solution through a Teflon stopper. The solution contained 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.0.

Electron microscopy: Specimens were prepared[17] with a slightly modified Kleinschmidt technique.[26] A Petri dish (19 cm diameter), covered with a new sheet of parafilm with each preparation, was used instead of a Langmuir trough, while the spreading was initiated from a sheet of freshly cleaved mica (4 × 1 cm) wetted in advance with the hypophase instead of from a paraffinized bar. The magnification of each film was calibrated with a replica of a grating having 2160 lines per mm.

Analytical methods: DNA and protein were determined as described previously.[27]

Results.—Properties of the sheep-heart mitochondria: The mitochondrial preparations used contained 0.8–1.1 μg DNA/mg mitochondrial protein. The amount of true mitochondrial DNA in mammalian mitochondria was estimated earlier to be about 0.5 μg/mg protein.[5] Therefore, it seemed likely that approximately 50 per cent of the DNA in our mitochondrial preparations was derived from contaminating nuclei or nuclear fragments. Attempts to remove the contaminating DNA by preincubation of the mitochondria with DNase, a procedure used routinely by us with mitochondria from other animals, were unsuccessful because no DNA at all was recovered from preincubated preparations. Apparently, these sheep-heart mitochondria were damaged during isolation and consequently they had become permeable to DNase. The poor integrity of these mitochondria was also evident from the virtual absence of oxidative phosphorylation and the high adenosinetriphosphatase activity.

Physicochemical characterization of the DNA, isolated from sheep-heart mitochondria and sheep-liver nuclei: In Figure 1, densitometer tracings are presented of UV-absorption photographs of DNA from sheep-heart homogenate and sheep-liver nuclei, and of sheep-heart mitochondrial DNA at different stages of purification, centrifuged to equilibrium in a CsCl gradient. There is no difference in the CsCl patterns of whole-tissue homogenate DNA or nuclear DNA. Since the isolation procedure used yields nuclei with less than 5 per cent mitochondrial contamination on a DNA basis, the 1.714 DNA must be present in the nucleus. The pattern obtained with the homogenate is also similar to that of the crude mitochondrial fraction, with definitely no enrichment of satellite DNA. This might mean either that mitochondrial DNA bands at the same density as the bulk of nuclear DNA and that the satellite band present in this DNA represents nuclear contamination, or that the true mitochondrial DNA has indeed a density corresponding to the satellite DNA, but that nearly all of it has been specifically lost during extraction and purification.

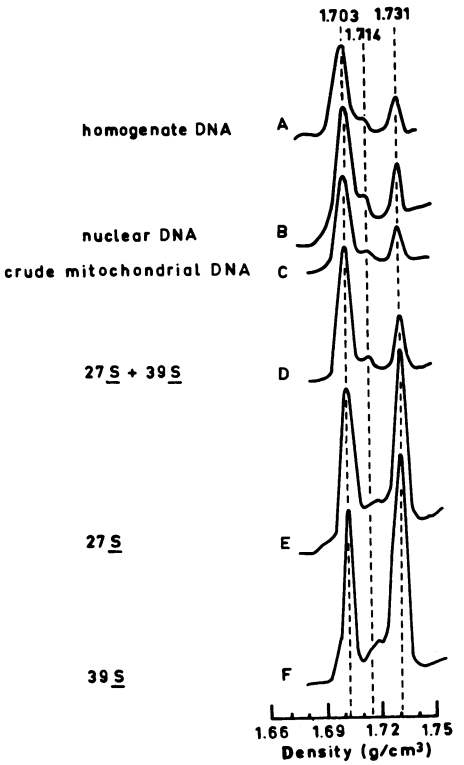

FIG. 1.—Densitometer tracings of UV-absorption photographs of sheep DNA, centrifuged to equilibrium in CsCl gradients. The tracings are matched at the *Micrococcus lysodeikticus* marker at 1.731 gm/cm³. The crude mitochondrial, nuclear, and homogenate DNA were prepared as described under *Methods;* 27*S*, + 39*S*, 27*S*, and 39*S* refer to fractions of sheep-heart mitochondrial DNA fractionated on a sucrose gradient as described in the text.

To exclude directly the latter possibility, the crude mitochondrial DNA was fractionated on a sucrose gradient to see whether fractions could be obtained with the characteristics of mitochondrial DNA but free of satellite material.

Mitochondrial DNA from chick, duck, and rat liver contains two components with sedimentation coefficients of 39*S* and 27*S*, respectively.[5, 18, 19] As illustrated in Figure 2, these represent the intact hypertwisted circular mitochondrial DNA and the open circular form derived from this by scission of one or more covalent bonds. Since more than 85 per cent of chick-liver mitochondrial DNA can be obtained in the 39*S* form,[19] it is likely that the variable amounts of 27*S* material are due to damage during purification. Figure 3 presents a comparison of sedimentation profiles of mitochondrial DNA from sheep heart and chick liver in two tubes of the same sucrose gradient. The sheep DNA gives a small peak of material which coincides exactly with the 39*S* peak of chick DNA. The bulk of the sheep DNA sediments in a broad band, the front of which coincides with the 27*S* peak of the chick DNA.

The DNA in the first half of the main peak (27*S* region) and in the 39*S* peak was either combined or separately concentrated on a methylated albumin-on-kieselguhr column[23] and subsequently analyzed in a CsCl equilibrium density gradient (Fig. 1, *D–F*). The satellite DNA (ρ = 1.714) is not enriched in either of these fractions, whereas most if not all of this DNA satisfies the sedimentation criteria of mitochondrial DNA. In fact, satellite DNA is not detectable in the 39*S* region of the gradient (Fig. 1, *F*). If the density of the mitochondrial DNA were 1.714 gm/cm³, comparatively little mitochondrial DNA would be found in the combined 27*S* + 39*S* and nothing in the 39*S* DNA, according to the results of Figure 1. This was investigated using the criteria of renaturation and circularity.

It has been shown in this laboratory[5,18] that mitochondrial DNA is very homogeneous in base sequence and that it consequently renatures rapidly under conditions where *unfractionated* nuclear and even bacterial DNA do not renature at all. (Unfractionated should be stressed in this context as we have done previously,[18] because the nuclear satellite components of ox and mouse tissues do renature under appropriate conditions.[14, 29]) Figure 4 shows a thermal de- and renaturation profile

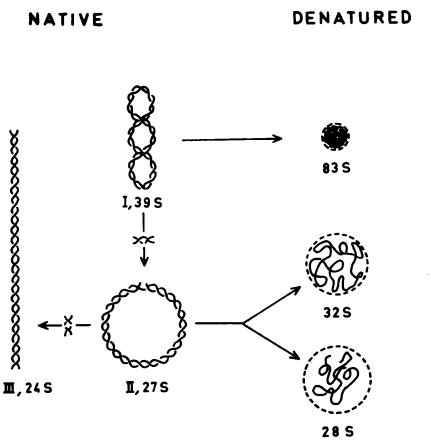

FIG. 2.—Diagrammatic representation of the various forms of mitochondrial DNA, modified from a paper by Vinograd *et al.*[28] on polyoma viral DNA. The dashed circles around the denatured forms indicate the relative hydrodynamic diameters. The identification of the various forms of mitochondrial DNA is based on previously published experiments[17] and more recent band sedimentation experiments with DNase-treated native DNA and DNA heat-denatured in the presence of formaldehyde and analyzed on 3 *M* CsCl bulk solution.[19]

of the DNA from the combined 27*S* and 39*S* regions of the sucrose gradient. About 65 per cent of this material rapidly renatures, whereas whole-heart DNA does not. Moreover, the mitochondrial DNA melted again sharply with the same melting temperature in a second denaturation cycle. The melting temperature (87°) is in good agreement with a guanine + cytosine content of 44 per cent calculated from the buoyant density in CsCl,[30] assuming that no unusual bases are present. From the total DNA of the mitochondrial preparations, about 40 per cent, i.e., 0.2–0.25 μg/mg protein was rapidly renaturable. This shows that about 40 per cent of the DNA isolated from sheep-heart mitochondrial preparations has both the sedimentation characteristics and the renaturation behavior of mitochondrial DNA from other mammals,[5, 13, 18] although in these preparations less than 5 per cent of the DNA had the equilibrium density of satellite DNA in CsCl gradients. Therefore, the bulk of the mitochondrial DNA of sheep heart must have an equilibrium density of about 1.703, like mitochondrial DNA from beef heart.[5, 12, 13]

Morphological characterization of the DNA, isolated from sheep-heart mitochondria: The conclusion drawn in the previous section was confirmed and extended by electron microscopy. We have shown earlier that mitochondrial DNA from chick, mouse, and ox consists of circular molecules with a narrow length distribution and a mean contour length of 5.4 μ.[5, 17]

Circular molecules of the same contour length were observed in DNA from sheep-heart mitochondria and the length distribution of open circles was the same as that obtained with chick-liver mitochondrial DNA.[5] A semiquantitative estimate of the relative amount of linear molecules, open circles, and twisted circles in different preparations gave the following results:

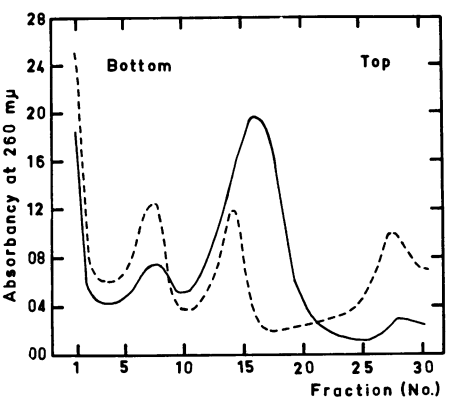

FIG. 3.—Fractionation of crude mitochondrial DNA from sheep heart and mitochondrial DNA from chick liver on a sucrose gradient. The fractionation procedure is described under *Methods;* ——, sheep DNA; - - - - -, chick DNA.

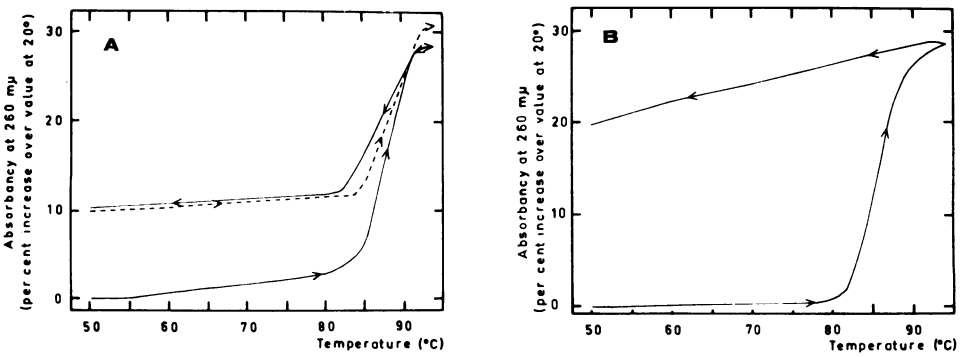

FIG. 4.—The thermal denaturation and renaturation of DNA from sheep-heart mitochondria (*A*) and sheep-heart whole-tissue homogenate (*B*). The change in absorbancy as a function of temperature was measured as described under *Methods*. Arrows to the right represent change in absorbancy during heating; arrows to the left, change in absorbancy during cooling. After cooling the mitochondrial DNA to 50° it was heated for a second time, as indicated by the dotted line.

In the crude sheep-heart mitochondrial DNA (cf. Fig. 1, *C*) about 20 per cent of the molecules were circular and most of these circles were open circles. The purified 27*S* DNA contained predominantly open, circular molecules, while in purified 39*S* DNA 70 per cent of all molecules were twisted circles. The length of the linear molecules was in most cases about 5.4 μ or shorter and these can be interpreted, therefore, either as fragments of mitochondrial DNA or as contaminating nuclear DNA. Figure 5 is an over-all picture of the 39*S* DNA, with examples of open, half-open, and twisted circles and of linear molecules.

Discussion and Conclusions.—The results presented in this paper show that over 95 per cent of the DNA extracted from sheep-heart mitochondrial preparations has an equilibrium density in CsCl close to that of the main component of nuclear DNA. Since 40 per cent of this DNA displays the sedimentation characteristics and the renaturation behavior of mitochondrial DNA from the other mammals and birds studied, it is clear that the bulk of sheep-heart mitochondrial DNA bands at about 1.703 gm/cm^3 in CsCl and not at 1.714 gm/cm^3. It seems likely that the 1.714 satellite DNA is not a mitochondrial component at all for the following reasons:

(*a*) The 1.714 DNA is not enriched in DNA extracted from mitochondria and a specific loss of a large fraction of mitochondrial DNA during extraction and purification is unlikely, since the amount of DNA with the characteristics of mitochondrial DNA recovered is about 0.20–0.25 μg/mg protein, which is in the same order of magnitude as the amount maximally recovered by the same procedures from chick-, duck-, guinea-pig-, or rat-liver mitochondria (0.25–0.30 μg/mg protein).[5, 19]

(*b*) The presence of appreciable amounts of 1.714 DNA of the characteristic size of mammalian mitochondrial DNA is excluded by the observation that the 1.714 component is not detectable in purified 39*S* DNA.

(*c*) The relative concentration of satellite DNA is the same in DNA from sheep-heart or liver whole-tissue homogenates and from purified sheep-liver nuclei.

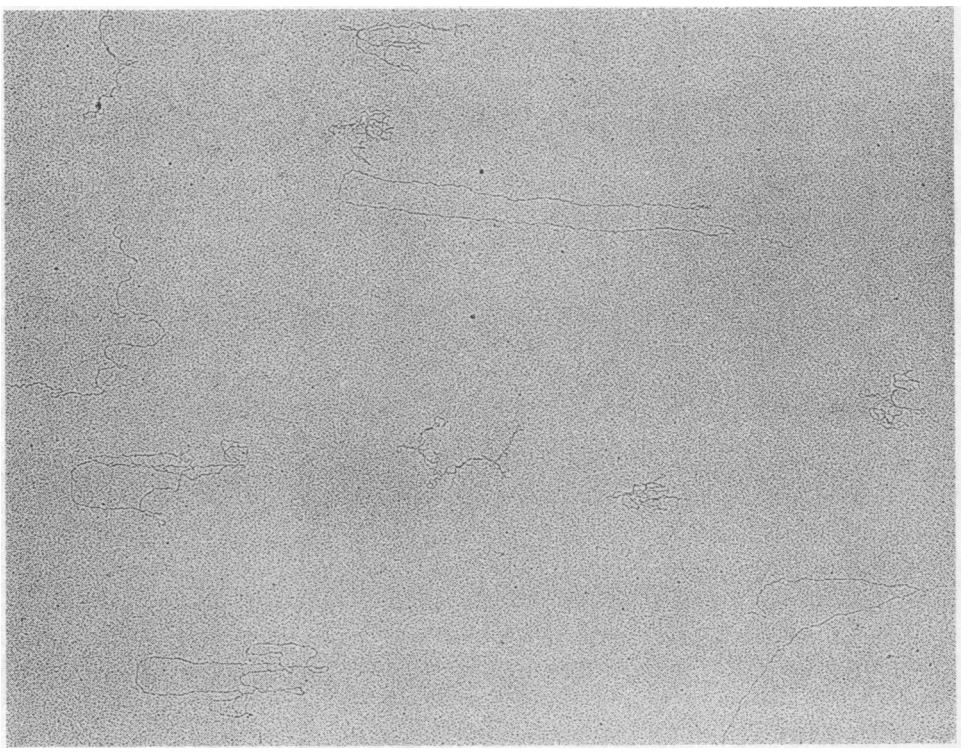

FIG. 5.—Electron micrograph of purified 39S DNA from sheep-heart mitochondria, prepared as described under *Methods*. Magnification: ×20,240.

This shows that most, if not all, satellite DNA is present in the nucleus, as in the other mammals investigated.[5]

(*d*) For ox and mouse tissues it was shown that satellite DNA is completely absent in DNA extracted from mitochondrial preparations, preincubated with DNase.[5, 18] In the absence of DNase pretreatment small amounts of satellite DNA could be detected in DNA from all ox-heart mitochondrial preparations, and we have suggested earlier[11] that this was derived from nuclear fragments which could not be completely eliminated from the mitochondrial preparation by the standard isolation procedure for beef-heart mitochondria. A similar contamination problem should be expected with mitochondrial preparations from sheep heart.

These arguments strongly suggest that the small amount of 1.714 DNA that we have found in crude DNA from sheep-heart mitochondrial preparations is derived from nuclear material contaminating the mitochondria. Since essentially identical results were obtained with five different preparations, we conclude that the intact mitochondrial DNA *sensu stricto* of sheep is a circular molecule with a contour length of about 5.4 μ, a melting temperature of 87° in 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.0, and an equilibrium density of 1.703 gm/cm^3 in CsCl, properties it shares with mitochondrial DNA from other mammals studied until now.

It is difficult to assess why other workers have reached the completely different conclusion that sheep mitochondrial DNA has an equilibrium density of 1.714 gm/cm^3 in CsCl. The details of the experiments of Corneo *et al.*[13] have not yet

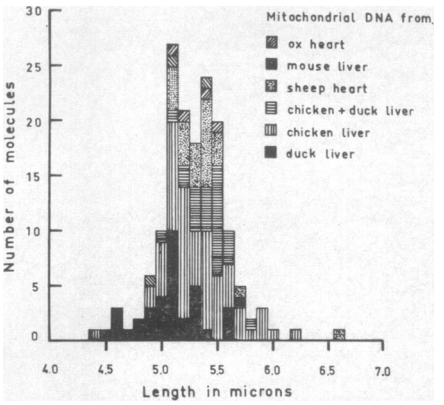

FIG. 6.—Length distribution of circular mitochondrial DNA from various animals. Chick + duck refers to a preparation containing equal amounts of mitochondrial DNA from chick and duck liver.

been presented. We have recalculated, however, the equilibrium density of sheep-heart mitochondrial DNA reported by Kalf and Grece[16] from their published densitometer tracings, and we arrive at a value closer to 1.703 gm/cm^3 than to 1.714 gm/cm^3, unless we assume that very different experimental conditions were chosen for different CsCl gradients. Kalf and Grece also demonstrated that the DNA of partially purified sheep-heart nuclei contained about 5 per cent satellite DNA and they suggested that this DNA was derived from mitochondria contaminating the nuclear preparation. However, this cannot be the case, since mitochondrial DNA in mammals comprises at most about 1 per cent of total cell DNA,[9, 19] while about 5 per cent of total cell DNA is present as satellite DNA.

In earlier papers[5, 17] we have shown that the contour length of mitochondrial DNA from chick liver, mouse liver, and ox heart was very similar. These limited data can now be supplemented by more extensive values for sheep heart and duck liver, summarized with the older values in Figure 6. It is clear from this histogram that within the limits of accuracy the mitochondrial DNA of the five animals studied is identical in size. Recently, Sinclair and Stevens[14] also reported the presence of circular molecules in mitochondrial DNA from mouse liver, and the average contour length of their circles was 5.0 μ. In view of the inherent uncertainty in these contour measurements and the problems involved in accurate calibration, we do not consider this significantly different from our values.

Although the similarity in size of mitochondrial DNA from two birds and three mammals invites generalization (cf. ref. 5), the range of animals studied is very limited. We have recently found[19] circular DNA in crude preparations of frog-liver mitochondria and frog eggs which appears to be substantially larger than 5.4 μ. Whether this DNA is indeed mitochondrial DNA is under investigation.

The most intriguing aspect of mitochondrial DNA, namely, whether it contains genetic messages indispensable for the biosynthesis of mitochondria, remains to be solved. Experiments with hybridization techniques are in progress to tackle this problem.

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[1] Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; EDTA, ethylenediaminetetraacetate; DNase, pancreatic deoxyribonuclease; *S*, Svedberg unit of sedimentation.

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

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

[4] Luck, D. J. L., in *Probleme der biologischen Reduplikation*, ed. P. Sitte (Berlin: Springer Verlag, 1966), p. 314.

[5] Borst, P., A. M. Kroon, and G. J. C. M. Ruttenberg, in *Symposium on the Structure and Function of Genetic Elements*, Warsaw, 1966, in press.

[6] McCarthy, B. J., *Progr. Nucleic Acid Res.*, **4**, 129 (1965).

[7] Suyama, J., and W. D. Bonner, Jr., *Plant Physiol.*, **41**, 383 (1966).

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

[9] Borst, P., and G. J. C. M. Ruttenberg, in *Regulation of Metabolic Processes in Mitochondria*, ed. J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater (Amsterdam: Elsevier, 1966), BBA Library, vol. 7, p. 454.

[10] Schneider, W. C., and E. L. Kuff, these PROCEEDINGS, **54**, 1650 (1965).

[11] Kroon, A. M., M. D. thesis: *Protein Synthesis in Mitochondria*, Amsterdam, Drukkerij Noordholland, Hoorn, 1966.

[12] Kroon, A. M., in *Regulation of Metabolic Processes in Mitochondria*, ed. J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater (Amsterdam: Elsevier, 1966), BBA Library, vol. 7, p. 397.

[13] Corneo, G., C. Moore, D. R. Sanadi, L. I. Grossmann, and J. Marmur, *Science*, **151**, 687 (1966).

[14] Sinclair, J. H., and B. J. Stevens, these PROCEEDINGS, **56**, 508 (1966).

[15] du Buy, H. G., C. F. T. Mattern, and F. L. Riley, *Biochim. Biophys. Acta*, **123**, 289 (1966).

[16] Kalf, G. F., and A. Grece, *J. Biol. Chem.*, **241**, 1019 (1966).

[17] van Bruggen, E. F. J., P. Borst, G. J. C. M. Ruttenberg, M. Gruber, and A. M. Kroon, *Biochim. Biophys. Acta*, **119**, 437 (1966).

[18] Borst, P., and G. J. C. M. Ruttenberg, *Biochim. Biophys. Acta*, **114**, 645 (1966).

[19] Borst, P., G. J. C. M. Ruttenberg, E. F. J. van Bruggen, and A. M. Kroon, in preparation.

[20] Crane, F. L., J. L. Glenn, and D. E. Green, *Biochim. Biophys. Acta*, **22**, 475 (1956).

[21] Widnell, C. C., and J. R. Tata, *Biochem. J.*, **92**, 313 (1964).

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

[23] Sueoka, N., and T. Y. Cheng, *J. Mol. Biol.*, **4**, 161 (1962).

[24] Schildkraut, C. L., J. Marmur, and P. Doty, *J. Mol. Biol.*, **4**, 430 (1962).

[25] Marmur, J., and P. Doty, *Nature*, **183**, 1427 (1959).

[26] Kleinschmidt, A. K., D. Lang, D. Jacherts, and R. K. Zahn, *Biochim. Biophys. Acta*, **61**, 857 (1962).

[27] Kroon, A. M., *Biochim. Biophys. Acta*, **108**, 275 (1965).

[28] Vinograd, J., J. Lebowitz, R. Radloff, R. Watson, and P. Laipis, these PROCEEDINGS, **53**, 1104 (1965).

[29] Polli, E., E. Ginelli, P. Bianchi, and G. Corneo, *J. Mol. Biol.*, **17**, 305 (1966).

[30] Marmur, J., and P. Doty, *J. Mol. Biol.*, **5**, 109 (1962).