Mitochondrial Deoxyribonucleic Acid of *Tetrahymena*. Its Partial Physical Characterization*

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ABSTRACT: Deoxyribonucleic acids (DNA's) were isolated from whole-cell and mitochondrial fractions of each of the following strains of *Tetrahymena pyriformis:* syngens 1, 4, 5, 6, 9, and a strain, GL. CsCl density-gradient equilibrium centrifugation analyses revealed that mitochondrial fractions of 4, 9, and GL contained DNA species which differed from the corresponding whole-cell DNA. The estimated densities (g cm^{-3}) of the mitochondrial and nuclear DNA's of these strains were: 4 (1.686, 1.692); 9 (1.684, 1.690); GL (1.684 1.688).

In addition to the DNA bands associated with mitochondrial fractions, DNA's isolated from whole cell of 4, 6, and GL exhibited additional minor bands in CsCl density gradient; the origin of this DNA is currently under investigation. Further studies made on the purified mitochondrial DNA from strain ST of *T. pyriformis* by sedimentation velocity and sucrose-gradient centrifugation analyses showed a major sedimenting component of $s^0_{20,w}$ = 41.6 S and a molecular weight of approximately 40×10^6. A second component found in varying amounts in our preparations possessed a molecular weight of approximately 1×10^6, which presumably represents an enzymatically degraded product of the major component. In attempts to estimate the molecular size of native DNA with minimum mechanical shearing, mitochondrial lysates were layered over sucrose gradients before and after phenol treatment. No DNA appeared which moved significantly faster than the 40×10^6 component, suggesting that this was the molecular size of the mitochondrial DNA in its native state.

The presence of deoxyribonucleic acid (DNA) in mitochondria has been well established in recent years and should be regarded as a universal phenomenon existing in cells of higher organisms. Although the function of this DNA is still unknown, several interesting features have emerged from studies of its structure. For example, electron microscopy has revealed that DNA fibrils of mitochondria apparently exist as a "nucleoid"[1] structure of bacteria, but differ from a chromosome of higher organisms (Nass and Nass, 1963a,b; Swift, 1965). CsCl density-gradient centrifugation analysis on purified DNA from various organisms revealed that the density of mitochondrial DNA generally differs from that of the corresponding nuclear DNA (Luck and Reich, 1964; Rabinowitz *et al.*, 1965; Suyama *et al.*, 1965; Swift, 1965; Tewari *et al.*, 1965). Although few generalizations can be made as to the difference of densities between mitochondrial and nuclear DNA's at the present time (Suyama *et al.*, 1965), these differences may present some interesting problems concerning the evolution of extrachromosomal DNA. Finally, the amount of DNA within a mitochondrion has been estimated to be relatively constant in a variety of organisms (Suyama and Preer, 1965; Suyama and Bonner, 1966). The *Tetrahymena* and *Phaseolus* mitochondrion contains approximately $3\text{–}5 \times 10^{-10}$ μg of DNA, which is equivalent to two to three times the amount of DNA contained in a single bacteriophase T2. Smaller amounts of DNA have been reported for rat liver (Nass *et al.*, 1965) and yeast (Tewari *et al.*, 1965) mitochondria. However, the molecular size of mitochondrial DNA in its native state has not yet been determined.

Whole-cell DNA of various syngens and strains of *Tetrahymena* have been examined previously and density differences have been detected (Sueoka, 1961). In addition, strain GL was shown to exhibit a satellite band in CsCl density gradient which was suspected to be of mitochondrial origin (Suyama and Preer, 1965). Because of the relative ease of obtaining mitochondria and mitochondrial DNA from *Tetrahymena* (Suyama and Preer, 1965), this organism was considered to be ideal for studying relationships between mitochondrial and nuclear DNA's and for characterizing the structure of the mitochondrial DNA. This paper reports preliminary results of CsCl density-gradient centrifugation analysis on DNA isolated from mitochondrial fractions of six different strains of *Tetrahymena pyriformis*, and the physical characterizations of DNA of mitochondria in a strain of *T. pyriformis*.

Materials and Methods

Culture. The following cultures of *T. pyriformis* used in these studies were obtained from Dr. D. L. Nanney.

* From the Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania. *Received January 31, 1966.* This research was supported by a grant [AT(30-1)-3588] from the Atomic Energy Commission.

[1] For detailed description of the term "nucleoid," see Fuhs (1965).

Syngens and stock identifications (in parentheses) of these were: 1 (A-1464), 4 (UM981), 5 (UM30), 6 (UM-1060), 9 (TC), and a strain, GL. A strain of *T. pyriformis* previously reported (Suyama and Preer, 1965), was also included in the present studies. This strain, of unknown origin, has been in use in the laboratories of this university for some time; it will hereafter be designated as "ST."

The stock culture was maintained at room temperature in culture tubes containing about 5 ml of 1% polypeptone or proteose–peptone liquid medium. For obtaining cells in quantity, a 4- to 5-day-old culture was prepared in 5 l. of medium (1% polypeptone or proteose–peptone) in a 5-gal carboy. Cells were harvested with an International oil testing centrifuge.

Preparation of Mitochondria. Methods used were essentially the same as those previously described (Suyama and Preer, 1965) for strain ST. However, the same methods could not be applied to all other strains due to the presence of a large amount of viscous substance in these cell extracts causing the cell debris and particles to clump together. As a result, even a low-speed centrifugation at 3000*g* precipitated all the cellular particles including mitochondria. In attempts to improve this condition, the following media were used, but with little success: (1) 0.5 M sucrose, 0.01 M Tris–HCl, pH 7.4, 1 mM EDTA; (2) 0.03 M mannitol, 0.05 M Tris–HCl, pH 7.4, 1 mM EDTA, 0.05% cysteine, 0.1% bovine serum albumin (BSA);[2] (3) (A) 0.2 M raffinose, 1 mM potassium phosphate buffer, pH 6.2, 0.25% BSA; (B) 0.2 M raffinose, 1 mM potassium phosphate buffer, pH 7.0, 0.25% BSA.

The modified methods for preparing mitochondrial fractions using medium 3A as in earlier experiments follow. The cells were suspended in 10 times the volume of the isolation medium 3A and crushed through a cream homogenizer. The homogenate was spun at 5000*g* for 6 min and the resulting supernatant was removed by suction. The pellet was resuspended in the same amount of buffer using care not to include the bottom layer. The same centrifugation step was repeated twice on this material and the final pellet was resuspended in 20 ml of saline–EDTA (0.15 M NaCl, 0.1 M EDTA, pH 8.0). While the mitochondrial fraction was reasonably pure, some cilia and cellular debris of unknown origin were present.

Isolation of DNA. The methods of isolating DNA from both a mitochondrial fraction and a whole cell were essentially the same as those described previously (Suyama and Preer, 1965). But the details of several minor modifications are described below. For isolation of mitochondrial DNA, mitochondria isolated from 25 ml of the original cell homogenate were suspended in a 20-ml volume of cold saline–EDTA (0.15 M NaCl, 0.1 M EDTA, pH 8.0). The mitochondrial suspension was then lysed with the addition of 2 ml of 5% sodium deoxycholate solution. The crude lysate was mixed with an equal volume of phenol solution (a 1-ml volume of saline–EDTA was added to a 9-ml volume of distilled phenol previously made 90% with distilled water). The pH of this phenol solution was then adjusted to 9.0 with concentrated NaOH. The mixture was placed in a 125-ml flask in an ice bath, and shaken on a rotary shaker (*ca.* 100 rpm) or manually for a period of 5 min. The phenol layer was separated by centrifugation in an International clinical centrifuge. The top layer was pipetted out and poured into dialysis tubing and subjected to dialysis *vs.* BPES (0.006 M Na_2HPO_4, 0.002 M NaH_2PO_4, 0.001 M Na_2 EDTA, and 0.179 M NaCl) as described previously (Suyama and Preer, 1965). After this dialysis, the contents of the tubing were treated with ribonuclease (RNAase) (5 μg/ml for 20 min at room temperature) which was preheated at a concentration of 1 mg/ml in BPES for 10 min in a boiling water bath. The same phenol treatment and subsequent dialysis were repeated on this material.

For isolating whole-cell DNA, packed cells were resuspended in cold saline–EDTA and immediately lysed with additions of 10% sodium lauryl sulfate to a final concentration of 0.5%. The lysate was quickly mixed with an equal volume of the phenol solution as described above. The mixture was shaken in the cold for 5 min. After centrifugation, the aqueous layer was removed. Twice the volume of cold ethanol was added to this layer and the contents were quickly mixed. Thread-like molecules were wound on a glass rod and dissolved in BPES. This solution was treated with RNAase (5 μg/ml, 20 min at room temperature), and the same phenol and alcohol treatments were repeated. The final solution was then centrifuged at 8000*g* for 10 min to remove denatured protein material. The solution obtained from the ST strain at this stage is usually turbid due to an unknown high molecular weight contaminating material. Centrifugation for 1.5 hr at 45,000*g* was sufficient to remove this turbidity.

Density-Gradient Centrifugation. For CsCl density-gradient centrifugation, the methods of Meselson *et al.* (1957) and Schildkraut *et al.* (1962) were followed. Each cell was equipped with a 1° negative upper window and two cell operations were employed as before (Suyama and Preer, 1965). The rotor speed of 44,770 rpm and the temperature setting of 20° were used throughout. DNA (ρ = 1.725 g cm^{-3}) from *Rhodopseudomonas palustris* calibrated *vs. Bacillus subtilis* DNA (ρ = 1.703 g cm^{-3}) was used as a reference density standard in determining the buoyant density of DNA. Due to a slight calibration error, values reported previously for densities of DNA's from whole cell (ρ = 1.688 g cm^{-3}) and from mitochondria (ρ = 1.682 g cm^{-3}) of the strain ST were corrected in the present studies to 1.692 and 1.686 g cm^{-3}, respectively.

Sucrose gradient centrifugation followed the general directions described by Martin and Ames (1961) using an average speed of 24,000 rpm in a SW 25.1 rotor as determined by odometer readings, and a 7-hr centrifugation time. For preparing tubes, a 2-ml sample solution was layered over a 5–20% linear sucrose (in

[2] Abbreviations used: BSA, bovine serum albumin; BPES: Na_2HPO_4, NaH_2PO_4, Na_2EDTA, and NaCl; GC, guanosine and cytidine; dAT copolymer, copolymer of deoxyadenylate and deoxythymidylate.

BPES) gradient. Upon conclusion of the run the contents of tubes were run through a flow cell (path length/volume = 10 mm/0.10 ml) and continuously monitored at 260 mμ. Fractions of approximately 1-ml sample each were collected for use in further analysis.

Sedimentation-Boundary Velocity Analysis. Centrifugation was made in a Spinco Model E centrifuge at the equilibrated temperature of 25° using ultraviolet absorption optics and a standard aluminum center piece. The sedimentation coefficient of DNA was measured in BPES and corrected to the density and viscosity of water at 20° (Svedberg and Pederson, 1940).

Concentrations. Protein determination was made by the methods of Lowry *et al.* (1951), using bovine serum albumin as a standard. The DNA determination was made by indole assay of Keck (1956) using as a standard *B. subtilis* DNA of a concentration previously calibrated *vs.* calf thymus DNA.

Determination of Melting Temperature. The methods used are essentially the same as those described by Marmur and Doty (1962). A DNA solution (OD_{260} = 0.200) made in BPES (0.195 M Na^+) was used for this analysis.

Results

Heterogeneity of Whole-Cell and Mitochondrial DNA. CsCl density-gradient equilibrium analyses made with DNA's isolated from whole-cell and mitochondrial fractions of six established strains of *T. pyriformis* revealed that mitochondrial fractions of 4, 9, and GL contained DNA species which differed from the corresponding whole-cell bulk DNA (Table I). As reported by Sueoka (1961) in the GL strain, whole-cell DNA preparations of GL as well as 4 and 9 exhibited satellite bands. A ratio of this satellite DNA to the major DNA was greatly enhanced in DNA isolated from a mitochondrial preparation, *e.g.*, a ratio of 1:1 in mitochondrial DNA as compared to approximately 1:10 in whole-cell DNA. This suggests that the minor bands are associated with mitochondria of these organisms. The fact that the DNA corresponding to the major band was not completely eliminated from the mitochondrial DNA preparations is perhaps accounted for by the general difficulty in obtaining a clean preparation of mitochondria. It is unlikely that this is due to internal heterogeneity of the DNA itself since mitochondria of the strain ST of *Tetrahymena* (Suyama and Preer, 1965) and all others (Luck and Reich, 1964; Rabinowitz *et al.*, 1965; Suyama and Bonner, 1966) were shown to contain only one density species of DNA. All other strains presumably possessed DNA in their mitochondria, but density differences between mitochondrial and nuclear DNA's were not detected. It is interesting to note that the densities of mitochondrial DNA's showed less variation than that of nuclear DNA's.

TABLE I: Densities (g/cm^{-3}) of DNA Isolated from Whole-Cell and Mitochondrial Fractions of Various Strains of *Tetrahymena* Determined by CsCl Density-Gradient Equilibrium Centrifugation.

	Whole Cell	Mitochondrial	Unknown[b]
GL[a]	1.688	1.684	1.693
1	1.685	1.685	
4[a]	1.692	1.686	1.700
5	1.687	1.686	
6	1.685	1.685	1.698
9[a]	1.690	1.684	

[a] Densities of mitochondrial DNA differ from that of the corresponding nuclear DNA. [b] Origins of these bands formed in CsCl gradients are not known.

During the course of these studies, it was noted that the whole-cell DNA preparations of syngen 4 exhibited a DNAase-resistant band in CsCl density gradients, the property of which is similar to that previously reported for the ST strain (Suyama and Preer, 1965). The material found in preparations of whole-cell DNA of ST could be removed by centrifugation at 45,000*g* for 1.5 hr and was resistant to DNAase and RNAase. Although the exact nature of this material is unknown, the similarity of the densities and the existence of such material in both strains suggest that ST is perhaps identical with syngen 4. However, as described in Materials and Methods, the cell extracts of these strains behave differently.

It is also noteworthy that whole-cell DNA preparations of 4, 6, and GL exhibited additional minor bands in CsCl density gradient (Table I). These bands represent less than one-half the amount of the satellite band of mitochondrial DNA. The bands were eliminated by DNAase treatments and hence might be regarded as DNA in nature. The origin and nature of this DNA is currently under investigation.

Sedimentation Analyses. It was shown previously that DNA could be isolated from mitochondria of ST strain with relative ease (Suyama and Preer, 1965). The isolated DNA showed a single band in CsCl gradient and the density of this DNA differed from that of whole-cell DNA. Further sedimentation velocity analyses made on this material at a rotor speed of 31,410 rpm demonstrated a major sedimenting component, $s^0_{20,w}$ = 41.6 S, calculated from the $1/s^0_{20,w}$ *vs.* concentration plot shown in Figure 1. It was reported (Eigner *et al.*, 1962) that S values of high molecular weight DNA vary with rotor speed, but that a single measurement of S of the same DNA at 44,770 rpm fell within the close range of values determined at 31,410 rpm. On the basis of an equation, $s^0_{20,w} = 0.034M^{0.405}$ for DNA over 4,000,000 mol wt (Eigner and Doty, 1965), the molecular weight of the purified mitochondrial DNA was calculated to be 42 $\times 10^6$. An equation by Crothers and Zimm (1965) which is applicable to the molecular range from 2×10^5 to 1.3×10^8, $0.445 \log M = 1.819 + \log (s^0_{20,w} - 2.7)$,

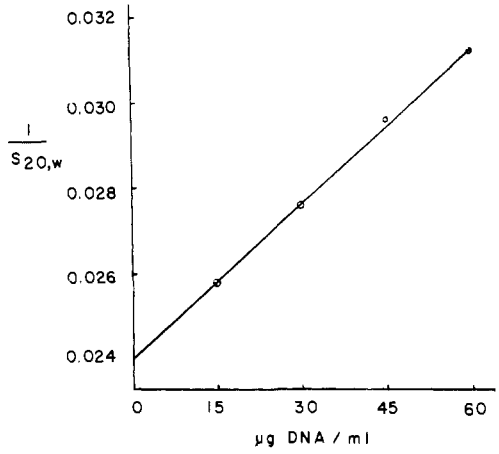

FIGURE 1: Reciprocal of the sedimentation coefficient, $s_{20,w}$ of mitochondrial DNA, plotted *vs.* concentration. Rotor speed = 31,410 rpm.

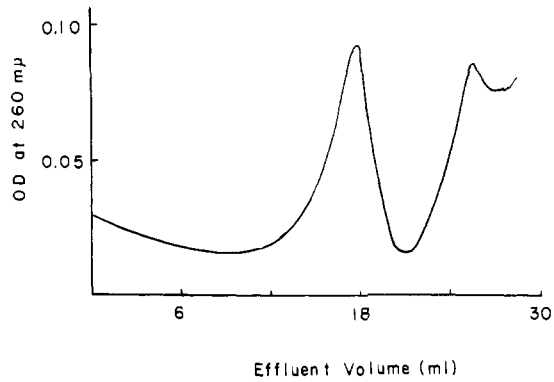

FIGURE 2: Sucrose gradient centrifugation of partially purified mitochondrial DNA. The contents of the tube were drained through a flow cell and monitored continuously at 260 mμ. The total volume = 28 ml; the initial OD_{260} input = 1.5.

gave a calculated value of 45×10^6 daltons for the same S value. In this paper, the size of this molecule will be an approximation referred to as 40,000,000 daltons.

In addition to the major component, a second component of an approximate molecular weight of 1×10^6 was found in our preparation in varying amounts (up to 50%). In order to clarify the nature and origin of this heterogeneity, the following experiments were performed. First, a DNA preparation was centrifuged in a sucrose gradient. Figure 2 shows the separation into heavy and light components. Fractions corresponding to each component were collected, pooled, and repurified separately. CsCl density-gradient analysis on the repurified components showed the density of the heavy component to be 1.686 g cm^{-3} and that of the light, 1.688 g cm^{-3} (Figure 3). Secondly, a mitochondrial preparation was treated with DNAase before lysis and washed with saline-EDTA prior to the isolation of mitochondrial DNA. Subsequently, DNA was isolated and subjected to the same sucrose-gradient centrifugation. The results showed that the amount of the light component was largely eliminated (down to 5%) from the preparation of DNA.

The similarity of the densities of both components together with the above results indicate that the light component would perhaps represent an enzymatically degraded product of the heavy component. This could be a result of rupture of mitochondria, which renders its DNA accessible to intracellular or intramitochondrial nuclease attack.

The foregoing experiments demonstrated that the isolated DNA from mitochondria has a molecular weight of 40×10^6. On the other hand, it was reported (Suyama and Preer, 1965) that the amount of DNA estimated to be present in the mitochondrion was equivalent to a molecule of approximately 2.4×10^8 daltons. In attempts to clarify the above discrepancy and to obtain a molecular size of the native DNA with minimum shearing, mitochondria were lysed by 0.5% sodium deoxycholate and centrifuged at low speed to remove any unlysed material. It was immediately layered over a sucrose gradient using maximum care in delivering the lysate from the orifice (diameter, 1 mm) of a 5-ml pipet. After a 7-hr period of centrifugation, the OD_{260} profiles of the contents were obtained through a

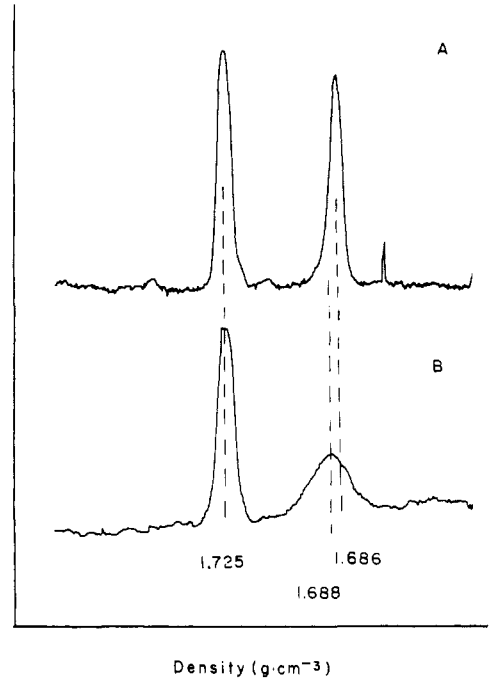

FIGURE 3: Microdensitomer tracings of the ultraviolet absorption photographs of mitochondrial DNA in equilibrated CsCl density gradients formed by centrifugation at 44,770 rpm for 24 and 56 hr, respectively, for (A) heavy component and (B) light component. *R. palustris* DNA (ρ = 1.725 g cm^{-3}) was used as a density reference.

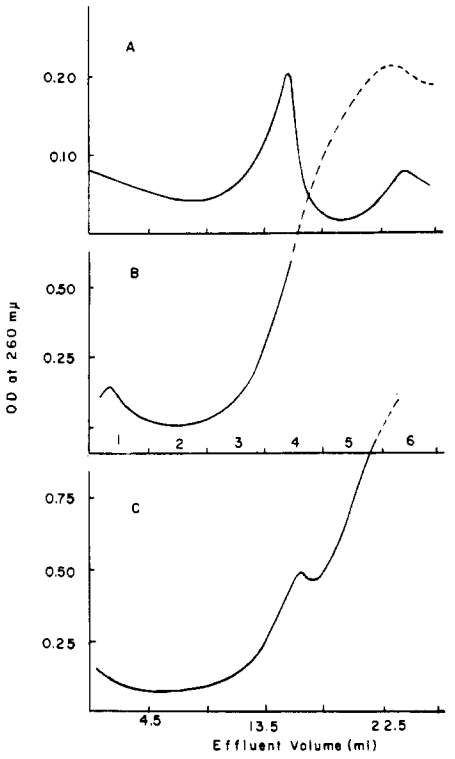

FIGURE 4: Sucrose gradient centrifugation analyses of (A) purified mitochondrial DNA, (B) mitochondrial lysate, and (C) mitochondrial lysate treated once with phenol. The continuous OD_{260} profiles were obtained by monitoring the contents through a flow cell. The total input materials were equivalent to OD_{260} = 1.0 for (A), 40 mg of mitochondrial protein for (B), and OD_{260} = 15 for (C). This is equivalent to 60 μg of DNA. The dotted lines indicate the readings beyond scale.

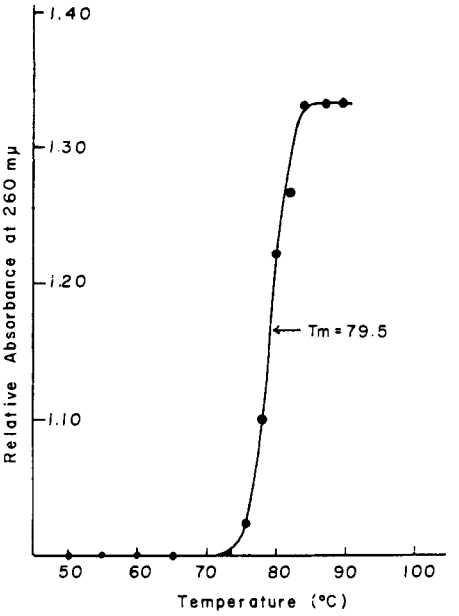

FIGURE 5: The OD_{260} increase as a function of the temperature of solutions (0.195 M Na^+) containing mitochondrial DNA. Initial OD_{260} = 0.200.

flow cell. The results presented in Figure 4B show that no peak appeared which was moving significantly faster than the 40 × 10^6 component as seen in the control run (Figure 4A) of the purified DNA mentioned above. In order to further verify this, six fractions, as indicated in Figure 4B by 1–6, were collected and subjected to the further purification of DNA. As shown in Table II, a significant amount of DNA appeared first in fraction 3, where the 40 × 10^6 DNA would be expected to appear. The nature and quantity of the DNA in fractions 1–4 were further examined in CsCl density-gradient centrifugation. Fractions 3 and 4 contained DNA, the density of which coincided with that of the mitochondrial DNA. However, 1 and 2 did not show any band in CsCl gradients, indicating that the total amount of DNA in 1 and 2 was much less than 0.3 μg,[3] as contrasted to the values obtained with the chromogenic assay (Table II).

TABLE II: Distribution of DNA in Six Fractions Obtained from the Sucrose Gradient of Mitochondrial Lysate.[a]

Fraction[b]	Total Vol (ml)	DNA (μg/ml)[c]	Total DNA (μg)
1	2.26	3.0	6.8
2	2.40	1.5	3.6
3	2.55	5.0	12.2
4	3.30	20	66
5	2.50	30	75
6	2.90	25	73

[a] Mitochondrial lysate equivalent to a total of 80 mg of protein. [b] See Figure 4B for these fractions. [c] The DNA determination was made by the methods of Keck (1956) on 1 ml of each sample. The determination is not too reliable below the DNA concentration of 5 μg/ml.

Since it was feared that the large amounts of protein and lipid materials in the lysate might have interfered with the movements of DNA, the mitochondrial lysate was treated with phenol (see Methods) and dialyzed *vs.* BPES. It was hoped that the phenol treatment alone would not cause any appreciable break in the DNA (Thomas and Berns, 1961; Hershey and Burgi, 1963; Berns and Thomas, 1965). The results of centrifugation of such material showed a peak nearly corresponding to the 40 × 10^6 DNA. Again, no significant amount of ultraviolet absorbing materials sedimented more rapidly

[3] This estimation is based on the assumption that 0.10 μg of DNA can be satisfactorily resolved by CsCl density-gradient centrifugation analysis.

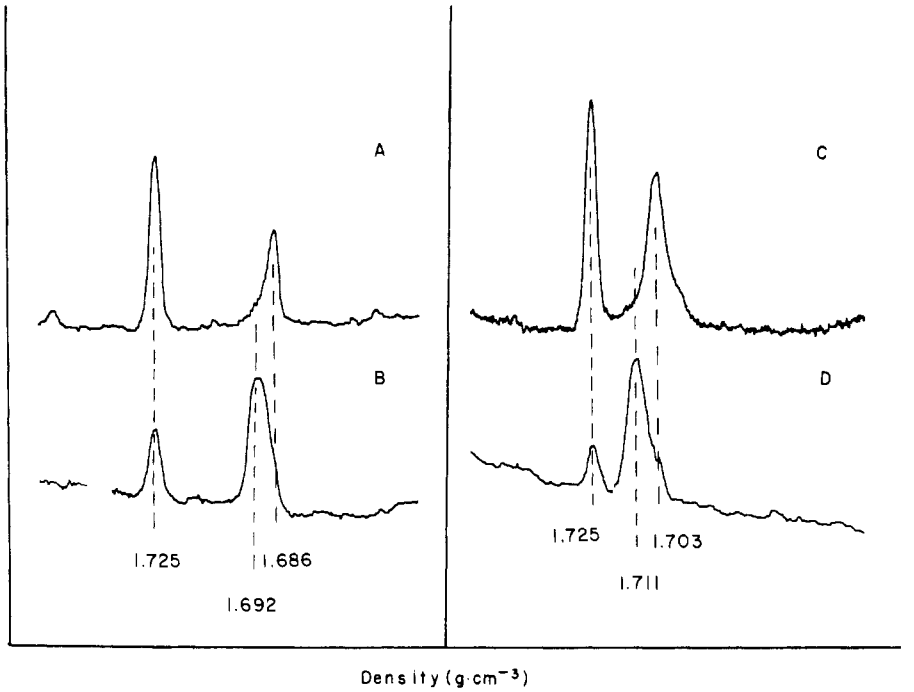

FIGURE 6: Microdensitometer tracings of the ultraviolet absorption photographs of various DNA's in equilibrated CsCl density gradients formed by centrifugation at 44,770 rpm for 24 hr; (A) native mitochondrial DNA, (B) native whole-cell DNA, (C) heat-denatured mitochondrial DNA, and (D) heat-denatured whole-cell DNA. *R. palustris* DNA (ρ = 1.725 g cm^{-3}) was used as a density reference.

than the component (Figure 4C). The concentration of DNA in this tube was adjusted to be approximately the same as in 4A, in order to minimize any artifacts resulting from concentration changes (Hershey and Burgi, 1963).

The above results suggest that the molecular size of the DNA in the mitochondria would not be much larger than that of the purified DNA. However, the present studies did not exclude the possibility that fragmentation of DNA was brought about by nuclease which might be present within the mitochondrion.

Denatured DNA. The mitochondrial DNA, that is, the 40 × 10^6 component purified from the bulk mitochondrial preparation by sucrose-gradient centrifugations as described, showed hyperchromicity upon heating as expected for a double-stranded native DNA (Figure 5). The optical density increased by 33%,[4] which is slightly lower than the average values generally observed (Marmur and Doty, 1962), but the melting temperature profile shows no anomaly and the melting temperature (T_m = 79.5°) of this DNA in 0.195 M Na^+ is in good agreement with the value expected for a GC content estimated from the density of this DNA. The calculated GC content of the DNA of ρ = 1.686 g cm^{-3} is 26% [ρ = 0.098(GC) + 1.660 (Schildkraut *et al.*, 1962)] and the T_m in 0.2 M Na^+ is 79.9° [T_m = 69.3 + 0.41(GC) (Marmur and Doty, 1962)]. This heat denatured DNA bands in CsCl density gradients at ρ = 1.703 g cm^{-3} whereas the heat denatured nuclear DNA at ρ = 1.711 g cm^{-3} (see Figure 6). The density increment observed for the mitochondrial DNA (Δ_ρ = 0.017 g cm^{-3}) is essentially the same as those values expected from bacterial DNA (Doty *et al.*, 1960; Schildkraut *et al.*, 1961), but the value for the nuclear DNA (Δ_ρ = 0.019 g cm^{-3}) is slightly higher than expected. These results provide strong evidence that the isolated mitochondrial DNA is not a complex of the nuclear DNA with some material of unknown nature, nor does it appear to be a RNA–DNA complex as postulated to be present in the rat liver mitochondrial DNA preparation (Nass *et al.*, 1965).

Discussion

It has been observed that DNA extracted from higher organisms, unlike that of most bacteria, contains a small amount of so-called "satellite" bands in CsCl density gradients. In *T. pyriformis*, Sueoka (1961) noticed such a band in the GL strain among seven strains examined. Upon examination of a *Tetrahymena* strain (ST), DNA of the mitochondrial fraction was found which differed from the bulk whole-cell DNA. It was postulated that perhaps minor bands observed in these cases are mainly attributable to the mitochondrial DNA. Although final proof is not yet available in the present studies, the minor band of the GL strain is greatly enhanced by isolating the mitochondrial

[4] The same optical density increment was obtained with pancreatic DNAase digestion.

fraction of this strain, suggesting that this band is of mitochondrial origin. Two other strains, 4 and 9, similarly possessed a distinct species of DNA in their mitochondrial fractions. In each case, the density of the DNA was found to be similar to that of the respective whole-cell (nuclear) DNA. This, in contrast to the previous speculation (Suyama and Preer, 1965), presents the idea that DNA of the subcellular particles evolves around the nuclear DNA. It is interesting, however, that densities of the mitochondrial DNA's of various syngens of *Tetrahymena* are very similar and showed less variation than those of the nuclear DNA's. This is somewhat analogous to the situation found in several plant materials so far examined, where all the mitochondrial DNA exhibited the same density (Suyama and Bonner, 1966).

The present studies further support the previous demonstration of the existence of DNA in mitochondria as revealed by autoradiography (Parsons, 1965; Stone and Prescott, 1964), as well as by electron microscopy (Swift, 1965), but contradict the evidence presented by Parsons and Dickson (1965) that syngen 6 of *T. pyriformis* possesses mitochondrial DNA, with a density different from that of the nuclear DNA. Although it might be possible that different stocks of the same syngen exhibit differences in DNA composition, the reported density (ρ = 1.671 g cm^{-3}) based on *Escherichia coli* DNA (ρ = 1.710 g cm^{-3}) of the mitochondrial DNA may be too low to be regarded as a pure native DNA at the present time. The density of dAT copolymer is reported to be 1.679 g cm^{-3} (Schildkraut *et al.*, 1962), which is the lowest density for all the DNA known thus far. Although all the available evidence suggests the existence of DNA within mitochondria, caution must be exercised in interpreting these data prematurely, since it has been shown (see Gibor and Granick, 1964; Preer, 1966) that some other subcellular structures such as plastids, kinetoplasts, kinetosomes, as well as other particles like κ of *Paramecium*, contain DNA. It is of particular interest that some strains of *Tetrahymena* apparently contain satellite DNA other than that associated with mitochondria.

The present studies of purified DNA from mitochondria of ST strain of *Tetrahymena* revealed that a molecular weight of this DNA is 40 $\times$ 10^6. Sucrose-gradient centrifugation analyses of both mitochondrial lysate and lysate treated only with phenol suggested a similar molecular weight range for presumably less mechanically degraded DNA. This, without further evidence, may be taken as an indication that this size represents the molecular size of the mitochondrial DNA in its native state. If this is the case, the amount of DNA previously estimated to be present within the mitochondrion would accommodate several molecules of this size.

As to the physical nature of DNA associated with mitochondria of rat liver, Nass *et al.* (1965) have presented evidence that the mitochondrial DNA might be partially complexed with RNA. Furthermore, DuBuy *et al.* (1965) observed that the kinetoplast DNA isolated from *Trypanosoma* showed a remarkably fast banding property unusual for normal DNA, and suggested that the DNA has a unique conformation facilitating its fast diffusion in CsCl. On the contrary, our preparation of mitochondrial DNA exhibited no such anomalies, and all the physical properties are consistent with the expectation for a regular double-stranded native DNA. However, whether the DNA is circular or not remains to be determined.

Acknowledgment

The author wishes to thank Dr. D. L. Nanney for making these *Tetrahymena* cultures available for these experiments. The author is also indebted to Dr. J. R. Preer, Jr., for his continuing interest and encouragement and also for critical reading of the manuscript.

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Titration of Salt-Extracted Human Skin Collagen*

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ABSTRACT: Hydrogen ion titrations were done on the undenatured and the heat-denatured (40°, 30 min, pH 6.5) forms of a purified and physically characterized salt-extracted collagen. Exposure to low pH of the undenatured soluble collagen resulted in the unmasking of 7.5 carboxyl groups/10^5 g. The release of these groups was not associated with detectable loss of helical structure. Either heat or high pH denaturation resulted in the unmasking of 5.0 carboxyl groups/10^5 g, 1.0 imidazole or α-amino groups/10^5 g, and 5.5 ϵ-amino groups/10^5 g.

The release of these groups was associated with loss of helical structure and separation of the protein to random coils. The unmasking of these ionizable groups is thought to result from the disruption of interchain bonds involving these groups. The precise chemical nature of these bonds cannot be determined from the titration data.

Titration studies have been done previously on collagen systems. Bowes and Kenton (1948) titrated intact collagen which had been extracted with 10% sodium chloride. Ames (1952) and Kenchington and Ward (1954) did extensive titrations of purified gelatin solutions. Jansson and Weaver (1964) have reported, in abstract form, titrations on collagen and gelatin extracted from rat tail tendon by the method of Dumitru and Garrett (1957), but these titrations were carried out only from pH 1 to 5. At the present time, however, no full pH range titrations have been reported for soluble collagen molecules in the undenatured and denatured states. The major objective of this work was to compare the numbers of groups titrated in the undenatured state with the numbers of groups titrated in the denatured state for collagen.

Salt-extracted molecules were chosen for this investigation because these molecules apparently represent an early phase in the maturation of collagen (Jackson and Bentley, 1960.) During the extraction procedure, intermolecular bonds were broken. Titration curves were obtained on the undenatured solubilized collagen molecules. During heat denaturation, the interchain bonds were broken. Titration curves were then obtained on the component polypeptide chains. Differences between the kinds and numbers of groups titrated in the two states gave the type and numbers of groups unmasked.

Experimental Procedure and Characterization of Soluble Collagen

Extraction Procedure. Body skin was obtained at autopsy within 8 hr of death from full-term newborns. In no specimen was gross or microscopic evidence of decomposition present. All preparatory procedures were carried out at 5° or less. The epidermis and subcutaneous fat were scraped off with a sharp blade; microscopic sections confirmed complete removal. The dermis was fragmented with a mechanical grinder and washed thoroughly and repetitively with large amounts of triple-distilled water for 48 hr, to remove readily soluble material. The washed dermis was extracted with 1.0 M NaCl (1:4, wet weight:volume) pH 7.4 for 48 hr, and the supernatant fluid was collected following centrifugation at 59,000*g* for 1 hr. The dissolved soluble collagen in the supernatant was precipitated by dialysis *vs.* at least 1000 times its volume of 5 M NaCl and collected by centrifugation at 59,000*g* for 1 hr. The precipitate was dissolved in 1 M NaCl and dialyzed *vs.* at

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