A Compact Form of Rat Liver Mitochondrial DNA Stabilized by Bound Proteins

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A highly folded, rapidly sedimenting form of rat liver mitochondrial DNA has been released from the organelles with BRIJ 58 and sodium deoxycholate in the presence of 0.5 M NaCl and isolated by sedimentation velocity in sucrose gradients. Under these conditions a majority of the mitochondrial DNA labeled in vitro sedimented beyond 39 S, the sedimentation coefficient of a highly purified mitochondrial DNA supercoil, and appeared as a stable, heterogeneous population of species ranging in s values between 42 S and about 70 S. Under formamide-spreading conditions most of the rapidly sedimenting forms appeared in the electron microscope as single genome length rosettes constrained at the center in a dense core. Except for an occasional δ-loop, no extraordinary structural features were evident along the smooth loops projecting radially from the central core. In sucrose gradients containing various amounts of ethidium bromide, the sedimentation velocity of the folded DNA changed in a biphasic fashion in response to increasing amounts of dye. At a dye concentration of 0.5 μg per ml the DNA species present reached s value minima, but two major peaks sedimenting at 32 S and 42 S were present at this point. Thus, although these species were similar in superhelix density, there appeared to be additional constraints superimposed upon their tertiary structure that folded these forms to differing degrees of compactness. Direct chemical analyses showed that proteins were bound to the folded DNA at a protein to DNA ratio of about 0.3. Separation of the bound proteins on SDS-polyacrylamide gels revealed an array of proteins ranging in molecular weight between 11,000 and 150,000. Several of the lower molecular weight proteins co-migrated with proteins from the inner mitochondrial membrane, but the major DNA-bound band (Mr = 58,000) was undetectable among the proteins from any other submitochondrial fraction. Digestion of the compact DNA structure with proteinase K under various conditions indicated that the DNA was maintained in the compact conformation by the tightly bound proteins and that the portions of these proteins directly involved in stabilizing the folded DNA were proteinase insensitive unless digestion was carried out in the presence of a disulfide reductant at elevated temperatures.

Animal mtDNA is a covalently closed, circular molecule having a contour length of about 5 μm corresponding to a molecular weight of approximately 9 × 10^6 (see reviews, Refs. 1 and 2). Upon isolation and purification of mtDNA using a deproteinizing procedure, several forms of circular molecules are obtained. The major species is the covalently closed supercoiled molecule having a sedimentation coefficient of about 39 S (3-5). The open circular form sedimenting at 27 S (3-5) is present to a lesser extent. Its presence can result either from the introduction of at least one strand scission into the covalently closed circle (3), or as in the case of mouse cells (6, 7), as a result of the insertion of a displacing strand (6, 8) that stabilizes the winding deficiency of the parental duplex, thus considerably reducing the number of supercoils (9). In addition to these species, catenated molecules containing two, or more, interlocked circles are found. Catenated dimers sediment between 36 S and 51 S depending upon whether the constituent monomers are open or closed circles. Catenanes are present at a frequency of between 3 to 9% of the mtDNA molecules from normal animal tissues (10). Using electron microscopy, Nass et al. (11) demonstrated that in thin tissue sections stained with osmium tetroxide, the mtDNA appeared in a compact state as a small, rodlike structure in the matrix. It was apparent from this work that the mitochondrial genome, like that of the bacterial chromosome (12, 13), must exist inside the organelle in a packaged, physical state that is far more compact than an isolated supercoil. However, the specific nature of the packaged form and the mechanisms for maintaining the supramolecular state of animal mtDNA in vivo have remained obscure.

Additional early work by Nass (14) and later by Van Tuyle and Kalf (15) provided evidence for the association of animal mtDNA with the mitochondrial membrane. More recently, Albring et al. (16) demonstrated that most of the HeLa circular mtDNA released from the organelles by Triton X-100 in the presence of low salt was associated with a proteinaceous structure which varied in appearance in the electron microscopy between a 10- to 20-nm knob and a 100- to 500-nm membrane-like patch. This structure was found to be bound near the origin of replication. In all these studies, however, the presence of an isolated membrane/DNA structure that existed in a compact, packaged state was not observed. Pinon et al. (17) have indicated that some of the DNA released from Xenopus laevis oocyte mitochondria by gentle lysis in the presence of spermidine was presumably associated with proteins forming a complex having a sedimentation coefficient of 58 to 60 S. In the electron microscope these structures appeared as small relaxed circles consisting of globules connected by a thin filament.

The work presented here describes a stable compact form of mitochondrial DNA isolated from rat liver. This DNA structure is visualized as a constrained circle forming a rosette with a dense central core. The structure is maintained in the highly folded state by associated proteins. Evidence is also...
provided which suggests that portions of the bound proteins are stabilized in a tight proteinase-insensitive form by disulfide bonds. Portions of this study previously presented in abstract form (18) are given in detail and extended in this communication.

**EXPERIMENTAL PROCEDURES**

**Materials**

Male Sprague-Dawley rats weighing 150 to 250 g were purchased from Flow Laboratories, Inc. Dublinia, Virginia. [Met,Ser-H3]Thd was obtained from Amersham/Searle, Arlington Heights, Ill. Prenson free of nucleases came from Calbiochem, La Jolla, Calif. Proteinase K was a product of EM Laboratories, Inc., Dartmund, Germany. BRJ 58 was obtained from Atlas Chemical Industries, Wilmington, Del. and Aqualosa was purchased from New England Nuclear, Boston, Mass. Nonidet P-40 came from Partite Data, Inc. in Ellicuurst, Ill. and Sarkosyl was purchased from ICN-K&K Laboratories, Cleveland, Ohio. Collidion (4%) in amyl acetate was obtained from Ladd Research Industries, Burlington, Vt. Specially pure sodium lauryl sulfate, formamide, and uranyl acetate came from BDH Chemicals, Ltd., Pool, England. Acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine, ammonium persulfate, and Coomassie brilliant blue R-250 were from Bio-Rad Laboratories, Richmond, Calif. Sigma Chemical Company, St. Louis, Mo., was the supplier of the following: electrophoretically purified pancreatic DNAse (EC 3.1.4.5), pancreatic RNAse A (EC 2.7.7.16), trypsin (EC 3.4.4.4), cytochrome c, β-galactosidase, phosphorylase a, ovalbumin, soybean trypsin inhibitor, bovine serum albumin, diithiothreitol, sodium deoxycholate, digitonin, Lubrol WX, ethidium bromide, Triton X-100 and the unlabelled ribonucleoside and deoxyribonucleoside triphosphates. Calf thymus DNA was purchased from Worthington Biochemical Corporation, Freehold, N.J. We are indebted to Dr. Francis Macrina of the Department of Microbiology, Medical College of Virginia, Virginia Commonwealth University, for the kind gift of [3H]labeled R6K plasmid.

**Methods**

**Isolation of Mitochondria**—Mitochondria were isolated from 40 to 50 g of rat livers essentially as described previously (19) except that the medium contained 0.3 M sucrose and 1 mM disodium EDTA, pH 7.4, at 4°C. The final pellet was suspended in isolation medium at a protein concentration of 20 mg/ml. The total yield of mitochondrial protein isolated under these conditions was between 700 and 900 mg.

**The Isolation of Submitochondrial Fractions**—The separation and isolation of the mitochondrial inner membrane, matrix, and outer membrane was carried out essentially as described by Schmutzmann and Greenawalt (20). A weight ratio of digitonin to mitochondrial protein of 0.075 was used for the isolation of the outer mitochondrial membrane. The outer mitochondrial membrane was removed from the low speed supernatant containing digitonin and any contaminating soluble proteins by centrifugation at 45,000 rpm for 1 h in a Beckman 65 rotor. The pellet was resuspended with homogenization in 1 ml of isolation medium. During the preparation of the inner mitochondrial membrane and matrix fractions, a digitonin to protein ratio of 0.080 was used to remove the outer mitochondrial membrane.

**The Resulting Imak Inner Mitochondrial Membrane Plus Matrix Preparation** was lysed by the addition of 0.1 mg of Lubrol WX per mg of protein. The inner mitochondrial membrane matrix fraction in the lysate were then separated by centrifugation at 45,000 rpm for 1 h in a Beckman 65 rotor. The supernatant fluid containing the soluble proteins was retained as the matrix fraction, and the pelleted inner mitochondrial membrane fraction was resuspended with homogenization in 1 ml of isolation medium. The degree of contamination of each submitochondrial component with proteins from the remaining two fractions was monitored by marker enzyme assays as shown in Table I. In each case the bulk of the site-specific total enzyme activity resided with the appropriate fraction. The cross-contamination in terms of specific activities among the submitochondrial components was below 10% in all cases except in the outer membrane which contained cytochrome oxidase activity at about one-fifth the level found in the inner mitochondrial membrane fraction.

**Marker Enzyme Assays**—The endogenous enzymes monoamine oxidase, cytochrome oxidase, and glutamate dehydrogenase were assayed as markers specific for the outer mitochondrial membrane, inner mitochondrial membrane, and matrix fractions, respectively. In all cases the mitochondria and submitochondrial fractions were activated with 0.3 mg of Lubrol WX per mg of mitochondrial protein for 15 min at 4°C. Monoamine oxidase was assayed as described by Tabor et al. (21). Cytochrome c oxidase was assayed polarographically according to Higgins and Friend (22) using N,N,N',N'-tetramethylethylenediamine as a reductant of ferricytochrome c. Glutamate dehydrogenase was assayed as described by Rogers (23), except that 0.10 mm anilal was present to inhibit reoxidation of NADH by the respiratory chain.

**Labeling of Mitochondrial DNA In Vitro**—The composition of the incubation mixture was the same as previously reported (5) except that 0.15 M sucrose was present and the four ribonucleoside triphosphates were each added at a concentration of 15 μM. The labeled precursor in all cases was 0.25 μM [methy]-H3Tnd (40 to 50 Ci/ mmol). The mixture was incubated 45 min at 37°C and chilled to 0°C in ice water to stop the reaction. The mitochondria were pelleted at 10,000 × g and, unless otherwise noted in the figures, resuspended with gentle homogenization in a medium containing 0.5 M NaCl, 10 mM disodium EDTA, 10 mM Tris, pH 8.0 at 4°C. The final concentration of resuspended mitochondria was 30 to 35 mg of mitochondrial protein per ml.

**The Isolation and Resedimentation of [H3]mDNA**—Unless otherwise noted, the labeled, resuspended mitochondria were lysed by the addition of an equal volume of 1% (w/v) BRJ 58 and 0.4% (w/v) sodium deoxycholate, 0.5 M NaCl, 10 mM disodium EDTA, 10 mM Tris, pH 8.0 at 4°C (12). The lysate was allowed to stand in ice for 20 to 30 min and then centrifuged at 27,000 × g for 20 min to remove any undissolved particulate material. The cleared lysate was layered onto 5 to 20% linear sucrose density gradients in 0.5 M NaCl, 10 mM Tris, and 1 mM disodium EDTA, pH 8.0 at 4°C (0.5 M STE buffer). Generally, 36-ml gradients were used, and 3 ml of lysate (about 50 mg of mitochondrial protein) were layered on each. The gradients were centrifuged in a Beckman SW 27 rotor for 16 h at 20,000 rpm at 4°C. One-mililter fractions were collected from the bottom after piercing

### Table 1

| Submitochondrial fraction | Total protein (mg) | Monoamine oxidase activity | Cytochrome oxidase activity | Glutamate dehydrogenase activity |
|---------------------------|-------------------|---------------------------|----------------------------|--------------------------------|
| Outer mitochondrial membrane | 3.8               | 83.3                      | 3.2                        | 0.01                           |
| Matrix (0.08 mg of digitonin per mg of protein) | 57.9             | 10.8                      | 86.3                      | 2.0                            |

"Based on whole mitochondria.

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**Distribution of marker enzymes in submitochondrial fractions**

The procedures for the isolation of the submitochondrial fractions and for the assay of the constituent marker enzymes are given under "Experimental Procedures."
the tube with a 30 gauge needle. Radioactivity in 50- to 100-μl samples from each fraction was counted by the filter paper disc method (19).

In all cases, the relative s values of the mtDNA species were determined by comparison with Form I (51 S) and II (38 S) of 14C-labeled R6K plasmid (24) used as external sedimentation markers. The mtDNA species of interest were isolated from pooled fractions by centrifugation in a Beckman 50.2 Ti rotor for 4 h at 45,000 rpm. The pelleted DNA was resuspended in a small volume of 0.15 M NaCl, 10 mM Tris, and 1 mM disodium EDTA, pH 8.0, at 4°C (0.15 M STE buffer). Where indicated, the [3H]mtDNA was further purified by resedimentation on a second sucrose density gradient containing 5 to 20% linear sucrose gradients containing various concentrations of detergent sodium deoxycholate in the presence of 0.5 M NaCl. SDS, and an equal volume of freshly distilled phenol saturated with 0.15 M STE buffer by gently swirling and stored at 4°C.

Although a pellet was hardly visible, greater than 80% recovery of the labeled DNA was achieved with this procedure.

**Chemical Assays**—The amount of protein in mitochondria and in the submitochondrial fractions was determined by the biuret method. The quantitative analysis of proteins bound to mtDNA was carried out by the method of Bensadoun and Weinstein (25) for assay of microgram quantities of proteins in the presence of interfering materials. Crystalline bovine serum albumin was used as the standard. The amount of DNA in equivalent aliquots was measured as described by Burton (26). Calf thymus DNA was used as a standard.

**Purification of mtDNA by Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis**—The isolated mtDNA sample was made 1% with respect to SDS, and an equal volume of freshly distilled phenol saturated with 0.15 M STE buffer was added. The phases were mixed by rotating the tube end over end for 30 min, and the aqueous phase was collected after a 10-min centrifugation. The phenol procedure was then repeated a second time.

**Elution Reomide Sedimentation**—The effect of increasing amounts of ethidium bromide on the s value of mtDNA was determined by layering 100 μl (less than 1 μg of DNA) of the DNA solutions onto 5 to 30% linear sucrose gradients containing various concentrations of ethidium bromide. The gradients were centrifuged in a Beckman 50.1 rotor for 16 h at 45,000 rpm at 4°C. The DNA was resuspended in a small volume of 0.15 M STE buffer by gently swirling and stored at 4°C.

**Electron Microscopy of mtDNA**—The samples of mtDNA visualized by electron microscopy were isolated and subjected to further purification by resedimentation as described above. The pellets were resuspended in 0.15 M NaCl and subjected to sucrose density gradient centrifugation for 2% h at 80 V and a constant current of 55 mA. The gel was fixed and stained overnight in 10% (v/v) acetic acid, 25% (v/v) isopropyl alcohol, and 0.6 mg/ml of Coomassie brilliant blue R-250, and destained in 10% (v/v) acetic acid.

**Results**

**Isolation of a Rapidly Sedimenting Form of mtDNA**—Mitochondria were labeled in the DNA in vitro and lysed by treatment with the nonionic detergent BRIJ 58 and the ionic detergent sodium deoxycholate in the presence of 0.5 M NaCl. After removing any undissolved particulate material from the suspension by centrifugation, the cleared lysate was analyzed on a 5 to 20% sucrose density gradient. The radioactivity profile of a typical gradient is shown in Fig. 1. The labeled DNA sedimented as a heterogeneous population of species having s values ranging from about 24 S to greater than 70 S. A majority of the labeled DNA sedimented as several broad zones (bracketed material) beyond 39 S and ranged from about 42 S to more than 70 S. This population of DNA molecules, sedimenting beyond the 39 S position, has been designated ">39 S mtDNA." This rapidly sedimenting material has been examined more carefully to elucidate its structural characteristics. The broad, but apparently discrete, peaks in the >39 S region of the gradient have been fairly consistent in mean s value in different preparations. However, the degree of labeling of >39 S mtDNA, relative to 39 S and to 27 S DNA (seldom detectable), varied somewhat from preparation to preparation. When the material in the fractions from the lower region of the gradient of Fig. 1 (bracketed material) was pooled, pelleted by centrifugation, and a portion was resedimented on a second sucrose density gradient (Fig. 2A), the DNA again sedimented beyond 39 S as a rather broad band composed of species of apparently heterogeneous nature. Virtually no conversion to the 39 S or 27 S forms was observed. Subsequent experiments indicated that >39 S mtDNA could be stored frozen for several months with little or no conversion to slower sedimenting forms. Pancreatic RNAse had no effect on the s value, or the shape, of the band of >39 S mtDNA (not shown), but pancreatic DNase degraded it to small fragments that remained at the top of the gradient (Fig. 2B).

![Fig. 1. Preparative sucrose density gradient of [3H]DNA from mitochondria lysed with BRIJ 58/deoxycholate. The mitochondria were labeled in vitro, lysed with BRIJ 58 and deoxycholate in the presence of 0.5 M NaCl and subjected to sucrose density gradient analysis as described under "Experimental Procedures."](http://www.jbc.org/)

**Fig. 1.** Preparative sucrose density gradient of [3H]DNA from mitochondria lysed with BRIJ 58/deoxycholate. The mitochondria were labeled in vitro, lysed with BRIJ 58 and deoxycholate in the presence of 0.5 M NaCl and subjected to sucrose density gradient analysis as described under “Experimental Procedures.”
When another portion of the pooled material was treated by a SDS/phenol purification step, nearly complete conversion to the 39 S form was observed (Fig. 2C). These results suggest that the >39 S mtDNA is maintained in a rapidly sedimenting, compact state by tightly bound non-DNA constituents and that the high s value of this form is not a property inherent in the structure of the DNA itself. They also rule out the possibility that the >39 S mtDNA consists primarily of topological multimers, because catenanes and concatenanes would not revert to the monomeric form as a result of a deproteinizing purification step.

Summary of Various Lysing Procedures that Yield >39 S mtDNA

In most of the experiments reported here, the mtDNA was released by lysis of the labeled mitochondria with BRIJ 58 and deoxycholate in the presence of 0.5 M NaCl. To demonstrate that the >39 S mtDNA does not arise as a result of this specific lysing procedure, numerous other procedures listed in Table II have been used successfully to isolate this form. The agents enumerated in Table II are either nonionic, or rather gentle ionic detergents such as deoxycholate and Sarkosyl. It was found that when mitochondria were lysed by any of these agents in concentrations of NaCl below 0.3 M, most of the mtDNA was degraded to small fragments ranging in size between 4 S and 10 S. However, in concentrations of NaCl between 0.3 M and 1 M, no degradation was seen, and regardless of the detergent used, essentially identical sucrose density gradient profiles were observed, and >39 S mtDNA was evident. Apparently, these gentle detergents released active nucleases associated with the mitochondrial preparation resulting in the degradation of the exposed mtDNA when the NaCl concentration was below 0.3 M. However, elevated levels of NaCl, known to inhibit mitochondrial-associated nuclease activities (30, 31) allowed the isolation of intact DNA after lysis by these detergents.

It is noteworthy that >39 S mtDNA could be released from the mitochondria by treatment of the intact organelles with pronase. Visible clearing of the suspension revealed that pronase-dependent lysis had occurred. In this case, the yield of total mtDNA was reduced compared to that obtained by detergent lysis. However, the sucrose gradient profile of the released DNA species (Fig. 3) revealed well resolved >39 S mtDNA peaks.

Summary of Various Lysing Procedures that Yield >39 S mtDNA

Table II

| Summary of Lysing Agents that Yield >39 S mtDNA |
|--------------------------------------------------|
| In all cases the labeled mitochondria in the lysate were present at a concentration of 20 mg/ml in a solution containing NaCl (at the concentrations indicated), 10 mM Tris, 10 mM EDTA, pH 7.8, at 4°C, plus the detergents at the concentrations noted. The lysed samples were analyzed on sucrose density gradients as described under "Experimental Procedures." The specific procedures used in B are given in Fig. 3. |
| A. In the presence of 0.5 M NaCl |
| 1. 0.5% BRIJ 58 + 0.2% deoxycholate (normal method) |
| 2. 0.5% Triton X-100 + 0.5% Nonidet P-40 |
| 3. 0.5% Triton X-100 |
| 4. 0.5% Nonidet P-40 |
| 5. 0.5% Sarkosyl |
| 6. 0.5% BRIJ 58 |
| B. In the presence of 0.1 M NaCl |
| 1. Pronase (50 μg/ml) |

Fig. 3. Sucrose density gradient analysis of [1H]mtDNA released from mitochondria by lysis with pronase. The mitochondria were labeled as described under "Experimental Procedures," pelleted from the incubation medium by centrifugation at 10,000 × g, and suspended at a concentration of 15 mg of mitochondria protein/ml with gentle homogenization in 1 ml of 0.1 M NaCl, 10 mM Tris, and 5% (v/v) sucrose, pH 7.4 at 4°C. The suspension was incubated at 37°C with 50 μg of pronase for 10 min. After cooling in ice water, any unlysed mitochondria and insoluble, particulate material were removed by centrifugation at 21,000 × g for 10 min. The cleared supernatant was analyzed on a sucrose density gradient as described in Fig. 2.

Fig. 2. Second sucrose density gradient analysis of isolated >39 S mtDNA. The rapidly sedimenting mtDNA seen in Fig. 1 (bracketed material) was pelleted and resuspended in 3 ml of 0.15 M STE buffer as described under "Experimental Procedures." One milliliter of the sample was incubated for 1 h at 37°C with 100 μg of electrophoretically purified pancreatic DNase in the presence of 5 mM MgCl₂. A second 1-ml portion was SDS/phenol extracted as described under "Experimental Procedures." The specific procedures used in B are given in Fig. 3.

TABLE II

| Summary of Lysing Procedures that Yield >39 S mtDNA |
|--------------------------------------------------|
| In all cases the labeled mitochondria in the lysate were present at a concentration of 20 mg/ml in a solution containing NaCl (at the concentrations indicated), 10 mM Tris, 10 mM EDTA, pH 7.8, at 4°C, plus the detergents at the concentrations noted. The lysed samples were analyzed on sucrose density gradients as described under "Experimental Procedures." The specific procedures used in B are given in Fig. 3. |
| A. In the presence of 0.5 M NaCl |
| 1. 0.5% BRIJ 58 + 0.2% deoxycholate (normal method) |
| 2. 0.5% Triton X-100 + 0.5% Nonidet P-40 |
| 3. 0.5% Triton X-100 |
| 4. 0.5% Nonidet P-40 |
| 5. 0.5% Sarkosyl |
| 6. 0.5% BRIJ 58 |
| B. In the presence of 0.1 M NaCl |
| 1. Pronase (50 μg/ml) |

Fig. 3. Sucrose density gradient analysis of [1H]mtDNA released from mitochondria by lysis with pronase. The mitochondria were labeled as described under "Experimental Procedures," pelleted from the incubation medium by centrifugation at 10,000 × g, and suspended at a concentration of 15 mg of mitochondria protein/ml with gentle homogenization in 1 ml of 0.1 M NaCl, 10 mM Tris, and 5% (v/v) sucrose, pH 7.4 at 4°C. The suspension was incubated at 37°C with 50 μg of pronase for 10 min. After cooling in ice water, any unlysed mitochondria and insoluble, particulate material were removed by centrifugation at 21,000 × g for 10 min. The cleared supernatant was analyzed on a sucrose density gradient as described in Fig. 2.
mtDNA indicating that although the pronase was active under these conditions, this enzyme had little effect on the integrity of the rapidly sedimenting form.

>39 S mtDNA is Not an Artifactual Aggregate—In spite of the above observation that different lysing agents and procedures could release a major portion of the mtDNA as a rapidly sedimenting form, there was the possibility that the >39 S mtDNA was an artifact resulting from the aggregation of several supercoiled molecules or from the spurious binding of clusters of non-DNA constituents to a single DNA molecule. Such theoretical aggregates could increase the effective mass of the DNA yielding artificial rapidly sedimenting species. To address these possibilities experimentally, highly purified deproteinized mtDNA that sedimented as a sharp peak of 39 S when analyzed alone was mixed into the BRIJ 58/deoxycholate-lysing medium. The mixture was then added to half of a typical preparation of incubated, but unlabelled mitochondria. The other half of the mitochondrial preparation was labeled and lysed with the same medium without exogenous DNA.

Fig. 4 presents the profiles of the 3H-labeled endogenous and exogenous mtDNA species run on identical sucrose gradients. Clearly, the contact of the exogenous DNA with the constituents in a mitochondrial lysate caused no enhancement of the sedimentation rate of any portion of this DNA. A significant portion of the endogenous mtDNA, however, was in the rapidly sedimenting form (bracketed material). This result argues strongly against the likelihood that >39 S mtDNA is an artificial aggregation of several DNAs, or of DNA and other constituents in the organelle lysate, and suggests that this structure represents the natural state of much of the DNA in mitochondria.

The Interaction of >39 S mtDNA with Ethidium Bromide—The binding of ethidium bromide to covalently closed circular DNA causes a partial unwinding of the duplex resulting in a reduction in the number of negative superhelical turns (33). This event is reflected in a concomitant decrease in sedimentation rate. At a specific level of dye binding, the number of superhelices is reduced to zero yielding the closed relaxed form as represented by a s value minimum approximately equivalent to that of the nicked open circular form of the same DNA. Additional increases in dye concentration after the relaxed form is attained result in the introduction of positive superhelices into the molecule and accompanying increases in s value (33).

When nondeproteinized >39 S mtDNA was analyzed in sucrose density gradients containing increasing amounts of free ethidium bromide, a complex pattern emerged (Fig. 5). The apparent single peak seen in the absence of ethidium bromide (Fig. 6A) resolved into two slower sedimenting peaks at 0.4 to 0.7 µg dye per ml (Fig. 5). The actual profile seen at

Fig. 5. Sedimentation coefficients of >39 S mtDNA as a function of ethidium bromide concentration before and after treatment with SDS/phenol. The material in the >39 S region of a preparative sucrose gradient was pelleted and resuspended in 0.15 M STE buffer. Half of this sample was further purified by SDS/phenol extraction. The s values of the deproteinized and untreated mtDNA species were determined as a function of ethidium bromide concentration in sucrose density gradients as described under "Experimental Procedures." ○, untreated >39 S mtDNA; ●, >39 S mtDNA after SDS/phenol treatment.
an ethidium bromide concentration of 0.5 µg/ml is shown in Fig. 6B. The two peaks had sedimentation minima of 32 S and 42 S, respectively. At higher dye concentrations, these peaks reconverged into one band again and continued to increase in mean s value until a profile essentially identical with that seen in the dye-free gradient was achieved (Figs. 5 and 6).

If, however, the >39 S mtDNA was first purified by SDS/phenol extraction prior to such an analysis, the DNA behaved as a single species throughout the ethidium titration curve (Fig. 5). The response to increasing amounts of ethidium bromide was uniform and typically biphasic. In the absence of dye, the deproteinized form sedimented at 39 S, decreased to a minimum of about 27 S at an ethidium bromide concentration of 0.5 µg/ml, and returned to near 39 S in the presence of still higher amounts of dye. Such a response would be expected of a closed circular DNA that had no extraordinary constraints imposed upon its superhelical structure.

The observation that both the deproteinized form and the several species of untreated >39 S mtDNA reached s value minima at approximately the same dye concentration (Fig. 5) indicates that all these forms had similar superhelix densities. Perhaps more importantly, the fact that none of the untreated species was able to untwist to the completely relaxed 27 S form indicates that there were additional constraints superimposed upon these molecules such that they were maintained in a form that is more compact than a supercoil.

Electron Microscopic Visualization—Fig. 7 is an electron photomicrograph of >39 S mtDNA that was fixed in 0.1% glutaraldehyde and relaxed in formamide prior to application to the grid. Eighty-two of the 96 molecules examined were seen as compact rosettes that were constrained at the center in what appeared to be a rather dense core. The portions of each molecule projecting radially from the central region appeared as relaxed smooth loops. Closer examination of these molecules revealed that the loops were variable in length, and except for several D-loops (arrows), no unusual structural features were obvious along their contour. Moreover, the dense cores did not appear as mere supercoiled regions of the DNA. In some cases their knob-like appearance was suggestive of the presence of non-DNA material at these sites.

Estimates of the length of the DNA in the rosettes ranged between 4.68 and 4.99 µm. Acknowledging the difficulties in making these measurements, the contour lengths obtained agreed very well with previously reported values of about 5 µm for the length of single rat liver mtDNA molecules (34, 35). The larger rosette marked (C) in Fig. 7 measured to be about 9.62 µm indicating that two genome length molecules were present in this form. The nature of their attachment to each other, however, was undiscernible from the electron photomicrograph. Only three of the 96 molecules examined were dimers.

In addition to the tightly constrained rosettes in Fig. 7, one of the molecules shown (B) appears loosely constrained, having little or no evidence of a central, dense core. This molecule may belong to the subpopulation of >39 S mtDNA that was seen previously to be more responsive to ethidium bromide titration (Figs. 5 and 6).

Fig. 8 presents an electron photomicrograph of >39 S mtDNA that had been SDS/phenol extracted prior to preparation for electron microscopic examination. All of these molecules were in the relaxed form, and none possessed a rosette-like appearance. Comparison of the molecules in Figs. 7 and 8 suggests that deproteinization with SDS/phenol, a procedure that should have no effect on the structural characteristics of the DNA per se, probably removes proteins that stabilize the DNA in the tightly folded conformation that gives rise to the >39 S forms.

The Association of Proteins with the >39 S mtDNA—To confirm the presence of proteins bound to the rapidly sedimenting DNA and to establish the relative amounts of protein and DNA in the complex, direct chemical quantitative measurement of protein and DNA in resedimented particles from three different preparations revealed a protein to DNA weight
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FIG. 8. Electron micrographs of formamide-relaxed, SDS/phenol-treated >39 S mtDNA. The procedures used for preparation of the DNA for visualization are given under “Experimental Procedures.”

The ratio ranging between 0.24 and 0.35. Such low values are consistent with the idea that the rapid sedimentation rate of the particle does not result merely from the increased effective mass conferred by the bound proteins and lends credence to the probability that the high s value is predominantly due to its compact folded nature.

SDS-polyacrylamide slab gel analysis of the proteins bound to the >39 S DNA is shown in Fig. 9D. The bound array consists of polypeptides ranging in molecular weight between 150,000 to about 11,000. The most intense band among those from the >39 S mtDNA (M, 58,000) is undetectable in any of the other submitochondrial fractions (Fig. 9, A, B, and C). This protein’s exclusive appearance in the DNA-bound array signals its exceptional specificity for the DNA molecule. In subsequent experiments it was shown that if the DNA was degraded with pancreatic DNase prior to the second sucrose density gradient centrifugation, the fractions of the gradient that correspond to the >39 S region, where the DNA would have sedimented had it not been degraded, contained no detectable protein bands. Thus, any proteins from the mitochondrial lysate not bound to the DNA do not co-sediment as free aggregates in detectable amounts into the middle and lower regions of the gradients.

Further comparisons of the DNA-bound polypeptides with those of the other submitochondrial fractions revealed that the most consistent co-migration existed with the bands obtained from the inner membrane, particularly in the molecular weight range below 30,000. It is clear, however, that not all of the major inner membrane proteins were represented in the

![Figure 9. SDS-polyacrylamide slab gel electrophoresis of the proteins associated with >39 S mtDNA and with the submitochondrial fractions. The procedures for the isolation of >39 S mtDNA, the separation of mitochondrial inner membrane (IM), matrix, and outer mitochondrial membrane (OM) fraction, and the electrophoresis of the constituent proteins are given under “Experimental Procedures.” The gel contained proteins from the (A) inner mitochondrial membrane, (B) matrix, (C) outer mitochondrial membrane, and (D) >39 S mtDNA.](http://www.jbc.org/)

**TABLE III**

The effect of various treatments on the s value of >39 S mtDNA

>39 S [3H]mtDNA was isolated and purified by resedimentation as described under “Experimental Procedures.” Except where 2 M NaCl was used, samples of less than 10 µl of the DNA solution were diluted to 100 µl with 0.15 M STE buffer. The diluted samples were treated as indicated in the table and analyzed along with untreated controls on 5-ml gradients of 5 to 20% sucrose in 0.5 M STE buffer. The gradients were centrifuged for 90 min at 50,000 rpm in a Beckman SW 50.1 rotor, and 10-drop fractions were collected directly onto filter paper discs and counted as described under “Experimental Procedures.” Where 2 M NaCl was used, both the DNA dilution buffer and the sucrose gradients contained 2 M NaCl.

A. Treatments that have no effect on the high s value

1. Triton X-100 (1% w/v, 0°C for 1 h)
2. Brij-58 (1% w/v, 0°C for 1 h)
3. Sarkosyl (1% w/v, 0°C for 1 h)
4. 2 M NaCl 60°C for 1 h
5. Trypsin (50 µg/ml for 1 h at 37°C)
6. Pronase (100 µg/ml for 1 h at 37°C)
7. Proteinase K (100 µg/ml for 1 h at 37°C)
8. Proteinase K (100 µg/ml, 5 M urea 1 h at 37°C)
9. Proteinase K (100 µg/ml, 50 mM dithiothreitol 1 h at 37°C)
10. Heating at 65°C for 30 min
a. With no additions
   b. In the presence of 5 mM dithiothreitol
   c. In the presence of proteinase K (100 µg/ml)

B. Treatments that cause the conversion of >39 S mtDNA to the 39 S form

1. SDS (1% w/v, 0°C for 1 h)
2. Phenol extraction (no SDS present)
3. 2 M NaCl (37°C for 1 h)
4. Heating at 65°C for 30 min with 5 mM dithiothreitol and proteinase K (100 µg/ml)
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>39 S array. Furthermore, the relative intensities of the coelectrophoresing DNA-bound bands were different from the relative intensities of the corresponding proteins seen in the total inner membrane profile. Thus, if the lower molecular weight polypeptides associated with the >39 S mtDNA are in fact derived from the inner mitochondrial membrane, then only a specific portion, rather than a large random fragment, of this membrane remains bound to the DNA under these isolation conditions.

The Stabilizing Features of the >39 S mtDNA—Table IIIA contains an extensive list of treatments that were used to probe the structural integrity and stabilizing features of the >39 S mtDNA. Each treatment was chosen on the basis of its probable efficacy in altering protein structure in order to establish the minimal perturbation required to disrupt the DNA-protein interaction and thus unfold the >39 S particle to the 39 S form. It was surprising that none of the treatments listed in Table IIIA had any effect on the high s value of the >39 S mtDNA. It was particularly curious that none of the proteinase treatments affected the rapid sedimentation rate of the particle. Thus, in order to elucidate any role played by the bound proteins specifically in the maintenance of the folded structure, it was necessary to determine whether the enzymatic proteolyses used were digesting any of the proteins associated with the DNA, and if so, which proteins, or portions of proteins, remained bound to stabilize the rapidly sedimenting form. To this end, half of a preparation of >39 S mtDNA was treated with proteinase K prior to the second sucrose gradient purification step. The polypeptides remaining associated with the untreated and digested complexes are shown in Fig. 10. The pattern given by the untreated sample (Fig. 10B) was consistent with previous preparations (compare with Fig. 9D). In the proteinase K-treated sample (Fig. 10A), however, all of the high molecular weight proteins were missing, but one main low molecular weight polypeptide ($M_r = 10,500$) and a faint band ($M_r = 15,500$) were detectable under these conditions.

![Fig. 10](http://www.jbc.org/)

**Fig. 10.** Analysis of the polypeptides remaining bound to >39 S mtDNA treated with proteinase K prior to resedimentation. The >39 S mtDNA was isolated on preparative sucrose density gradients, pelleted, and resuspended in 2 ml of 0.15 M STE buffer. Half of the sample was incubated with proteinase K at a concentration of 100 μg/ml at 37°C for 1 h. The remaining half was incubated in like fashion, but without the proteinase K. Both samples were cooled in ice, resedimented on separate sucrose density gradients, and the constituent polypeptides were analyzed on SDS-polyacrylamide gels as described under “Experimental Procedures.” (A) proteinase K digested; (B) untreated control.

![Fig. 11](http://www.jbc.org/)

**Fig. 11.** The effect of dithiothreitol, heating at 65°C, and proteinase K treatment on the sedimentation velocity of >39 S mtDNA. The 3H-labeled >39 S mtDNA was isolated and resedimented as described under “Experimental Procedures.” Samples of less than 5 μl were diluted to a volume of 50 μl with 0.15 M STE buffer and incubated for 1 h under the conditions indicated on the figure. When present, the proteinase K and dithiothreitol were at a concentration of 100 μg/ml and 5 mM, respectively. The samples were cooled in ice and analyzed on sucrose density gradients as described in Table III.
conditions. In other experiments a second faint band with a molecular weight of about 18,000 was sometimes seen. Each of these bands appeared to be a discrete partially digested remnant of proteolysis, because none coelectrophoresed exactly with bands from the untreated sample. It was, therefore, apparent that one or more of these tightly bound remnants played a critical role in the maintenance of the compact supramolecular structure of the DNA.

Table IIIIB presents a listing of the rather severe treatments that were required to unfold the compact structure to the 39 S form. Each of these treatments presumably acts to alter the structural integrity of the stabilizing proteins such that they are no longer able to maintain the back folding of the DNA domains. Although the requirement for such severe treatments is indicative of the extreme stability of the complex, it is clear that covalent modification is not necessary for conversion to the 39 S form. The final entry in the table indicating the efficacy of digestion of the complex with proteinase K in the presence of dithiothreitol at 65°C confirms that proteins do indeed play a central role in the maintenance of the compact structure. Furthermore, as seen in Fig. 11, if any of these three conditions (i.e. digestion with the proteinase, the presence of a disulfide reductant, or elevated temperature) is not met, then unfolding to the 39 S form does not occur. Thus, it can be deduced that portions of the proteins that are crucial to the maintenance of the compact structure can be classified as "tight" (36) polypeptides known to be insensitive to proteolysis. This would, therefore, be a major difference between the mitochondrial folded genome and the Escherichia coli nucleoid in which both RNA (13, 39, 40) and protein (40) have been implicated in the maintenance of the tertiary folding. There remains the possibility, however, that RNA may still be a stabilizing component of the >39 S mtDNA, but it may be so highly sequestered in the interstices of the dense DNA core that it is inaccessible to enzymic degradation.

From the work reviewed by Borst and Kroon (41) and by Nass (1) it can be calculated that rat liver contains between 2 to 10 mtDNA molecules per mitochondrion. The specific organization of the several copies of mtDNA inside each organelle is at present unknown. It is possible that several of these circular molecules exist naturally as dimeric or oligomeric clusters that are bound to each other by some non-DNA liaison such as a common membrane binding site (14-16) or other macromolecular coupler. Because of the gentle nature of our isolation procedure, it was postulated that >39 S mtDNA may be the isolated form of such a natural cluster. In the electron microscope analyses, however, no large clusters of molecules were seen. Most of the rosette-like structures were of single genome size. The few larger structures seen were roughly 10 μm in length, and it was assumed that these dimers were interlocked circles because they were present at a frequency well within the range noted for normal rat liver mtDNA isolated by more conventional methods (10). Thus, this evidence suggests that >39 S mtDNA is an isolated packaged form of individual mtDNA molecules.

The biphasic response of the compact DNA to ethidium bromide titration shows that superhelical domains are present in the structure. The reason why there were several species in the untreated >39 S mtDNA that responded differently to ethidium bromide is unclear at this point. Several plausible explanations exist. It is possible that the constraints on the DNA were less stringent on certain molecules in the population. These "looser" structures may have been more easily disrupted and unfolded by the dye than those that were more tightly contained. For example, it is conceivable that molecules actively transcribing RNA may undergo a relaxation mechanism resulting in a loosening of the folded structure to allow RNA synthesis to occur (38). In addition, the compact structure of molecules that are in the process of nascent strand expansion during replication of the DNA must be unraveled and unfolded in order to achieve separation of the parental strands in the replicating regions. The validity of either of these possibilities has yet to be confirmed.

The results indicating that some of the DNA-bound proteins may be derived from the mitochondrial membranes are consistent with other reports (15, 16) of the isolation of DNA/membrane complexes from animal mitochondria using similar gentle lysis methods. However, the observation that the major protein associated with the >39 S mtDNA is undetectable in any of the other submitochondrial fractions clearly demonstrates the specificity of this protein for binding the DNA and suggests that it is either a nonmembrane protein or present in the membranes in very low concentration.

The compact DNA structure from rat liver mitochondria described here contrasts markedly to the chromosome-like structure isolated by Pinon et al. (17) from X. laevis oocyte mitochondria. Although these workers used the same lysing detergents, the lysing medium contained 0.15 M NaCl and the polyamine spermidine. Under those conditions a rapidly sedimenting form of the mtDNA was isolated. This structure was seen in the electron microscope as a 1.54-μm open circle having no apparent central dense core and exhibiting a thin fibril studded with globules presumed to be proteins. Their estimates of the protein to DNA ratio were about 3 times higher than those calculated here for >39 S mtDNA, and
their structure was sensitive to treatment with 1% Sarkosyl. The reason for these major differences between the two similarly isolated structures is not yet clear and must await further studies.

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