

MITOCHONDRIAL DNA FROM PROTOZOA¹

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IT is known from the extensive genetic studies made in yeast (EPHRUSSI 1952) and *Neurospora* (WAGNER and MITCHELL 1955) that certain defects associated with mitochondrial proteins are inherited under the control of a cytoplasmic element. However, the chemical nature and the specific location of such an element in the cytoplasm have not yet been elucidated.

In recent years, evidence has been accumulating that the mitochondrion contains deoxyribonucleic acid (DNA) and that this DNA might be capable of functioning in the synthesis of some proteins in the mitochondrion (LUCK and REICH 1964; and see NASS, NASS, and AFZELIUS 1965). Thus, it is likely that the DNA found in the mitochondrion (mitochondrial DNA) is indeed the vector of the cytoplasmic element, mutations of which may result in alteration of some mitochondrial proteins. Attractive as this possibility may be, there is no direct experimental evidence to prove this point. It was however felt that detailed quantitative as well as qualitative studies of the mitochondrial DNA might lead to the elucidation of the function that this DNA possibly implements within the mitochondrion. Furthermore, comparative studies of mitochondrial DNA particularly in related organisms are of special interest in light of the previous finding that the mean base compositions of DNA from related organisms are similar and thus the phylogenetic relations are reflected in DNA base composition (SUEOKA 1962; SCHILDKRAUT, MARMUR and DOTY 1962). It was hoped that mitochondrial DNA also exhibits such a relationship.

In this paper are reported the isolation and partial characterization of mitochondrial DNA's from two related protozoa, *Tetrahymena* and *Paramecium*.

MATERIALS AND METHODS

Culture: A strain of *Tetrahymena pyriformis* was grown in 2 liter Erlenmeyer flask containing 1 liter medium [2% proteose peptone (Difco) and 0.1% Basamin-Busch (Anheuser-Busch)] for 4 days at 27°C. A strain (51) of *Paramecium aurelia*, kappa free, was grown in 2.5 gallon jugs containing an infusion of Scottish grass by the methods described previously (PREER and PREER 1959).

Both *Tetrahymena* and *Paramecium* cells were concentrated by a commercial cream separator at 1000 ×g. The concentrated cell suspension was further centrifuged with an International oil testing centrifuge tube as described previously (PREER and PEER 1959).

Preparation of mitochondria: All operations were carried out at melting ice temperature. Packed cells were suspended in four volumes of the medium found suitable for isolating intact

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mitochondria by PREER and PREER (1959). The medium consists of 0.2 M raffinose, 0.25% bovine serum albumin (fraction V, Calbiochem), and 1 mM potassium phosphate buffer at pH 6.2. The cells suspended in this medium were disrupted by forcing through a milk homogenizer. The homogenate was centrifuged for 6 min at $500 \times g$ and the resulting precipitate was discarded. The supernatant was further centrifuged at $5,000 \times g$ for 6 min; the supernatant was decanted and the flocculent material running down the centrifuge tube wall was quickly wiped by cheesecloth. The pellet was resuspended in the same medium, but care was taken so as not to include the bottom layer representing cellular debris. This suspension was centrifuged again at $5,000 \times g$ for 6 min, and the pellet was similarly washed again with the same medium. The final pellet was then resuspended in a 40 ml volume of cold saline-EDTA (0.15 M NaCl, 0.1 M EDTA, pH 8) as described by MARMUR (1961).

For determining the number of mitochondrial particles, the mitochondrial suspension at this stage was properly diluted and mixed with a yeast cell suspension, the cell density of which was previously determined to be 2×10^8 cells/ml by hemocytometer counting. The mixture was then examined under the phase contrast microscope and the ratio of the numbers of mitochondria to yeast cells was determined. The total mitochondrial number was obtained by multiplying this ratio by 2×10^8 and also taking into consideration a proper dilution factor and the total volume of the mitochondrial suspension. Mitochondria in this condition are opaque and round in appearance and approximately 1μ in diameter; therefore, they can be easily identified and counted.

Isolation of DNA: The isolation methods of mitochondrial DNA were essentially the same as those described by LUCK and REICH (1964). To the mitochondrial suspension, 5% sodium deoxycholate solution was added to give a final concentration of 0.5%. A complete lysis of mitochondria was obtained by this treatment alone; the extra treatment such as freezing, thawing and subsequent heating at 60° for 10 min as described by LUCK and REICH (1964) is not necessary. The lysate was centrifuged at $8,000 \times g$ for 10 min to remove any unlysed material.

For isolation of DNA, the supernatant of the deoxycholate lysate was mixed with an equal volume of phenol saturated with saline-EDTA; the mixture was then shaken for a period of 20 to 30 min on a rotary shaker. The phenol layer was separated by centrifugation in an International clinical centrifuge. The top, aqueous layer was pipetted out and poured into dialysis tubing [1 cm flat-width tubing previously soaked and washed in BPES (0.006 M Na_2HPO_4 , 0.002 M NaH_2PO_4 , 0.001 M Na_2EDTA , and 0.179 M NaCl (CROTHERS 1964)]; the tubing was subjected to dialysis against 1 liter BPES with two or three 1 liter changes within a period of 48 hours. After this dialysis, the contents of the tubing were treated with RNase (Worthington Biochem Co.) at a concentration of $5 \mu\text{g/ml}$ for 20 min at room temperature. The phenol treatment and the subsequent dialysis were repeated on this material. The OD_{260} loss due to this RNase treatment is large; the total OD usually becomes less than 0.1 of the starting OD. After concentration of the contents by pervaporation and subsequent dialysis, the ultraviolet (UV) absorption spectrum ranging from 230 to $280 \mu\mu$ was obtained; usually a ratio of $\text{OD}_{260}/\text{OD}_{230} \geq 2.0$ with the absorption peak at a range between 255 and $260 \mu\mu$ was attained.

The Tetrahymena mitochondrial fraction was free of any cellular particles except cilia; the Paramecium mitochondrial fraction was occasionally contaminated with trichocysts, cilia, and bacteria. However, the number of each of these contaminants was estimated to be less than 10^8 , which is unlikely to contribute sufficient amount of DNA to interfere with the present analysis. Furthermore, the contaminating trichocysts and bacteria are all insensitive to deoxycholate, excluding the possibility that such are the sources of the DNA isolated from the mitochondrial fractions.

Whole cell DNA was isolated from both Tetrahymena and Paramecium as follows. Packed cells collected by the methods described previously were resuspended, 1 ml cells in 20 ml cold saline-EDTA, and immediately lysed with addition of sodium deoxycholate to a final concentration of 0.5%. Sometimes, cell lysis was effected by sodium lauryl sulfate at a concentration of 0.5% (this is much more effective than deoxycholate). The lysate was then quickly mixed with an equal volume of phenol and the mixture was centrifuged in order to separate the aqueous layer from the phenol layer. To the aqueous layer, twice the volume of cold ethanol was added and the contents were quickly mixed. The thread-like DNA molecules were then wound on a

glass rod and dissolved in BPES. This solution was treated with RNase (5 $\mu\text{g}/\text{ml}$, 20 min at room temperature), and the same phenol and alcohol treatments were repeated. The final solution was then centrifuged at $8,000 \times g$ for 10 min to remove denatured protein material.

CsCl density gradient centrifugation: The methods were those described by MESELSON, STAHL and VINOGRAD (1957) and SCHILDKRAUT *et al.* (1962). The amount of sample DNA equivalent to 1 to 4.5 μg as calculated on the basis of 50 μg DNA/OD_{260 m μ} with a standard reference DNA (see below) were mixed with 11 molal solution of CsCl containing 0.02 M Tris buffer, pH 8.0. The refractive index of the mixture was adjusted to $N_D=1.4000$ at 25° and the solution was centrifuged at a speed of 44,770 rpm in a Spinco Model E centrifuge for more than 19 hr at the temperature setting of 20°C. The centrifuge cell was equipped with a 1° negative wedge upper window. For the two-cell operation, a 1° side wedge lower window was used in one of the cells. From the mixed image of both cells seen at the front opening of the camera-lens housing cylinder, each cell image was effectively separated by means of a handmade mask. UV absorption photographs were taken separately for each cell.

Determination of the mean density ($\text{g}\cdot\text{cm}^{-3}$) of each DNA band was made against the reference DNA (N^{15}H_2 *B. subtilis* DNA) isolated from *B. subtilis* grown in the Spizizen's minimal medium replacing the respective light components with (N^{15}H_4)₂SO₄ and deuterium oxide. The density ($\rho = 1.740$) of this DNA was previously calibrated against the light *B. subtilis* DNA ($\rho = 1.703$).

Determination of melting point temperature: 0.7 ml DNA solution containing 0.0195 M Na⁺ was placed in a 1 cm path 1 ml quartz cuvette. On top of it, 0.3 ml mineral oil was added to prevent evaporation during heating. The cuvette was heated in the Beckman DU monochromator cell compartment with circulating hot water within attached thermospacers by the methods of MARMUR and DOTY (1962), and the hyperchromicity at OD_{260 m μ} and temperature changes were recorded by a Gilford multiple sample absorbance recorder. Temperature raise was insured before each OD measurement.

RESULTS

Tetrahymena: Microdensitometer tracings of the UV absorption photographs of equilibrated DNA from *Tetrahymena* mitochondria and the whole cell are shown in Figure 1. The tracings are matched at the standard reference DNA peak, the density of 1.740. A single species of DNA of the density ($\rho = 1.682$) was obtained from the mitochondrial fraction, which was essentially free of any other DNA. This clean DNA can be obtained from the mitochondria which were not even treated with DNase before lysis. DNase treatment (10 $\mu\text{g}/\text{ml}$, 3mm MgCl₂, 30 min in ice), however does not affect the DNA within the mitochondria.

DNA fraction isolated from the whole cells of *Tetrahymena*, shows two peaks in CsCl density gradient centrifugation, one corresponding to the density, $\rho = 1.688$ and the other, $\rho = 1.680$. In the tracing shown in Figure 1, however, the lighter peak is seen to be split into two. This split peak is due to an optical artifact, since this effect in the tracing results from a very sharp remarkation line in the original photograph. In addition, this effect was observed only when an excess amount of the material was present. Since no such optical effect has been reported on DNA in CsCl, we investigated the matter further. It turned out that the material was resistant to RNase and DNase and that heating at 100°C and fast cooling did not alter the banding property of this material; thus this material is not nucleic acid, the exact nature of which is currently under investigation. On the other hand, the heavier DNA band ($\rho = 1.688$) was completely eliminated by a DNase treatment. Furthermore, heating (at 100°C for 3 min)

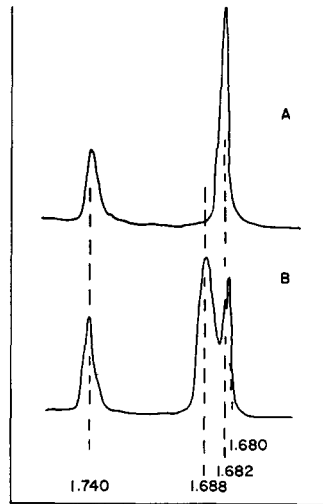


FIGURE 1.—Microdensitometer tracings of (A) *Tetrahymena* mitochondrial DNA ($\rho = 1.682$) and (B) whole-cell DNA ($\rho = 1.688$) in equilibrated CsCl density gradient formed by centrifugation at 44,770 rpm for 20 hr. The band at the far left is the standard reference DNA of density 1.740. The band with density of 1.680 seen in (B) results from a contaminating material of non-DNA nature (see Text and Figure 2); this was previously reported to be the nuclear DNA of *Tetrahymena* (SUYAMA *et al.* 1965).

and fast cooling resulted in a complete denaturation as judged by its density increase ($\Delta\rho = 0.020$). Figure 2 shows the microdensitometer tracings of the ultraviolet absorption photographs made on whole cell DNA treated with either RNase, DNase or heating and fast cooling in equilibrated CsCl density gradient. It is clear that the density ($\rho = 1.682$) of mitochondrial DNA does not correspond to either band obtained from the whole cell DNA fraction.

Paramecium: Microdensitometer tracings of the UV absorption photographs of DNA isolated from *Paramecium* mitochondria and from the whole-cell in the equilibrated CsCl gradient are presented in Figure 3. The density of the mitochondrial DNA was calculated to be 1.702; that of the whole-cell DNA was 1.689. This value for the whole-cell DNA corresponds to that reported by SMITHSONNEBORN, GREEN and MARMUR (1963) and furthermore to that of *Tetrahymena* whole-cell DNA. The densities of the *Paramecium* and *Tetrahymena* of whole-cell DNA's are almost identical, but those of mitochondrial DNA's are markedly different in these two organisms.

Since the *Paramecia* were grown in a semisterile condition and in a medium infected with bacteria, the possibility that the mitochondrial DNA might have originated from these contaminating bacteria should be considered. This possibility is unlikely because of the following reasons: (1) the total bacterial counts were less than 10^8 , (2) the bacteria were resistant to deoxycholate, (3) the DNA isolated from bacteria collected from the culture filtrates was identified and found quite different from the mitochondrial DNA. The density of the major band

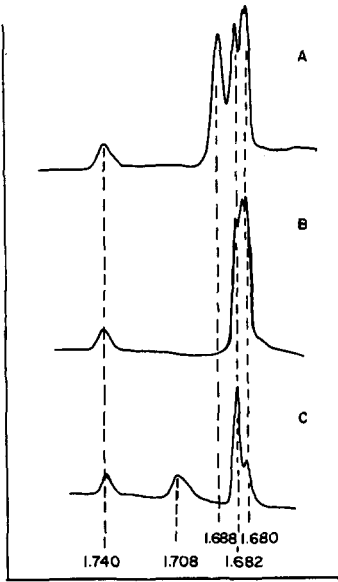


FIGURE 2.—Whole cell *Tetrahymena* DNA was treated with (A) RNase (B) DNase and (C) heating and fast cooling before CsCl density gradient centrifugation with added *Tetrahymena* mitochondrial DNA ($\rho = 1.682$) and $N^{15}H^2$ *B. subtilis* DNA ($\rho = 1.740$) as references. The microdensitometer tracings of the UV absorption photographs of these samples in equilibrated CsCl density gradient were matched at these reference peaks.

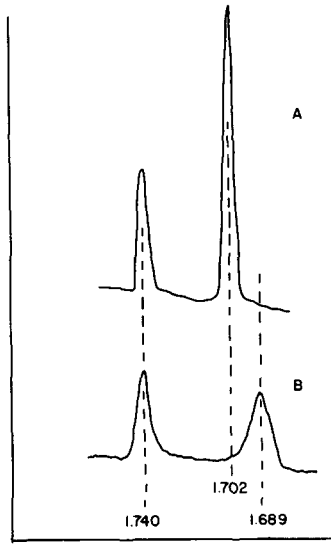


FIGURE 3.—Microdensitometer tracings of the UV absorption photographs of *Paramaecium* (A) mitochondrial DNA ($\rho = 1.702$) and (B) whole-cell DNA ($\rho = 1.689$) in equilibrated CsCl density gradient formed by centrifugation at 44,770 rpm for 19 hrs (A) and 20 hrs (B). $N^{15}H^2$ *B. subtilis* DNA ($\rho = 1.740$) was used as reference.

corresponded to 1.722, in addition three minor bands were identified and their densities were 1.710, 1.698 and 1.695.

Determination of the amount of DNA in a mitochondrion: A total of 9×10^{10} *Tetrahymena* mitochondria was mixed with 14 μg of $N^{15}H^2$ *B. subtilis* DNA before the lysis of the mitochondria. This mixture was then subjected to an entire isolation procedure for mitochondrial DNA as described in MATERIALS AND METHODS. After the purification of DNA, 0.09 $OD_{260\text{ m}\mu}$ unit of the final solution was mixed with CsCl solution and the mixture was centrifuged. For the quantitative estimation of the individual DNA, areas under the peaks were cut from the tracing paper, each of which was then weighed. The ratio of the average weights (three independent pieces cut from tracing papers) for $N^{15}H^2$ *B. subtilis* DNA to the mitochondrial DNA was 1/2.4; the total amount of DNA present in the 9×10^{10} mitochondria was $2.4 \times 14 = 33.6 \mu g$ which corresponded to approximately $3.7 \times 10^{-10} \mu g$ DNA per mitochondrion.

Determination of the amount of the *Paramaecium* mitochondrial DNA was not made at this time. However, the amount of DNA recovered after the entire isola-

TABLE 1

Recovery of DNA from mitochondria of Paramecium and Tetrahymena

		Total number of mitochondria	Final DNA solution		OD _{260 mμ}
			OD _{260 mμ}	Total volume	10 ¹¹ mitochondria
Paramecium		3.0×10^{11}	0.420	2.4 ml	0.33
Tetrahymena	i	0.75×10^{11}	0.274	1.8 ml	0.64
	ii	1.5×10^{11}	0.436	1.8 ml	0.52

tion procedure is available for both *Paramecium* and *Tetrahymena* mitochondria. The data presented in Table 1 suggest approximately an equal amount of DNA present in the mitochondrion of *Paramecium* as in the mitochondrion of *Tetrahymena*.

Melting point analysis of mitochondrial DNA: DNA of the mitochondria of both *Tetrahymena* and *Paramecium* were examined for hyperchromicity resulting from thermal denaturation of the double-stranded DNA. *Tetrahymena* mitochondrial DNA (0.044 OD₂₆₀) and *Paramecium* mitochondrial DNA (0.056 OD₂₆₀) contained in cuvettes were heated and changes in OD_{260 mμ} were recorded as described. The melting profiles of these DNA's are shown in Figure 4. T_m's for *Tetrahymena* and *Paramecium* mitochondrial DNA's were 60° and 70°, respectively; under the same condition of Na⁺ concentration, *B. subtilis* DNA (ρ = 1.703) shows T_m = 73°. These T_m's show a good agreement with those expected from the relationship between T_m values and G-C content and Na⁺ concentration obtained by MARMUR and DOTY (1962). Furthermore, OD₂₆₀ increases for the *Tetrahymena* DNA and the *Paramecium* DNA were 25% and 14%, and their OD transition was virtually complete within a period of several minutes, suggesting that they are typically double-stranded, native DNA molecules being relatively homogeneous throughout the length of DNA. However, the relatively poor OD increases observed here may be due to low molecular weight denatured DNA contaminating these preparations.

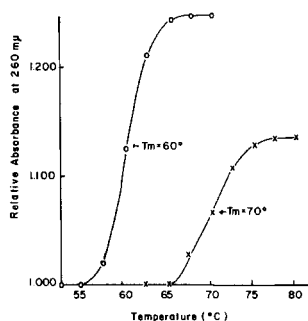


FIGURE 4.—OD_{260 mμ} increase as a function of the temperature of solutions containing mitochondrial DNA of *Tetrahymena* (O—O) and *Paramecium* (X—X).

DISCUSSION

The present studies establish that mitochondria from both *Paramecium* and *Tetrahymena* indeed possess DNA of double-stranded nature, thus supporting the finding previously made by LUCK and REICH (1964) in *Neurospora crassa*. However, that the presence of DNA in mitochondria is a universal phenomenon does not need to be newly emphasized here, as it has been amply demonstrated by NASS, NASS, and AFZELIUS (1965) from their electron microscopic studies on a variety of organisms. It is of interest, however, that it appears to be a general rule that mitochondrial DNA is different from the respective nuclear DNA in their mean densities (LUCK and REICH 1964; SUYAMA and BONNER 1965); this may account for some of the observations of the satellite DNA encountered in many species of higher organisms. (SUEOKA 1961; SCHILDKRAUT, MARMUR and DOTY 1962).

The amount of DNA present in the *Tetrahymena* mitochondrion was estimated to be approximately 3.7×10^{-10} μg . About the same amount of DNA was shown to be present in the *Paramecium* mitochondrion, and also in the mung bean (*Phaseolus aureus*) mitochondrion (SUYAMA and BONNER 1965). This amount of DNA is equivalent to several times the amount of DNA of a T2 bacteriophage; if all of it is present as a single molecule, this corresponds to a piece of DNA of mol wt 2.4×10^8 , which then should be able to code for more than several hundred proteins. These results would be expected if one assumes that mitochondrial DNA is responsible for the synthesis of many proteins known to be associated with mitochondria. However, there is no experimental evidence as to what function this DNA plays and what structure, haploid, diploid or polyploid it may assume within the mitochondrion. However, recent demonstrations of actinomycin D sensitive RNA synthesis with the isolated *Neurospora* mitochondria by LUCK and REICH (1964) and of protein synthesis studied by KROON (1963), together, allude to the possibility that this DNA is endowed with the essential function of determining some of the properties of mitochondria.

The extensive studies made on DNA's from various organisms have brought light to the question of the evolution of DNA. Emphasis has been given to the fact that the DNA's of related organisms possess similar mean G-C composition; thus DNA reflects the phylogenetic relations between the organisms (SUEOKA 1962). Here, essentially the same was found to be true with whole-cell DNA's isolated from *Paramecium* and *Tetrahymena*; they are essentially identical in terms of their mean densities. Superficially, it was expected that the mitochondrial DNA's in the related organisms should also exhibit the similar phylogenetic relationship as in whole-cell DNA's. The results obtained here were quite unexpected and difficult to explain on the basis of the simple mutation and selection hypothesis. However, within the framework of ideas presented by SUEOKA to account for a wide variation of bacterial DNA composition, the following are possibilities which would explain the present situation: either protein synthesis in the mitochondrion is not regulated by the same coding mechanism as in whole-

cell; i.e., the coding mechanism is not universal; or very little function is associated with the mitochondrial DNA, nonsense or redundancy is abundant. However, it is still possible that different intranuclear and intramitochondrial environments have led to independent evolution of base compositions. Therefore, before drawing any conclusion concerning these possibilities, it is necessary to examine density distribution of mitochondrial DNA of many more related organisms.

SUMMARY

DNA's were isolated from mitochondria of *Tetrahymena pyriformis* and *Paramecium aurelia*. Their buoyant densities ($\text{g}\cdot\text{cm}^{-3}$) determined by CsCl density gradient centrifugation were $\rho = 1.682$ and $\rho = 1.702$, respectively. On the other hand, the densities of whole-cell DNA were $\rho = 1.688$ and $\rho = 1.689$, both of which differ from that of the corresponding mitochondrial DNA's.

Mitochondrial DNA's of both organisms showed the hyperchromicity characteristic of double stranded native DNA upon heating; T_m 's (in 0.0195 M Na^+) were 60° and 70° , respectively. The *Tetrahymena* mitochondrion was estimated to contain 3.7×10^{-10} μg DNA; *Paramecium* was approximately the same.

The fact that mitochondrial DNA of related organisms differ may bear on the function of the mitochondrial DNA.

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