CHARACTERIZATION OF A MITOCHONDRIAL REPLICATION COMPLEX FROM PARAMECIUM AURELIA

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Submitted to the University of Edinburgh
as a thesis in fulfilment of the requirement
for the degree of Doctor of Philosophy

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August, 1981.

SUMMARY

A membrane-DNA complex has been isolated from Sarkosyl lysed mitochondria of Paramecium aurelia. Apart from DNA, which has been demonstrated to be of mitochondrial origin, the complex contains proteins involved in the process of DNA replication. When used as a sole source of enzymes and DNA template, the membrane-DNA complex was capable of synthesizing mitochondrial DNA in vitro. The incorporation of (^{3}H)dTTP was dependent on the presence of endogenous mitochondrial DNA template, magnesium ions and required four deoxyribonucleoside triphosphates for its maximal activity. Actinomycin and ethidium bromide were found to have an inhibitory effect on the incorporation of the label. It was demonstrated that the in vitro DNA synthesis by the membrane-DNA complex involved formation of known replicative intermediates (lariats and dimers) characteristic of the replication of mitochondrial DNA in vivo. It was concluded, therefore, that the membrane-DNA complex represents a mitochondrial replication complex.

The complex was further characterized in terms of protein content by SDS polyacrylamide gel electrophoresis. The electrophoretic comparison of complexes isolated from normally grown cells and cells exposed to drugs affecting mitochondrial DNA replication in Paramecium showed some protein variation. Preliminary attempts were made to remove the proteins not involved in the process of DNA replication by salt extraction.

Electron microscope studies of the replication complex revealed a novel chromatin-like structure of the mitochondrial genome. Biochemical studies of the putative mitochondrial chromatin showed the presence of five basic proteins which behaved in terms of their acid solubility and electrophoretic mobility similarly to histones extracted from nuclear chromatin. Evidence was obtained for protection of mitochondrial DNA

from random digestion by nucleases, thus implying the direct association of DNA with basic proteins. The evolutionary implications of the discovery of chromatin-like structure in mitochondria are discussed.

CONTENTS

INTRODUCTION

The existence of cytoplasmic genes was suggested as early as 1909 by Correns and Baur as a means of explaining certain nonmendelian genetic properties in plants. Although information which supported the idea of cytoplasmic genes slowly accumulated during the next fifty years, it was not until the early sixties that convincing evidence for the presence of DNA in chloroplasts (Ris and Plant, 1962) and mitochondria (Nass and Nass, 1963) was established. The discovery of DNA and of a protein synthesizing system in mitochondria has opened up new avenues for the investigation of mitochondrial formation and function. The use of genetic techniques coupled with the technical sophistication of molecular biology, biochemistry and electron microscopy has promoted a better understanding of the biogenesis of this complex organelle. Most recently the entire human mitochondrial genome has been sequenced (Anderson et al., 1981), which together with the analysis of mitochondrial transcripts (Montoya et al., 1981; Ojala et al., 1981) provides new insights not only into mitochondriogenesis but also into the evolutionary origin of the organelle. One might think that sequencing of mitochondrial DNA (mt DNA) would complete the study of mitochondrial biogenesis; in reality, however, it has stimulated several new questions such as those concerning unidentified reading frames (URF's) coding for as yet unknown proteins (Anderson et al., 1981; Borst and Grivell, 1981) and the mechanism of splicing and expression of split genes (e.g. in yeast - Lazowska et al., 1980; Bos et al., 1980). At the same time some of the old problems concerning replication of the mitochondrial genome, and the involvement of proteins and their precise role in this process, as well as their genetic control, still remain unanswered.

The application of genetic methods to the study of mitochondria

has generated many new ideas, stimulating researchers to dissect mitochondrial structure and function into its component parts in a way which would not have been possible by morphological or biochemical studies alone. The simplicity of the mitochondrial genetic system has proved to be particularly advantageous, making certain types of investigation possible. For example, studies of the mode of mt DNA replication are greatly facilitated by the small size of the mitochondrial genome, allowing intact DNA molecules to be isolated. In addition, it is presumed that the protein system involved in the replication of mt DNA is less complex and therefore easier to investigate than the analogous replicative machinery in the nucleus. Since a major part of mitochondrial biogenesis is controlled by the nucleus, an analysis of the regulation of the mitochondrial genetic system should also yield information about the more complex problem of nuclear control mechanisms in eukaryotic cells.

Paramecium aurelia is a unique organism from a genetical point of view. Over the last thirty years investigation into a variety of phenomena: kappa particles, mating types, serotypes and the pattern of organisation of surface structures, have all involved some form of cytoplasmic inheritance (Preer, 1968, 1971; Preer et al., 1974). These early experiments established Paramecium as an excellent organism with which to study cytoplasmic inheritance and nucleo-cytoplasmic interactions. The study of such a broad spectrum of cytoplasmic inheritance in Paramecium has been possible due to several advantages the organism offers for the investigation of extranuclear and in particular mitochrondrial genetics. (i) During conjugation - one of the sexual modes of reproduction in Paramecium - the extent of cytoplasmic exchange can be experimentally controlled, making it feasible to follow the transmission of mitochondrial markers. (ii) Paramecium contains several

thousands of mitochondria (Adoutte, 1974) and biochemical preparations of these are easily obtained. (iii) The large size of the cell (100μm) allows the handling of individual cells, and has allowed the development of microinjection techniques (Koisumi and Preer, 1966: Koisumi, 1974; Knowles, 1974). (iv) Populations of mixed mitochondria can be obtained either by isolating exconjugant animals from pairs in which cytoplasmic exchange has occurred or by mitochondrial injection.

The name Paramecium aurelia actually covers many genetically isolated "species", formerly denoted varieties or syngens. The microinjection technique facilitates the construction of interspecies hybrids in which mitochondria of one species can function and replicate in the nuclear environment of other species. Such hybrids provided a unique opportunity to study the degree of autonomy of mitochondria in relation to the nucleus. Paramecium aurelia has therefore proved a valuable organism in which to study the expression of mitochondrial mutations and mitochondrial selection.

No system used for experimental research is without some disadvantages, and this applies equally to Paramecium. In the laboratory, the organism is grown in a grass infusion seeded with a pure culture of the bacterium Klebsiella aerogenes. Consequently, in purifying mitochondria there is always the risk of bacterial contamination of the mitochondrial pellet. This problem may be overcome, if necessary, by growing Paramecium in axenic cultures (Sonneborn, 1970). Another disadvantage of Paramecium is the absence of detectable complementation and recombination between mitochondrial genes (Beale et al., 1972; Adoutte and Beisson, 1972; Beale, 1973) which precludes further studies in mitochondrial genetics. More recently a thorough search for recombination of various mitochondrial genes, including those controlling resistance

to various antibiotics, temperature sensitivity, and suppressors of the cl_1 mutant, has been carried out, again with negative results (Adoutte et al., 1979).

The development of Paramecium mitochondrial genetics essentially began a little over a decade ago with the demonstration by Beale (1969) of cytoplasmically inherited resistance to the drug erythromycin in P. primaurelia (formerly syngen 1). Further reports of similar phenomena soon appeared (Adoutte and Beisson, 1970; Beale, 1973; Adoutte, 1974). Beale et al. (1972) provided evidence that drug resistance arose through mitochondrial mutation by showing that the injection of mitochondria from resistant cells into sensitive cells led to the transformation of the recipients. In addition, it was shown that the change from sensitivity to resistance was accompanied by a change in one or more mitochondrial proteins. Experiments performed by Perasso and Adoutte (1974) and by Knowles (1974) suggested that during the process of transformation from drug sensitivity to resistance, an active multiplication of resistant genomes occurred under selective conditions where little or no cell division took place. Cummings et al. (1976) subsequently demonstrated by restriction enzyme analysis that following the injection of mitochondria from drug resistant cells into a sensitive strain, the development of resistance in the recipient was accompanied by the replacement of its own mt DNA by that of the donor.

During conjugation in P. aurelia, nuclear exchange normally occurs without cytoplasmic exchange. Therefore, where intraspecific genetic variation exists for mitochondrial proteins it is possible to determine whether variant phenotypes are determined by nuclear or mitochondrial genes. Thus Tait (1968; 1970) was able to show that the mitochondrial membrane enzyme 3-hydroxybutyrate dehydrogenase in P. novoaurelia and

the mitochondrial form of NADP-dependent isocitrate dehydrogenase in P. biaurelia were under the control of nuclear genes. The method can be extended to include interspecific genetic variation by making use of nucleo-mitochondrial hybrids produced by microinjection techniques. In this way Knowles and Tait (1972) added fumarase to the list of nuclear coded mitochondrial enzymes. A further extension of the approach to include mitochondrial ribosomal proteins allowed Tait et al. (1976a; 1976b) to demonstrate that some proteins, probably the majority, were coded by the nucleus while others were determined by the mitochondrial genome. With regard to mitochondrial ATPase there is evidence that in Paramecium at least two polypeptides are controlled by the mitochondrial genome (Beale and Tait, 1981). Furthermore, studies with hybrids produced by microinjection of mitochondria have shown that some of the membrane proteins (chloroform/methanol soluble fraction) are determined partly by mitochondrial genes (Tait et al., 1976b). Analysis of restriction endonuclease digests combined with hybridization studies of labelled 14S and 20S rRNAs revealed that in Paramecium, as in other organisms, two mitochondrial ribosomal RNA genes are coded by mt DNA (Cummings et al., 1980).

During the last few years studies of Paramecium mitochondria have been extended to include the elucidation of the mode of replication of mt DNA. In contrast to the circular molecules found in a variety of organisms the mitochondrial genome of Paramecium has been shown to be a linear molecule, 13.8μm in length (Goddard and Cummings, 1975). The mass of the Paramecium mt DNA is 27.1×10^6 daltons, as determined by electron microscopy (Goddard and Cummings, 1977) or restriction enzyme digestion (Maki and Cummings, 1977). The only other reported linear mt DNA was that of another protozoan organism, Tetrahymena pyriformis (Suyama and Miura, 1968; Borst, 1972). The mode of replication of

Paramecium mt DNA has been described by Goddard and Cummings (1975, 1977) and its schematic representation is given in Figure 1. Replication of the linear monomer is initiated by closure at a unique end, followed by unidirectional synthesis via a lariat intermediate, a form consisting of a circle and a tail. As replication continues, the circle becomes progressively larger, while the size of the tail decreases. The processing of DNA in this manner requires a strong linkage (a covalent cross-link) at the initiation end of the molecule which is maintained throughout the replication process. The replication results in the formation of a linear dimer in which the two monomer units are arranged in a head-to-head configuration. The final step is the conversion of the dimer to two semi-conservatively constituted monomer length molecules and this process occurs by an as yet unknown cleavage at the head-to-head initiation site. The dimer form of the initiation region for four species of Paramecium DNA has been cloned and characterized (Pritchard et al., 1980). Most recently, sequencing of the initiation region (Cummings et al., 1980) revealed that in the distal segments of this fragment the sequences are palindromic as expected for a head-to-head dimer molecule; however, in the central region there is an A+T rich nonpalindromic sequence. It was proposed that this nonpalindromic sequence constitutes the cross-link of the duplex strands at the initiation of replication. This form of replication so far remains unique for Paramecium since, interestingly, linear Tetrahymena mt DNA is replicated bidirectionally from the centre of the molecule resulting in the formation of an "eye" type intermediate (Upholt and Borst, 1974; Arnberg et al., 1974). The patterns of replication of both these linear forms of DNA contrast with the asymmetric unidirectional mode of replication leading to D-loop intermediates characteristic for circular mt DNAs in other organisms (Kasamatsu and Vinograd, 1974).

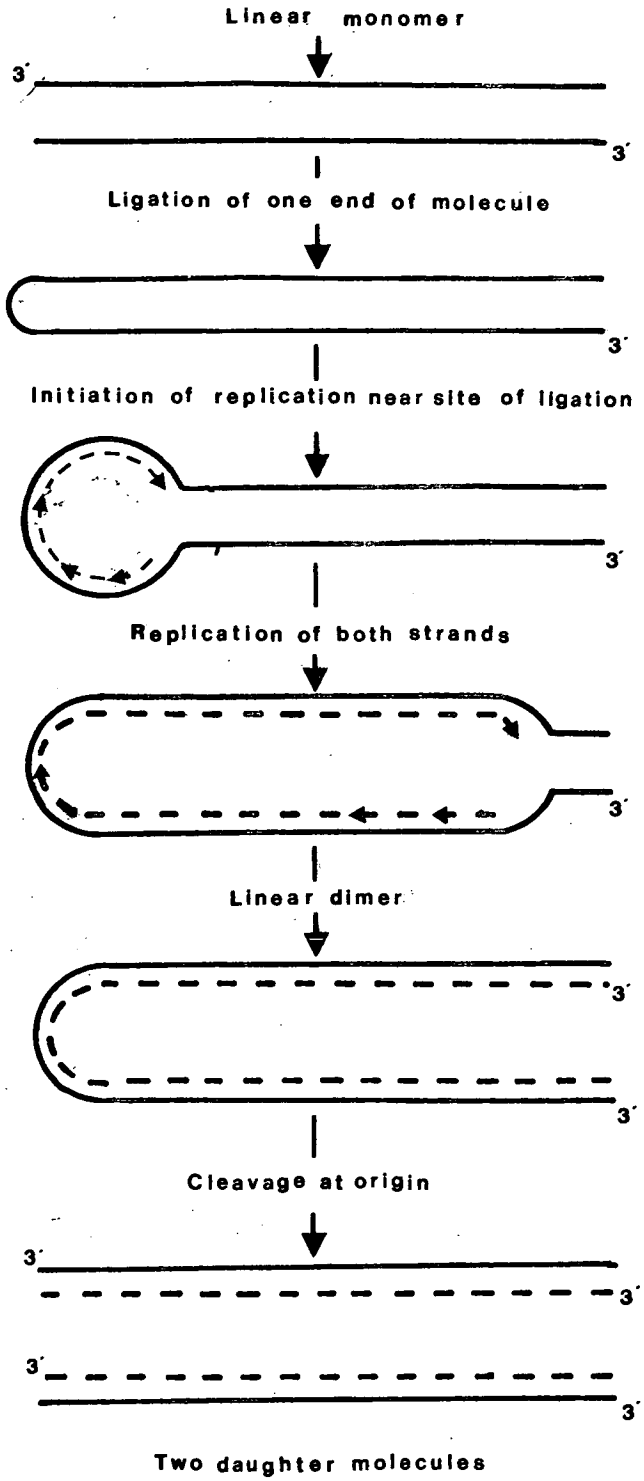


Figure 1. Replication of mt DNA of Paramecium aurelia (after Goddard and Cummings, 1975). The dashed lines represent the newly synthesized daughter strands.

Although the modes of replication of mt DNA in various organisms have been well defined, the extent of knowledge concerning the proteins involved in this process is still rather limited. The conceptual basis for replication of DNA in mitochondria is derived from prokaryotic models. It is generally accepted that DNA is replicated by a multi-enzyme complex incorporating a multiplicity of gene products. In prokaryotes, genetic analysis and the development of *in vitro* replication systems have permitted characterization and functional analysis of many components of the replication complex. The first attempts to identify and reassemble the protein components required for DNA replication were reported by the Kornberg group (Schekman *et al.*, 1974). These proteins were resolved with the aid of a simple viral DNA, whose replicative intermediates were well defined, in conjunction with cell free extracts obtained from a strain of *E. coli* carrying thermosensitive DNA replication mutants. Currently the basic list of protein components considered necessary for replication of prokaryotic DNA includes DNA polymerases, helix-destabilising protein, DNA unwinding enzymes, DNA ligase, and RNA primer synthesizing and erasing enzymes.

It seems a reasonable assumption that a multienzyme system involving similar protein components is responsible for the replication of mt DNA. At present there is only limited evidence available for the proteins involved in DNA replication in this organelle. Of the possible replication proteins in mitochondria, the DNA polymerases have been most extensively studied because of their clear involvement in DNA synthesis. From the first studies of Kalf and Ch'in (1968) and Meyer and Simpson (1968, 1970) it has been assumed that mitochondria possess a unique DNA polymerase and it has been shown that some of its properties are different from those of the nuclear enzyme. Rat liver (Meyer and Simpson, 1970; Fujisawa *et al.*, 1977), HeLa cells (Tibbetts and

Vinograd, 1973; Fry and Weissbach, 1973; Radsak et al., 1976), yeast (Wintersberger and Wintersberger, 1970; Wintersberger and Blutsch, 1976) and Xenopus laevis oocytes (Bazzicalupo, 1979) have been sources for mitochondrial polymerase. The rat liver enzyme has been partially purified but its low yield and high lability have delayed full purification and characterization. It is reported to be a salt stimulated enzyme with a molecular weight of about 150,000 (Probst and Meyer, 1973). Similar characteristics were described for the polymerase isolated from mouse liver mitochondria (Hecht, 1975). The enzyme from yeast mitochondria (Wintersberger and Wintersberger, 1970) and that obtained in one study from HeLa cells (Tibbetts and Vinograd, 1973) also have a molecular weight of about 150,000 but are not stimulated by the addition of salt. Another study with mitochondria isolated from HeLa cells claimed that two DNA polymerase activities were present (Fry and Weissbach, 1973). One of these was DNA polymerase γ and the other a new DNA polymerase, which had a molecular weight of about 106,000 and which was quite different from the other cellular enzymes. This latter activity was considered to be the mitochondrial DNA polymerase (Radsak et al., 1976) and was also found in preparations of mitochondria of KB cells (Wang et al., 1975) and mouse cell cultures (Radsak and Seidel, 1976). However, later work has shown that the 106,000-dalton enzyme was probably a mycoplasma-derived contaminant, as mitochondria from mycoplasma-free HeLa cells or from rat liver contain only one DNA polymerase activity, which is very similar to the corresponding cellular γ-polymerase (Bolden et al., 1977). This would imply that in higher cells there is no uniquely different class of mitochondrial DNA polymerases. A more recent report (Bazzicalupo, 1979) provided evidence that in Xenopus laevis oocytes, too, the enzyme that most resembles the γ-polymerase of other systems is the mitochondrial DNA polymerase. However, unlike the

enzyme in HeLa cells and rat liver, the polymerase in Xenopus oocytes seems to be present only in mitochondria.

Recently the presence of enzymes, other than polymerase, involved in the replication of mt DNA has been reported for rat liver mitochondria. These included a nicking-closing enzyme (Fairfield et al., 1979) and a DNA gyrase (Castora and Simpson, 1979), the contribution of which to helix unwinding in this system may be essential. Thus, while the evidence is still rather limited it does nevertheless suggest the involvement of a multienzyme complex in the replication of mt DNA.

The aim of this study therefore was to investigate the proteins associated with mt DNA, their involvement in DNA replication, and the site of their genetic control. There are several ways in which the problem of the proteins involved in replication of mt DNA can be elucidated. The most promising approach at present is through the use of methods designed to disturb the DNA-synthetic machinery as little as possible, thereby encouraging DNA replication in vitro by a process similar to that observed in vivo. This can be best achieved by using an intact replication complex isolated from mitochondria. Such a complex, apart from containing mt DNA as well as a complete set of replication proteins, should be capable of replicating the mitochondrial genome in vitro. This is in a way an analogous approach to Kornberg's studies (mentioned previously) of the proteins involved in the replication of E. coli DNA; the difference being the use of exogenous viral DNA (in the case of Kornberg) as opposed to the endogenous DNA in the case of mitochondria. The lack of mutants affecting DNA synthesis in mitochondria is compensated by the fact that in Paramecium the process of replication can be easily blocked at various stages by growth of the cell in presence of various drugs. Thus, the cell extracts of E. coli thermosensitive

mutants used by Kornberg, in the mitochondrial studies could be replaced by sets of proteins isolated from the replication complexes of drug treated cells.

It is thought that apart from elucidating some problems concerning the replication of mt DNA, the study involving the replication complex might also provide new insights into organisation of the mitochondrial genome. It is not known whether any mt DNA is organised in a chromatin-like structure that resembles that of the nuclear DNA. At present it is generally believed that mt DNA, like that of bacteria, is "naked", though this appearance could be due to the drastic methods used in preparing the DNA. One might expect that if the replication complex represented in vivo association between DNA and proteins it would provide suitable material for investigating the validity of the current view on the prokaryotic-like organisation of the mitochondrial genome.

In the study presented in this thesis mitochondria of Paramecium aurelia were chosen as a suitable model system for the isolation and characterization of the replication complex. This system has several advantages for such studies: (i) the mode of replication of the linear mitochondrial genome has been relatively well defined and its replicative intermediates are easily identified; (ii) the replication of mt DNA in Paramecium can be specifically blocked at the stage of lariats or dimers, thus leading to the accumulation of these intermediates (Goddard and Cummings, 1975); (iii) provided interspecies variation in proteins involved in replication of Paramecium mt DNA can be detected, this system offers a unique opportunity to determine their genetic control, since by the microinjection technique it is possible to obtain interspecies hybrids containing mitochondria of one species and nuclei of the other, and (iv) drug inhibition studies provide provisional evidence that at

least one of the replication proteins from Paramecium mitochondria is synthesized on mitochondrial ribosomes and therefore probably coded by the mitochondrial genome (Goddard and Cummings, 1975).

The work to be presented in this thesis is organised into three major sections:-

1. Methods of isolation of a membrane-DNA complex. Since the original technique for the isolation of such a complex was designed specifically for mitochondria of rat liver (Van Tuyle and Kalf, 1972), several modifications were required in order to apply such a method to the mitochondria of Paramecium. This section, therefore, deals with the development of the methodology for the isolation of the membrane-DNA complex (subsequently designated as the mitochondrial replication complex) from Paramecium aurelia.
2. Characterization of the complex. This section presents a description of the complex in terms of its origin, DNA and protein content and its ability to replicate mt DNA in vitro.
3. Mitochondrial chromatin. This section provides an electron microscopic and biochemical characterization of the novel chromatin-like structures of the mitochondrial genome, obtained from the replication complex.

MATERIALS AND METHODS

1. Materials

The following strains of *Paramecium* were used in these studies: *P. primaurelia* (syngen 1), stock 513; *P. septaurelia* (syngen 7), stock 227 and a hybrid strain obtained by Knowles and Tait (1972) using a microinjection technique. The hybrid strain was a stock derived from a cell of syngen 7 injected with mitochondria from syngen 1.

2. Methods

2.1 Culture conditions

Cells were cultured at 27°C in Scotch grass infusion containing 9mM $Na_2 HPO_4$ (pH 7.6 - 8.0) and inoculated with *Klebsiella aerogenes* (Sonneborn, 1970) or in an axenic medium (Soldo *et al.*, 1966). Culture volumes of 750ml were maintained in 30-60 Thompson bottles incubated in a horizontal position to ensure maximum aeration. Cell growth was initiated by the addition of cells to a starting density of 150-200 cells/ml. During exponential growth the cultures were supplemented twice with additional bacteria obtained by centrifugation of nutrient broth cultures and resuspension of the bacterial pellets in a small volume of the grass infusion. The total amount of bacteria used for feeding approximated to 1 litre of overnight bacterial culture per 10 bottles of *Paramecium*.

In some experiments the cells were grown in the presence of drugs (ethidium bromide or chloramphenicol). Ethidium bromide (4µg/ml) was added to exponentially growing cultures and the cells were harvested several generations later. A similar procedure was used for growth of cells in chloramphenicol, which was added to a final concentration of 25µg/ml during balanced growth, and the cells were harvested 15-20 hours later.

2.2 Harvesting of Paramecium cells

Paramecium cells were harvested when the culture had "cleared" (i.e. after disappearance of the initial cloudiness caused by the addition of concentrated bacteria) which normally took place about 40 hours after the initiation of the cell culture. The cultures were filtered through a layer of absorbent cotton wool to remove debris from the culture. The filtrate was concentrated to approximately 400ml by harvesting in an M.S.E. continuous flow centrifuge head (3,000rpm at a flow rate of about 800ml/min). The cells were subsequently washed twice with 400ml of Dryl's solution (2.0mM sodium citrate, 1.1mM $Na\ H_2PO_4 \cdot H_2O$, 1.0mM $Na_2\ HPO_4$, 1.4mM $CaCl_2$ pH 7.9 - Dryl, 1965) and then subjected to final packing by centrifugation at 1,700rpm in pear-shaped bottles in an M.S.E. oil testing centrifuge for 4 minutes. In general, a balanced growth culture yielded approximately 0.5ml of packed cells per litre of culture.

2.3. Preparation of mitochondria

The packed cells were resuspended in ten volumes of ice cold mannitol buffer containing 0.44M mannitol, 1mM MOPS*, 0.25% bovine serum albumin (BSA), pH 7.2. The resulting suspension was homogenised twice in a pre-cooled stainless steel homogenizer and the mitochondrial fraction was isolated and purified by differential centrifugation (Suyama and Preer, 1965) as follows. The supernatant obtained from centrifugation of the homogenate at 600xg for 6 minutes at 4^oC was collected, supplemented with EDTA to a final concentration of 2mM and recentrifuged at 5,000xg for 6 minutes. The resulting mitochondrial pellet was resuspended gently in mannitol buffer supplemented with 2mM EDTA and centrifuged at 5,000xg for another 6 minutes. This step was repeated twice giving the final fraction of purified mitochondria.

* morpholinopropane sulphonic acid

2.4 Isolation of a membrane-DNA complex

A membrane-DNA complex was prepared from mitochondria using a technique based on the procedure described by Shearman and Kalf (1975). The first step involving the removal of the outer membrane from mitochondria was achieved by treatment of mitochondria with digitonin as follows. Purified mitochondria suspended in mannitol buffer plus 2mM EDTA were mixed with one volume of digitonin solution (1mg of digitonin/ 10mg of mitochondrial protein). The mixture was stirred gently for 15 minutes at $0^{o}C$ and then diluted with 3 volumes of mannitol buffer containing 2mM EDTA. The separation of lysed mitochondrial outer membranes from the inner-membrane matrix (mitoplast) was achieved by centrifugation at 9,000xg for 10 minutes, followed by two washes of the pelleted mitoplasts. The final mitoplast pellet was resuspended in 8.5% sucrose-TMK buffer (10mM Tris-HCl, pH 7.4, 10mM magnesium acetate, 100mM KCl) and a 3ml sample of this suspension containing 15mg of mitochondrial protein was mixed gently with 0.1ml of 15% Sarkosyl. The mixture was immediately layered on a discontinuous sucrose gradient consisting of two 10ml layers of 15% and 35% sucrose-TMK buffer. The gradient was centrifuged at 23,000xg for 15 minutes in a swing out rotor. A membrane-DNA complex (visible as a sharp white band) was collected with a wide-bore pipette from the interface between the 15% and 35% sucrose. The complexes from multiple gradients were pooled and after concentration by centrifugation (23,000xg for 15 minutes) the sample was exhaustively dialysed for 21 hours against 10mM Tris, pH 8.5, 20% glycerol.

In the experiments where the membrane-DNA complex was obtained from intact mitochondria and not from an inner-membrane matrix preparation (see Results) the following steps of the technique described above were modified: (i) BSA was omitted from mannitol buffer used for the preparation of mitochondria; (ii) the step involving digitonin treatment

was omitted in its entirety and (iii) Sarkosyl was applied directly to the purified mitochondria.

In the later course of these studies an additional step involving purification of the membrane-DNA complex was introduced. In the case of the purification of the complex the original glycerol buffer used for dialysis of the complex was supplemented with 10mM EDTA. The dialysate was subsequently purified by differential centrifugation as follows. The supernatant collected after twice-repeated centrifugation at 7,500xg for 10 minutes was recentrifuged at 34,000xg for 15 minutes. The resulting supernatant contained the purified membrane-DNA complex. All operations of isolation and subsequent purification of the complex were carried out at $0^{o}C$.

2.5 Spectrophotometric assay of enzymes

Monoamine oxidase was assayed as described by Tabor et al. (1954). Samples were activated with 0.3mg Lubrol per mg mitochondrial protein for 15 minutes at $4^{o}C$. Oxidation of benzylamine (5mM) to benzaldehyde was recorded at 250nm in phosphate buffer, pH 7.6.

Kynurenine hydroxylase was measured as described by Bandlow (1972). Oxidation of NADPH was followed by monitoring the decrease in optical density at 340nm in the presence of L-kynurenine for a period of 20min.

Rotenone-insensitive NADH-cytochrome c reductase was measured by following the reduction of cytochrome c at 550nm. The assay mixture contained: 50mM phosphate buffer, pH 7.5, 0.3mM KCN, 0.1mM cytochrome c, 1.5µM rotenone and 0.1mM NADH used as a substrate (Werner and Neupert, 1972).

3-Hydroxybutyrate dehydrogenase was assayed as described by Robinson and Coon (1957). The assays were run in the direction of NAD reduction with 3-hydroxybutyrate as substrate.

Succinate dehydrogenase was determined according to Slater and Bonner (1952) by following oxidation of succinate at 400nm.

Malate dehydrogenase was assayed as described by Ochoa (1955) by measuring the decrease in absorbance of NADH at 340nm.

Glutamate dehydrogenase was determined according to the method of Strecker (1955).

Adenylate kinase was assayed as described by Sottocasa et al. (1967a) following the conversion of ADP to ATP + AMP and coupling the formation of ATP to the reduction of NADP with hexokinase and glucose--6-phosphate dehydrogenase. The absorption change was measured at 340nm.

The assays were carried out in cuvettes of 3ml volume and 10mm pathlength using a Pye Unicam SP 30 UV Spectrophotometer and a Pye Unicam DR 16 digital printer. The change in absorption over the first minute was calculated and taken to be a measure of the initial velocity. In the assays 0.1ml of mitochondrial extract or submitochondrial fractions (i.e. outer membranes or mitoplasts) were added to each cuvette, and diluted until an almost linear trace of transmittance with time was obtained.

2.6 Standard labelling procedures

The DNA polymerase activity of the membrane-DNA complex was examined in an incubation mixture similar to that described by Shearman and Kalf (1975). The complete system consisted of 100mM Tris-HCl pH 7.0; 10mM $MgCl_2$; 0.27mM dATP, dCTP, dGTP; 1μM (^{3}H)dTTP (specific activity 30μCi/nmole) and the membrane-DNA complex (100-200μg protein/ml). Incubation was carried out in duplicate with shaking at 30°C for various lengths of time (noted in the figure legends). Samples (100μl) were collected on the glass fibre disc filters and immediately placed in ice cold 10% trichloroacetic acid for 30 minutes. The filters were

additionally washed twice in 5% trichloroacetic acid for 20 minutes, in ethanol for 15 minutes and finally in ether for 15 minutes. After drying, the filters were placed in vials containing 10ml of scintillation solution (prepared by dissolving in 1 litre of toluene 5g of 2,5-diphenyloxazole (PPO) and 100mg of ρ-bis (2-(5-phenyloxazolyl)) benzene (POPOP) and acid insoluble radioactivity was determined in liquid scintillation counter. The background varied between 20-30cpm; efficiency for (^{3}H) was 33%.

Nuclei were labelled with (^{3}H)-thymidine and isolated by previously described techniques (Cummings et al., 1974; Cummings and Tait, 1975).

2.7 Extraction of DNA from a mitochondrial membrane-DNA complex

The technique used for extraction of DNA from the membrane-DNA complex was based on the method described by Gause et al. (1973). The membrane-DNA complex was lysed with 2% SDS and incubated with pronase (1mg/ml) for 2 hours at 30^{o}C. After cooling the samples, 7M CsCl was added to a final concentration of 1M and the insoluble caesium dodecyl sulphate was removed by brief centrifugation in a bench centrifuge. The DNA was extracted from the supernatant with water saturated phenol/chloroform (1:1). Extractions were repeated several times until a clear interface was observed. The DNA samples were dialysed overnight against 10mM Tris-HCl pH 7.5, 0.3M NaCl, 1mM EDTA at 4^{o}C and against 10mM Tris-HCl pH 7.5, 1mM EDTA for another 5 hours. In order to determine approximately the total yield of DNA the absorbance of the sample at 260nm was measured. In all experiments involving preparation of DNA sterile and siliconised glassware was used.

2.8 Density determination of DNA

The density of DNA extracted from the membrane-DNA complex was determined in the Beckman Model-E analytical ultracentrifuge using DNA from Micrococcus lysodeikticus as a density marker. The DNA (2-3µg) was mixed with CsCl (analytical grade) to give an initial density of 1.710g x cm^{-3} and centrifuged to equilibrium at 44,000rpm for 20 hours at 25°C. Photographs were scanned on the Joyce Loebl Microdensitometer and the buoyant density was calculated with reference to the marker DNA (ρ = 1.731g x cm^{-3}).

The density of in vitro labelled DNA from the membrane-DNA complex was determined by equilibrium centrifugation in CsCl. The DNA was labelled and extracted as previously described, except that a higher concentration of the membrane-DNA complex (400µg protein/ml) and (^{3}H)dTTP (4µM) were used. The incubation was carried out with shaking for 15 minutes at 30°C. The extracted DNA was mixed with CsCl to give an initial density of 1.7g x cm^{-3} and centrifuged to equilibrium at 90,000xg for 67 hours at 25°C. The gradient was fractionated (0.4ml per fraction) from the bottom of the tube, and the carrier DNA was added to a final concentration of 0.8mg/ml. Fractions were precipitated with trichloroacetic acid and radioactivity was determined as described previously under Section 2.6.

2.9 Sedimentation velocity

The DNA extracted from the membrane-DNA complex was gently layered on a 8-25% sucrose gradient in 10mM Tris-HCl pH 8.0, 1M NaCl, 1mM EDTA. The gradient was centrifuged at 48,000xg for 15 hours at 4°C. Fractions of 0.5ml were collected from the bottom of the tube and absorbance at 260nm was determined in each fraction using the 8% sucrose solution as a blank.

2.10 Electron microscopy

The purified replication complex was fixed in 1% glutaraldehyde for 15 minutes at 37°C, dialysed against 10mM Tris-HCl pH 7.5 and spread for electron microscopy using the cytochrome monolayer technique (Davis et al., 1971). The spread material was picked up on nickel grids coated with formvar film and carbon. They were stained in uranyl acetate and rotary shadowed with platinum. The grids were examined in an AEI EH6 electron microscope.

2.11 Digestion of DNA with restriction endonucleases

The standard conditions for digestion with EcoRI, BamI and HindIII were to incubate 50 to 100µg DNA/ml for 1 hour at 37°C in 100mM NaCl, 10mM mercaptoethanol, 10mM $MgCl_2$, 10mM Tris-HCl (pH 7.5). The same conditions were used for PstI, except that the mercaptoethanol concentration was 1mM.

The digests were analysed by 1.2% agarose slab gels in 90mM Tris-borate (pH 8.3), 1mM EDTA. The gels were run at 1.5V/cm for 16 hours, stained in ethidium bromide (1µg/ml) for 30 minutes and photographed under an ultraviolet lamp.

2.12 SDS polyacrylamide gel electrophoresis

SDS polyacrylamide slab gel electrophoresis was a modified method of Laemmli (1970). 7.5-15% or 12-20% polyacrylamide gradient gels were used throughout this study. To obtain e.g. 12-20% exponential gradient the same volumes (22ml) of 20% and 4% acrylamide solutions in the resolving buffer were mixed in a gradient maker. Apart from appropriate concentrations of acrylamide the resolving buffer contained: 0.4% BIS-acrylamide, 2mM EDTA, 0.1% SDS, 0.02% TEMED, 0.03% ammonium sulphate, 375mM Tris-HCl, pH 8.8 and 20% glycerol used to stabilize

the gradient. After polymerization of the gradient the stacking gel was applied containing 4.8% acrylamide, 0.1% SDS, 0.05% TEMED, 0.04% ammonium persulphate and 125mM Tris-HCl, pH 6.8. Samples were mixed with 70mM Tris-HCl, 1% SDS and run at 8 mA for 17 hours. The gel was stained for 1 hour with 0.25% Coomassie Brilliant Blue, 50% methyl alcohol, 10% glacial acetic acid and destained by diffusion in a solution of 5% methyl alcohol and 7.5% acetic acid.

2.13 Extraction and gel electrophoresis of basic proteins from a membrane-DNA complex and from nuclei

Basic proteins were prepared from the purified membrane-DNA complex by extraction with 0.25N HCl for 1 hour at $4^{o}C$. After centrifugation, the basic proteins were precipitated from the supernatant with 10 volumes of acetone for 24 hours at $-20^{o}C$. The precipitate was washed twice with acid acetone, twice with acetone and dried in vacuo. Histones from nuclei were prepared in the same manner except for mechanical homogenisation of whole nuclei prior to extraction with acid. All preparations of basic proteins were carried out in the presence of 0.05M sodium bisulfite.

Electrophoresis of histones was performed on 15% acrylamide slab gels containing 6.25M urea and 0.9N acetic acid according to Panyim and Chalkley (1969). The electrophoresis was carried out at 4mA for 14 hours. The gels were stained for 1 hour in 0.25% Coomassie Brilliant Blue, 45% methyl alcohol, 9% acetic acid in H_2O and destained by diffusion in a solution of 5% methyl alcohol and 7.5% acetic acid in H_2O.

2.14 Extraction and gel electrophoresis of DNA after micrococcal nuclease or endonuclease digestions

The conditions of the digests were basically as described by Seale (1976). In the case of micrococcal nuclease digestion mitochondria were incubated with 0.1 unit of enzyme per $10A_{260}$ of mitochondria for the times indicated. Prior to incubation with endogenous nuclease, mitochondria were treated with pancreatic DNasel (10µg/ml) for 20 minutes in ice after addition of 5mM $MgCl_2$ to the mannitol/MOPS/BSA buffer. After incubation the DNase was diluted out with 35ml of mannitol buffer plus 10mM EDTA followed by centrifugation at 5,000xg for 6 minutes. The digests of mitochondria with endogenous nuclease were carried out in the same manner as with micrococcal nuclease, except that the enzyme was omitted from the incubation mixture. The incubation was terminated by the addition of 3% SDS, 20mM EDTA. DNA was prepared from such digests in the same way as described for extraction of DNA from a membrane--DNA complex (Section 2.7) except that iso-amylalcohol/chloroform was used instead of phenol/chloroform. DNA was precipitated from the dialysate overnight at -20°C with 2.5 volumes of ethanol in the presence of 0.3M NaCl. The DNA precipitate was collected after centrifugation at 34,000xg for 30 minutes and dried to remove traces of ethanol. Products of digestion were analysed on 6% acrylamide slab gels containing 6M urea as described by Peacock and Dingman (1967). Heat denatured (5min at 100°C) DNA samples were subjected to electrophoresis at 24mA for 3 hours. Gels were stained with 10µg/ml ethidium bromide for 20 minutes and photographed under ultraviolet light.

2.15 Chemical Assays

Protein was determined by the method of Lowry et al. (1951) with crystalline BSA as the reference.

DNA determinations were carried out with diphenylamine (Burton, 1956) using deoxyribose as a standard.

RESULTS

The initial experiments in this study were concerned with the development of a technique for the isolation of a membrane-DNA complex from mitochondria of Paramecium aurelia. The experiments were based on the method described by Van Tuyle and Kalf (1972) for the preparation of such a complex from rat liver mitochondria. During the course of the study presented here it was necessary to introduce certain modifications to the above technique to cover specific requirements posed by Paramecium mitochondria. The following section of the Results describes various steps undertaken before a satisfactory method was finally adopted. The section is organised in three parts. The first deals with the rationale of the original technique and with its modifications. The second part presents an alternative method for the preparation of the mitochondrial membrane-DNA complex. The third part describes the purification procedure of the complex. The diagram of the final technique used is presented at the end of this section.

1. Methods of isolation of membrane-DNA complex.

1.1 Isolation of membrane-DNA complex from mitoplasts.

The technique for the isolation of the mitochondrial membrane-DNA complex involves three major steps:

Step 1. Treatment of purified mitochondria with the detergent digitonin to remove the outer mitochondrial membrane. As a result of this treatment the inner membrane-matrix preparation (mitoplast) is obtained.

Step 2. Treatment of mitoplasts with the detergent Sarkosyl in the presence of magnesium ions. This detergent causes lysis of the inner membrane and interacts with magnesium to form white crystals. Portions of the inner membrane adhere to the hydrophobic surface of the crystals and as a result of this interaction a complex is formed which apart from

magnesium-Sarkosyl crystals and inner-membrane fractions may also contain membrane attached DNA, plus membrane- and DNA-bound proteins. In further studies this complex will be referred to as the membrane-DNA complex.

Step 3. Separation of the membrane-DNA complex from the matrix on a discontinuous sucrose gradient. The entire complex, when centrifuged, sediments in a discontinuous sucrose gradient at the interface between 15 and 40% sucrose, forming a sharp white band. This band is subsequently collected from the gradient and used for further studies.

In the technique described above, removal of the outer membrane is achieved by treatment of the mitochondria with digitonin. This reagent shows a fair degree of specificity towards the outer membrane in its disruptive effects; however, above certain concentrations it, at least partially, solubilises and/or disrupts the inner membrane also (Schnaitman et al., 1967). In principle, it is necessary to use a certain ratio of digitonin to mitochondrial protein so that the outer membrane is preferentially broken but the inner one remains intact. Various organisms require different amounts of the detergent for removal of the mitochondrial outer membrane which could be due to differences in relative proportion of outer membrane vs. inner membrane and possibly also to differences in the composition of the outer membranes. It has been shown, for example, that isolated Neurospora mitochondria (Cassady et al., 1972) require a much higher ratio of digitonin to mitochondrial protein than rat liver mitochondria (Schnaitman and Greenawalt, 1968). It was essential, therefore, to determine the optimal ratio of detergent to mitochondrial protein, which when applied to Paramecium aurelia mitochondria would result in removal of the outer membrane, leaving the inner one largely intact. The disruptive action of the detergent can

be monitored by the assay of various marker enzymes which are associated with the outer or inner membranes, or localized in the intermembrane space. Figure 2 shows the release of enzymes from Paramecium mitochondria after treatment with varying amounts of digitonin. It can be seen that adenylate kinase (marker for the intermembrane space) is removed rapidly from mitochondria even with low concentrations of the reagent. Succinate dehydrogenase and glutamate dehydrogenase, both associated with the inner membrane-matrix fraction, require higher amounts of detergent for their release from mitochondria. Low activity of adenylate kinase does not necessarily signify that the outer membrane has been removed, as its rupture, which can occur even at a very low concentration of digitonin, is sufficient to release a significant amount of the enzyme from the intermembrane space. It would be more meaningful to monitor activity of enzymes such as kynurenine hydroxylase, monoamine oxidase and rotenone-insensitive NADH-cytochrome c reductase which are markers for the outer membrane in mammalian mitochondria (Okamoto et al., 1967; Schnaitman and Greenawalt, 1968; Sottocasa et al., 1967b). Although several attempts were made to assay these enzymes in Paramecium mitochondria, no significant activity has been detected (Table 1). This could be due either to their absence in Paramecium (e.g. yeast mitochondria lack monoamine oxidase; Bandlow, 1972) or due to non-optimal assay conditions used for these enzymes.

In view of these difficulties the optimal concentration of digitonin for removal of the outer membrane was determined on the basis of maximal release of adenylate kinase with minimal release of activity of enzymes associated with inner membrane-matrix and relatively high retention of mitochondrial proteins. From the data presented in Figure 2 a ratio of 1:10 of digitonin to mitochondrial protein was chosen as fulfilling the above conditions and therefore was adopted for future experiments.

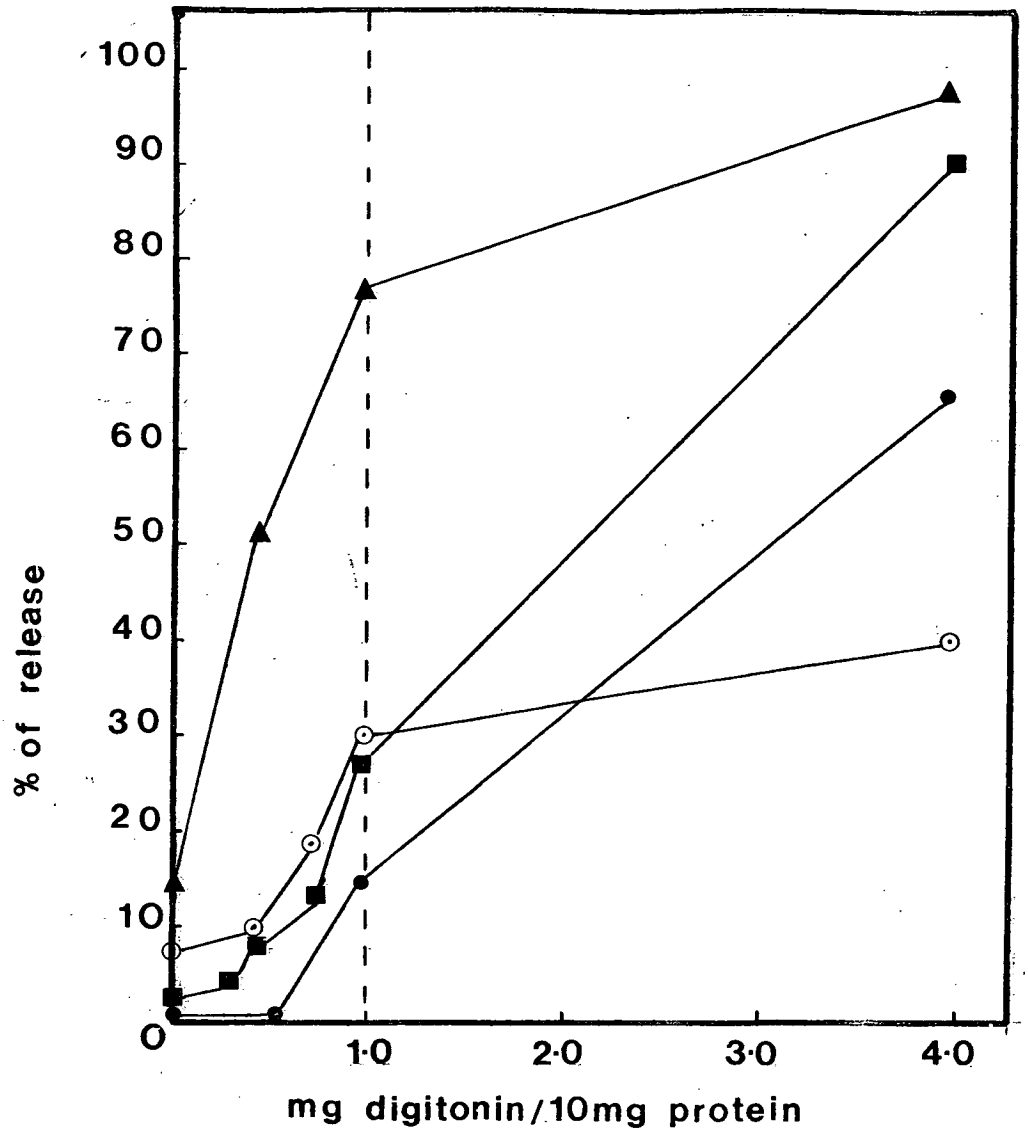


Figure 2. The release of mitochondrial enzymes by various concentrations of digitonin. Mitochondria lysed with digitonin were centrifuged at 9,000xg for 10 min and enzymatic activities were measured in pellets and supernatant fractions. The percentage of the activities released to the supernatants was calculated in relation to the initial total activity in mitochondria and plotted versus digitonin concentration. ●—●, succinate dehydrogenase; ■—■, glutamate dehydrogenase; ▲—▲, adenylate kinase; ○—○, proteins.

Table 1. List of marker enzymes assayed in mitochondria from *Paramecium aurelia*.

Enzymes tested	Marker specific for	Activity
Kynurenine hydroxylase	Outer membrane	-
Monoamine oxidase		-
Rotenone-insensitive NADH-cytochrome c reductase		-
3-Hydroxybutyrate dehydrogenase	Inner membrane	+
Succinate dehydrogenase		+
Adenylate kinase	Intermembrane space	+
Malate dehydrogenase	Soluble matrix	+
Glutamate dehydrogenase		+

+, activity detected; -, no activity detected.

1.2 Preparation of membrane-DNA complex from intact mitochondria without digitonin

In the later course of these studies it was found that the step involving digitonin treatment of mitochondria (Step 1) is not absolutely necessary for obtaining the membrane-DNA complex. However one condition must be fulfilled, namely the omission of bovine serum albumin (BSA) from the medium used for the isolation of mitochondria. It is thought that BSA, which is normally added to the medium, has a protective effect on mitochondria during their isolation from the cell homogenate and the subsequent purification steps. The omission of BSA from the medium results in preparation of "leaky" mitochondria. Therefore, the treatment with digitonin seems to be no longer necessary, as Sarkosyl on its own lyses the outer membrane sufficiently for the membrane-DNA complex to be obtained. The complex prepared in such a way, i.e. omitting BSA at the stage of isolation of mitochondria, which are directly treated with Sarkosyl without pre-treatment with digitonin, shows identical characteristics (amount of proteins, DNA and (^{3}H)dTTP incorporation) to the complex prepared from mitoplasts (Table 2). Therefore, treatment of mitochondria with digitonin was omitted in its entirety in later experiments, as its exclusion from the procedure greatly reduced the time of preparation of the complex, thus minimising the possible degradation of DNA which can occur during lengthy isolation procedures.

The membrane-DNA complex prepared by this method was used in some of the experiments involving DNA studies and in all the experiments concerned with characterization of the proteins from the complex and mitochondrial chromatin.

1.3 Purification of the membrane-DNA complex

Electron microscope studies of the membrane-DNA complex isolated from intact mitochondria revealed the presence of various structures from

Table 2. Comparison of membrane-DNA complexes isolated from mitoplasts (A) and intact mitochondria (B). Protein and DNA contents were estimated as described in Materials and Methods and the values given per 15mg of either mitoplast or mitochondrial protein loaded on the discontinuous sucrose gradient.

	Protein (mg)	DNA (μg)	Specific activity (^{3}Hcpm/mg protein)
A	1.065	14.08	6,250
B	1.177	14.97	7,687

the cell surface in the preparation. Figure 3 shows the most commonly occurring structures which were identified according to Jurand and Selman (1969), Selman and Jurand (1970) as fragments of pellicle, shafts and tips of extruded trichocysts. As these contaminants were sedimenting to the 15-35% interface of the discontinuous sucrose gradient, it was suspected that they were attached to the magnesium-Sarkosyl crystals together with the membrane-DNA. It was thought, therefore, that purification of the complex could be achieved by dissolving the crystals, thus releasing the surface structures. The first step of the procedure subsequently developed for purification of the complex involved solubilisation of magnesium-Sarkosyl crystals. It was found that EDTA caused the

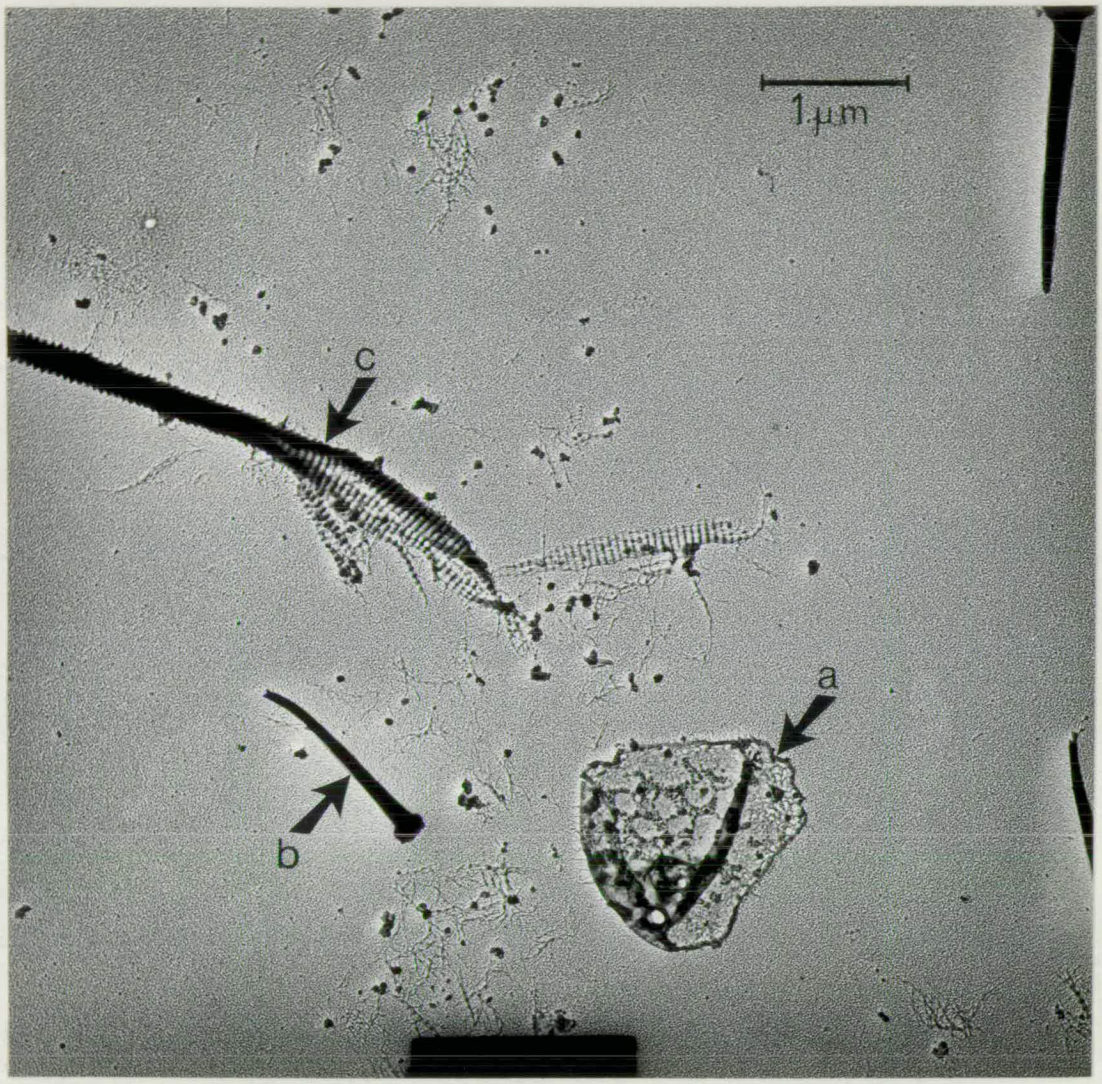


Figure 3. Electron micrograph illustrating the typical contaminants of the membrane-DNA complex prior to purification: fragment of pellicle(a), tip (b) and shaft (c) of extruded trichocyst.

solubilisation of the crystals due to the binding of magnesium ions by EDTA and release of free Sarkosyl. The next step involved the separation of the complex from the contaminating structures by differential centrifugation as described in Methods. The diagram of the final method for the isolation of the mitochondrial membrane-DNA complex adopted in this study is presented in Figure 4.

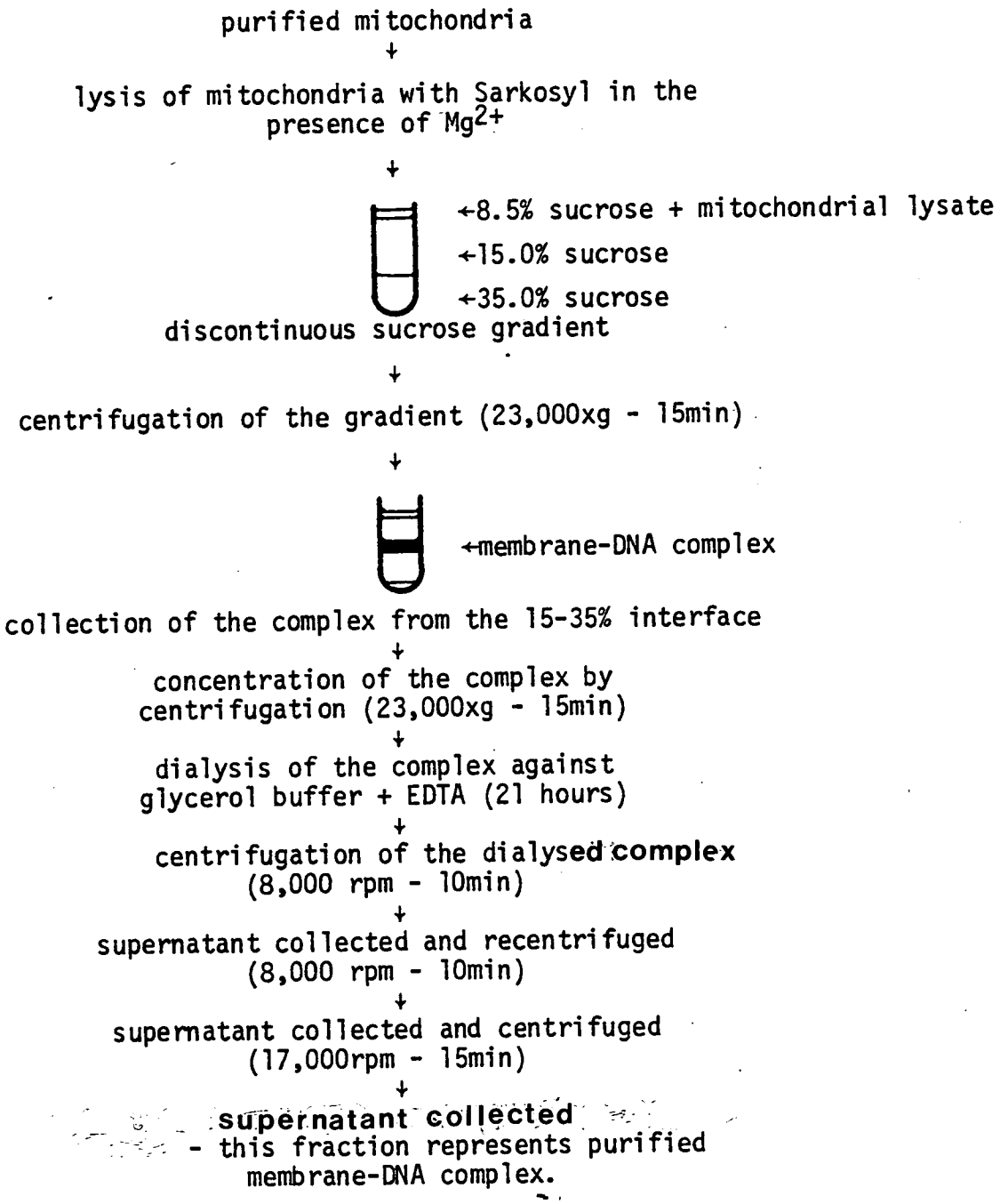


Figure 4. Diagram illustrating the final method used for the isolation of membrane-DNA complex from Paramecium mitochondria.

2 Characterisation of membrane-DNA complex

2.1 Origin of DNA in the membrane-DNA complex

It is necessary to prove that DNA present in the complex is indeed of mitochondrial origin. It is possible that contamination could arise from nuclear material as DNA released from nuclei during cell homogenisation might adhere to mitochondria and thus might be isolated together with mitochondrial fraction. Another possible source of contamination might be the bacteria (Klebsiella aerogenes) used to feed the Paramecium cultures. Although, according to Kalf and Ch'in(1968) bacterial cells are not subject to lysis by Sarkosyl unless the cell wall has been rendered fragile by treatment with EDTA and lysozyme, it is still necessary to prove that this is true for Klebsiella derived from food vacuoles in Paramecium cells. A possibility existed that bacterial cells might be damaged during the preparation of mitochondria such that a bacterial membrane-DNA complex would be formed together with the mitochondrial complex. To examine this possibility an attempt was made to prepare a membrane-DNA complex from a bacterial culture under conditions identical to those used with the mitochondrial preparations. Concentrated bacteria, resuspended in the buffer used for the isolation of mitochondria were homogenized and subjected to differential centrifugation as described previously for the preparation of mitochondria. After standard digitonin treatment bacteria were treated with Sarkosyl and the mixture centrifuged in a discontinuous sucrose gradient. In the fraction of the gradient collected from the interface between 15% and 35% sucrose (position of the mitochondrial complex) no proteins were detected by standard methods, indicating the absence of a bacterial membrane-DNA complex.

The problem of possible contamination of the mitochondrial membrane--DNA complex was further studied by other techniques. As the densities

of Klebsiella, and of nuclear and mitochondrial DNA of P. primaurelia are known (Goddard and Cummings, 1975), it seemed possible to establish the source of the DNA in the complex by measuring its density. DNA was therefore extracted from the membrane-DNA complex and analysed by analytical CsCl equilibrium centrifugation (Model E). Results from a typical experiment are presented in Figure 5. The optical density trace revealed two peaks. The major peak corresponded to a DNA density of 1.700g x cm^{-3} which is in agreement with the density found for mt DNA extracted from Paramecium (Goddard and Cummings, 1975). The second minor peak with ρ = 1.716g x cm^{-3} was identified as Klebsiella DNA (ρ = 1.718g x cm^{-3}). It should be pointed out that the level of Klebsiella DNA contamination (about 7%) detected by this method is insignificant when compared to mt DNA. No peak corresponding to nuclear DNA from Paramecium (ρ = 1.686g x cm^{-3}) was detected. It was concluded, therefore, that the major species of DNA in the membrane-DNA complex is of mitochondrial origin and that the complex is essentially free from contamination by either nuclear or bacterial DNA.

The possibility of contamination by nuclear DNA was further studied by preparing the membrane-DNA complex from a mixture of unlabelled cells and purified nuclei labelled with (methyl-^{3}H) thymidine. Samples were taken at various stages of the purification and the amount of DNA and TCA precipitable radioactivity was determined (Table 3). There was no detectable radioactivity in the membrane-DNA complex. Furthermore, knowing the specific activity of the nuclear DNA added (404 x 10^3 dpm/mg) and the ratio of unlabelled to labelled nuclei (7:1), one can calculate that if all the DNA from the complex were of nuclear origin, a radioactivity of 455dpm should be obtained:

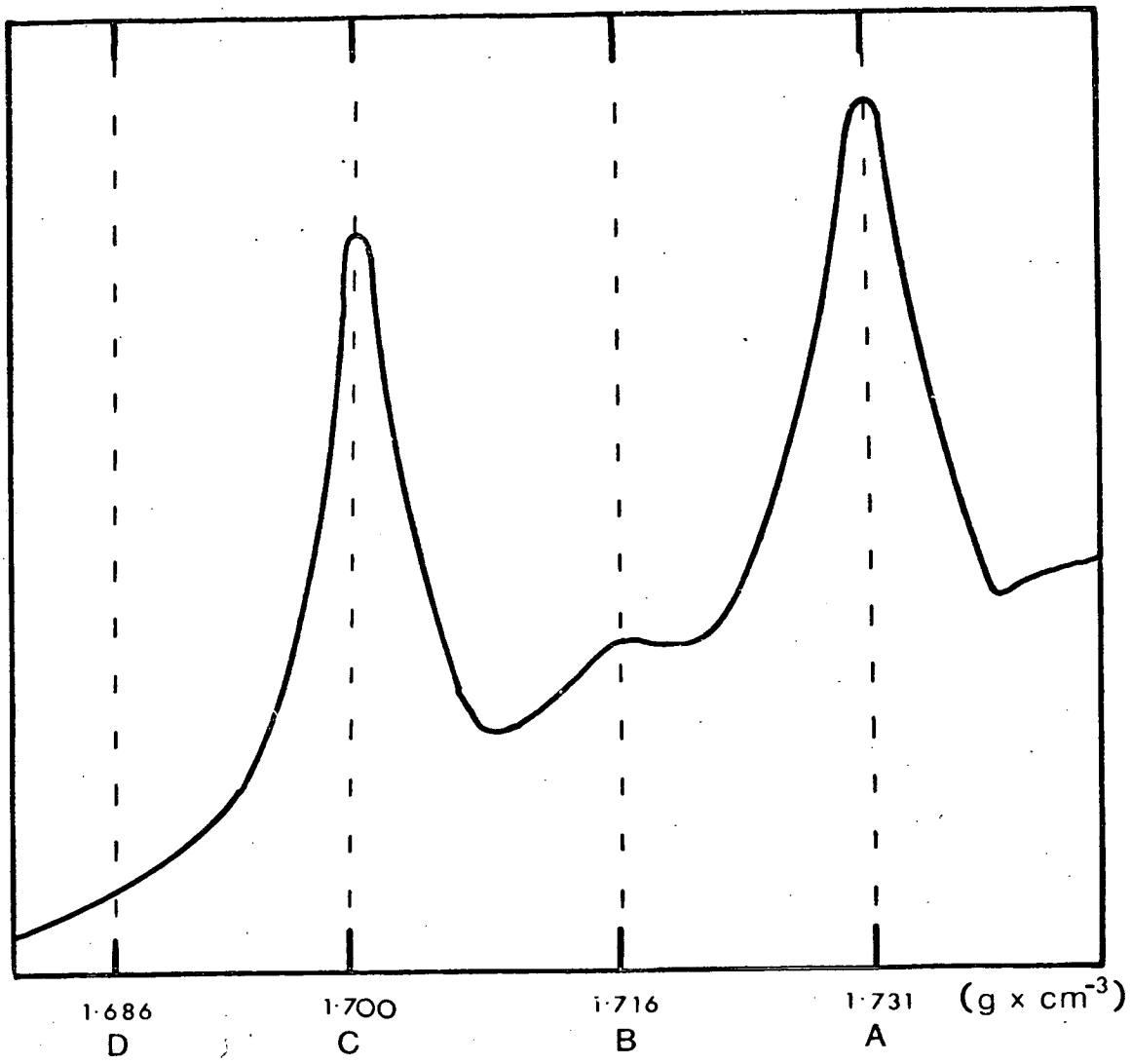


Figure 5. Determination of the density and purity of DNA, extracted from the membrane-DNA complex, by analytical CsCl equilibrium centrifugation. The figure presents the microdensitometer tracing of the gradient; positions of marker DNA from Micrococcus lysodeikticus (A), Klebsiella aerogenes DNA (B), Paramecium mitochondrial DNA (C) and nuclear DNA (D) are indicated.

$$\frac{404 \times 10^3 \text{dpm} \times 9\mu\text{g}}{10^3 \mu\text{g} \times 8} = 455\text{dpm}.$$

Thus, it can be seen that if a significant fraction of the DNA from the complex were nuclear it would be detected.

It has been demonstrated by density gradient equilibrium centrifugation of nuclear DNA, followed by hybridisation of the fractionated DNA with ribosomal RNA, that the density of ribosomal DNA in P. primaurelia coincides with that obtained for mt DNA (Cummings, 1975). In view of this data the density of the DNA cannot be used as the only criterion for the presence of mitochondrial and absence of nuclear DNA. Thus, in order to overcome any ambiguities concerning the origin of DNA in the membrane--DNA complex, additional experiments were undertaken. An attempt was made to digest the DNA isolated from the complex with restriction enzymes, but this approach did not give conclusive results. Instead of several bands of different molecular weight only one high molecular weight band (around 27×10^6 daltons) was observed on agarose gel electrophoresis (Figure 6). This result suggested the presence of possible inhibitors of restriction endonucleases in the complex. The fact that bacteriophage λDNA, when added to the incubation mixture, was restricted, implies that the inhibitor preventing the digestion of the DNA from the complex was not diffusible. Despite several attempts to further purify the DNA and the use of different restriction endonucleases (EcoR1, HindIII, PstI and BamI) no digestion was observed.

The several different kinds of data presented in this section, when taken together, rule out the possibility of nuclear or bacterial contamination and indicate the mitochondrial origin of the DNA in the membrane--DNA complex.

Table 3. The distribution of ^{3}H-labelled nuclear DNA during preparation of the mitochondrial replication complex.

Fraction	dpm ($x10^3$)	dpm/mg DNA ($x10^3$)	µg DNA
Homogenate*	222	32	7×10^3
Mitochondria	7.2	7	1×10^3
Replication complex	0	0	9

* labelled nuclei (404×10^3 dpm/mg DNA) mixed with unlabelled cell homogenate.

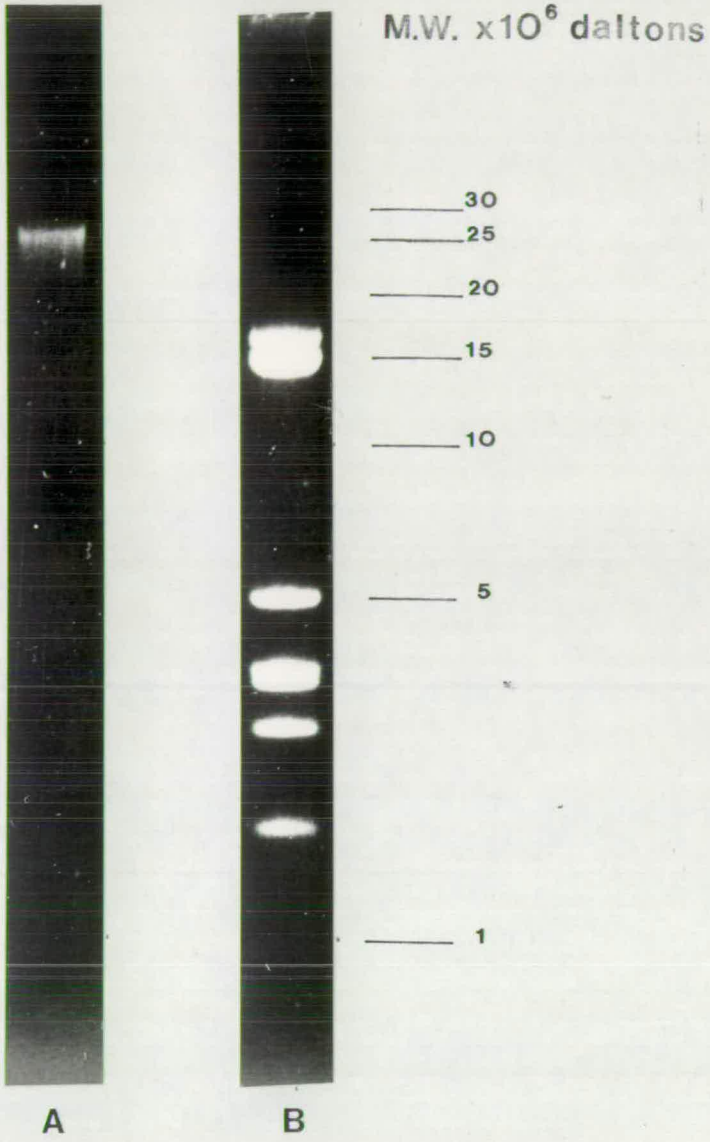


Figure 6. Digestion with EcoRI of DNA extracted from the membrane--DNA complex. Ethidium bromide-stained 1.2% agarose slab gel is shown: undigested mt DNA (A); product of EcoRI digestion of λDNA used as a molecular weight marker (B).

2.2 Incorporation of (^{3}H)dTTP by the membrane-DNA complex *in vitro*.

2.2.1 Deoxyribonucleoside triphosphate and template requirements.

To demonstrate that DNA synthesis occurs on the mitochondrial membrane--DNA complex, the complex was assayed for its ability to synthesise DNA *in vitro* using the complex as the sole source of enzymes and DNA template. DNA synthesis by the complex was assayed by the incorporation of deoxyribonucleoside triphosphates into an acid insoluble form using (^{3}H) thymidine triphosphate as the labelled substrate. Figure 7 demonstrates that the membrane-DNA complex is capable of incorporating (^{3}H)dTTP *in vitro* when incubated with (^{3}H)dTTP, 3 unlabelled deoxynucleoside triphosphates and magnesium. Incorporation of (^{3}H)dTTP starts without a lag and is linear as a function of time for approximately 20 minutes after which the rate of incorporation decreases. The incorporation is dependent on the presence of endogenous DNA of the complex as template, since the addition of pancreatic DNase 1 to the incubation mixture inhibited the incorporation by 92% (Table 4).

The membrane-DNA complex was found to require the presence of all four deoxyribonucleoside triphosphates for maximal incorporation of (^{3}H)dTTP. The effect of excluding three dNTP's is shown in Figure 7. With only (^{3}H)dTTP present in the assay, incorporation of the label is 28% of that with all four triphosphates present.

2.2.2 Effect of pH, divalent cations and inhibitors

The effect of pH on the (^{3}H)dTTP incorporation by the membrane-DNA complex is presented in Figure 8. The complex showed maximal activity at pH 7.0.

Synthesis of DNA by the membrane-DNA complex is dependent on the presence of magnesium ions as omission of this cation from the incubation

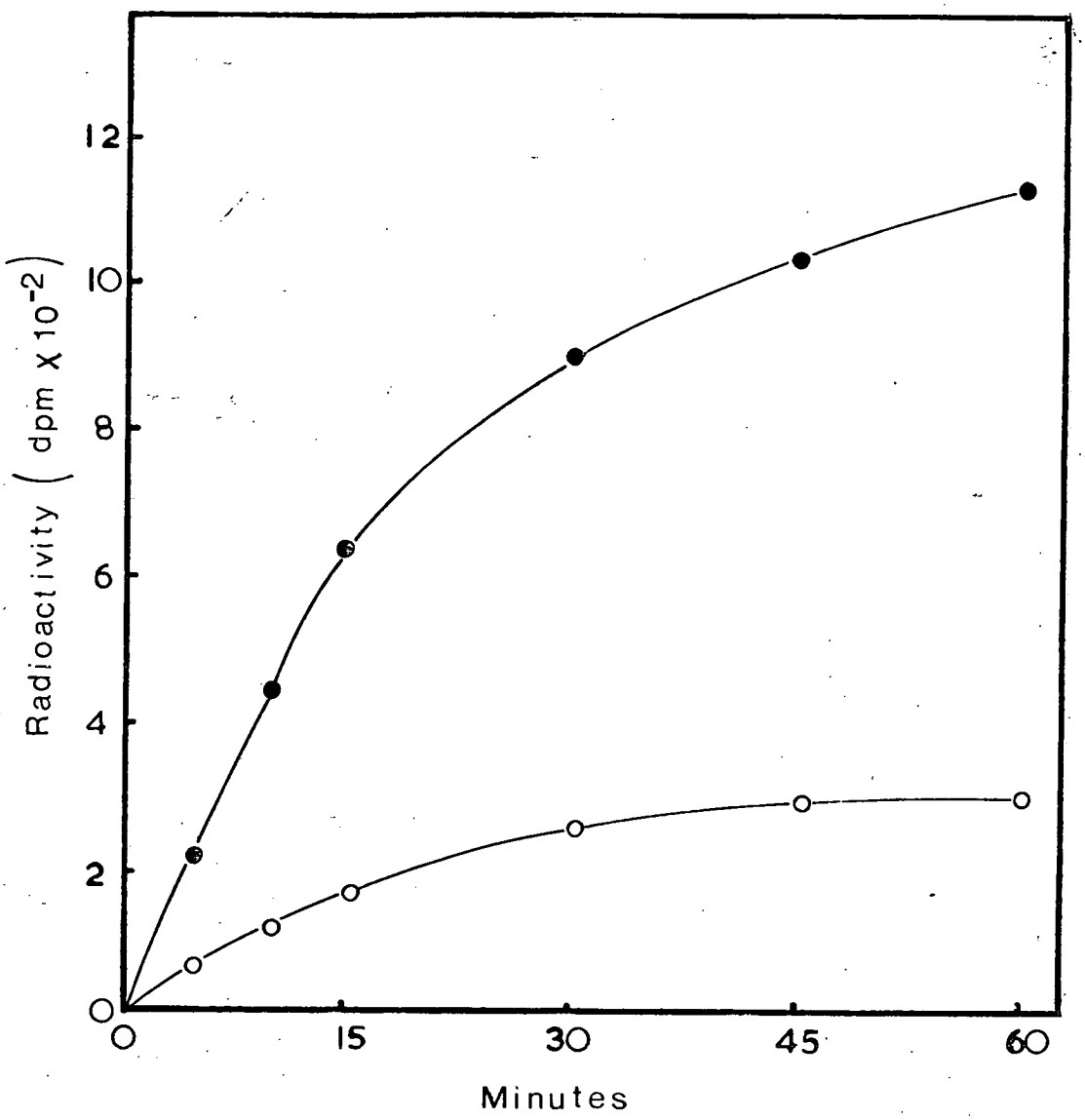


Figure 7. Incorporation of (^{3}H)dTTP by the membrane-DNA complex as a function of time. The incubation conditions were as described in Material and Methods. ●——● , complete assay; ○——○, assay with omission of 3 unlabelled deoxyribonucleotides.

Table 4. The effect of various compounds on the incorporation of (^{3}H)dTTP by the membrane-DNA complex in vitro.

System	(^{3}H)dTMP Incorporated (% of Control)
Complete system*	100
+ Pancreatic DNase 1 (200 µg/ml)	8
- 3 Unlabelled dNTP's	28
- $MgCl_2$	5
+ Actinomycin D (50 µg/ml)	30
+ Ethidium bromide (5 µg/ml)	50
(10 µg/ml)	22
(20 µg/ml)	0

* Standard assay conditions were used as described in Material and Methods. Incubations were carried out for 15 minutes.

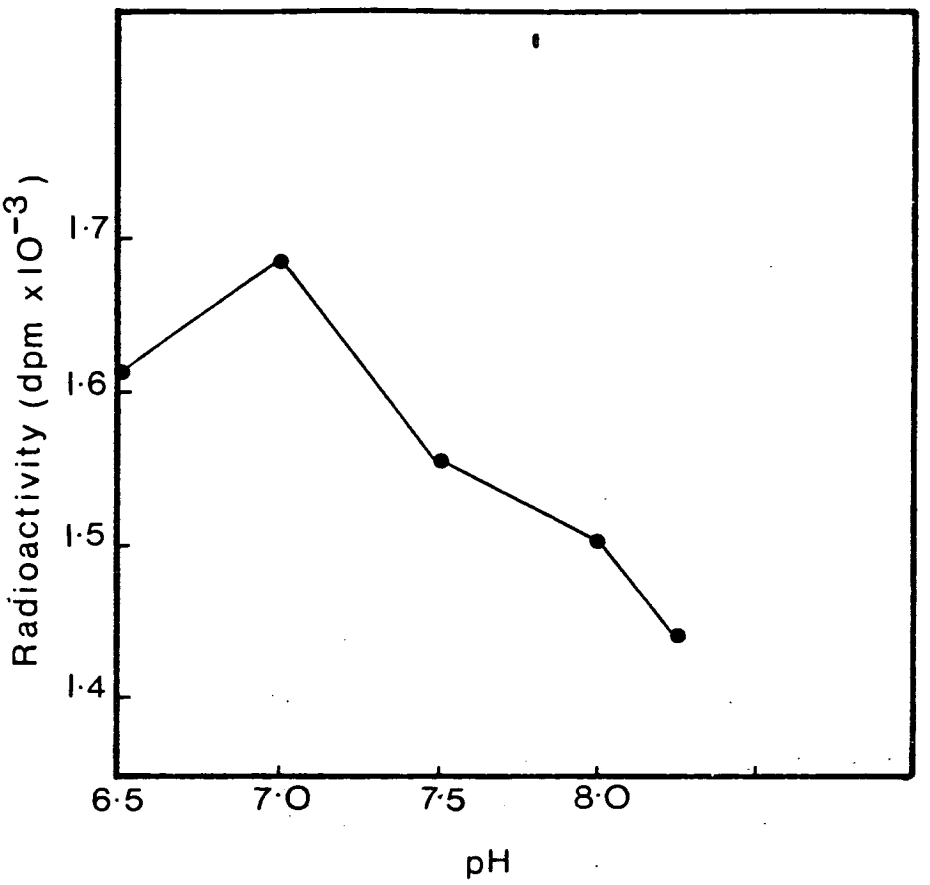


Figure 8. The effect of pH on the incorporation of (^{3}H)d TTP by the membrane-DNA complex. Incorporation was measured for 15 minutes under standard conditions using 100mM Tris-HCl buffers of appropriate pH (30^{o}C).

mixture reduces the incorporation of (^{3}H)dTTP by 95% (Table 4). Figure 9 shows (^{3}H)dTTP incorporation in the presence of various concentrations of Mg^{2+}. The optimal concentration of magnesium required by the complex was found to be 10mM; the incorporation decreasing rapidly with increasing Mg^{2+} concentrations. Incubation mixtures containing both magnesium and manganese ions show an inhibition of (^{3}H)dTTP incorporation by the complex (Figure 10). Addition of Mn^{2+}, even at low concentration (5mM), caused a 50% reduction in the activity of the complex.

The effect of actinomycin D and ethidium bromide on the incorporation of (^{3}H)dTTP by the complex is presented in Table 4. It can be seen that actinomycin D has an inhibitory effect, as its addition to the incubation mixture resulted in reduction of (^{3}H)dTTP incorporation to the level of 30% of the control (without actinomycin). Ethidium bromide has an inhibitory effect on the incorporation of (^{3}H)dTTP by the complex (Table 4). This compound suppresses the incorporation by 78% at 10µg/ml, while higher concentrations abolish the incorporation completely.

2.2.3 Evidence for incorporation of (^{3}H)dTTP into mt DNA.

Since analysis of the DNA extracted from the membrane-DNA complex revealed that in addition to mt DNA a trace of bacterial DNA is present (see Results - Section 2.1), it was necessary to prove that (^{3}H)dTTP is incorporated specifically into mitochondrial rather than bacterial DNA. Labelled DNA was extracted from the complex after *in vitro* incubation in the presence of (^{3}H)dTTP and analysed by CsCl equilibrium density centrifugation. The result obtained from this experiment (Figure 11) indicates that the labelled DNA was indeed mitochondrial as its density ($1.699g \times cm^{-3}$) was in agreement with that found for *Paramecium* mt DNA (Goddard and Cummings, 1975). Furthermore, in the fractions of the

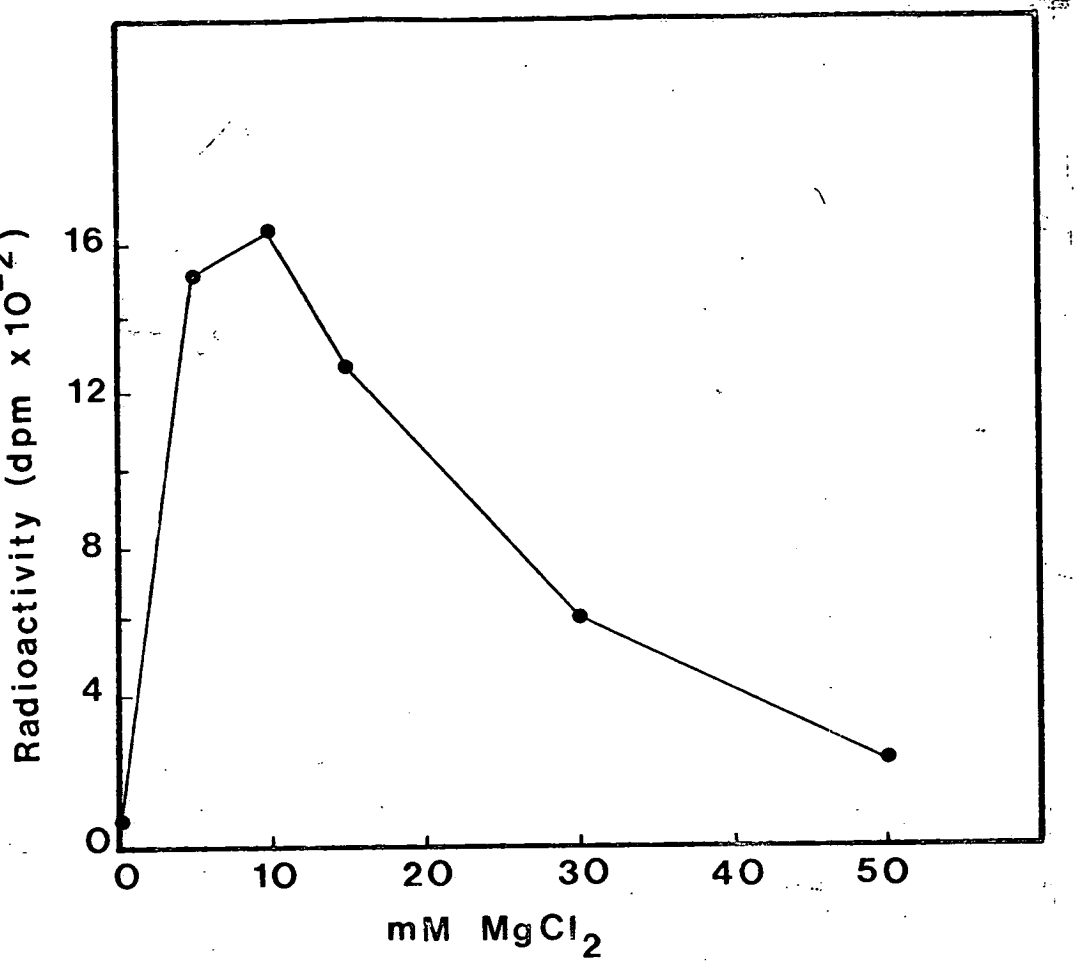


Figure 9. The effect of magnesium ions on the incorporation of (^{3}H)dTTP into the acid insoluble fraction by the membrane-DNA complex. The standard incubation mixtures were used except for varying concentrations of $MgCl_2$; the samples were incubated for 15 minutes.

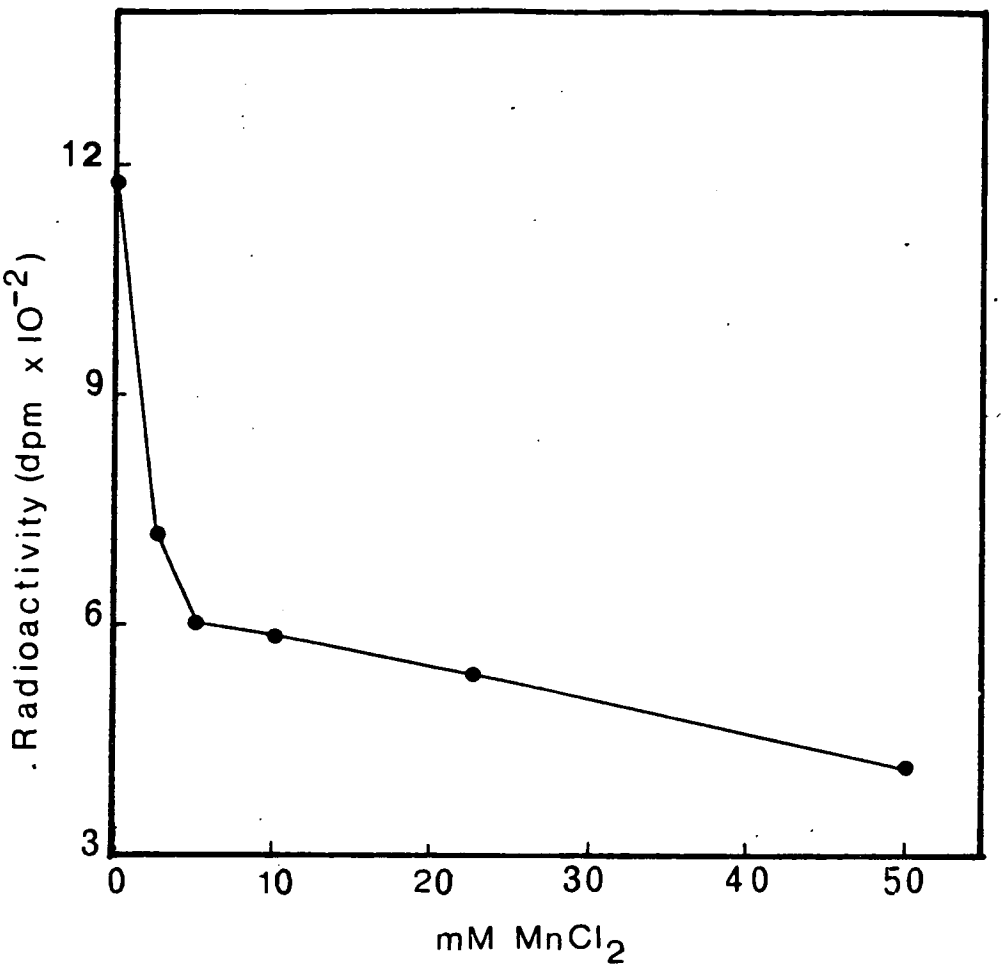


Figure 10. The effect of manganese ions on the incorporation of (^{3}H)d TTP into the acid insoluble fraction by the membrane-DNA complex. The standard incubation mixture was supplemented with varying concentrations of $MnCl_2$; incubation was carried out for 15 minutes.

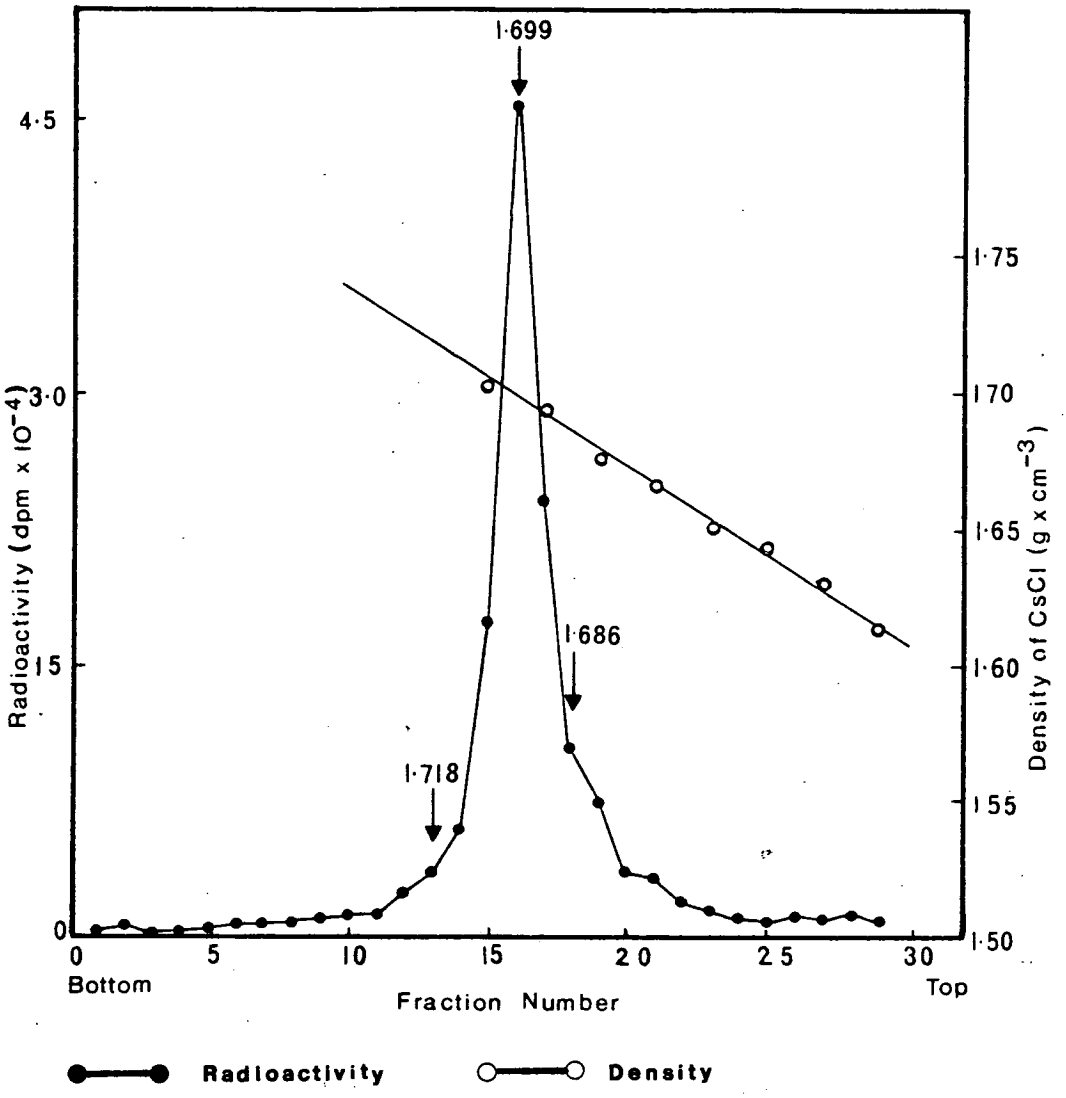


Figure 11. CsCl equilibrium density centrifugation profile of in vitro synthesized DNA by the membrane-DNA complex. The complex was labelled with (^{3}H)dTTP for 15 mins as described in Materials and Methods (Section 2.8). The gradient was fractionated and radioactivity and density of each fraction determined. Arrows indicate the positions corresponding to densities of bacterial (1.718g x cm^{-3}), mt (1.699g x cm^{-3}) and nuclear (1.689g x cm^{-3}) DNA.

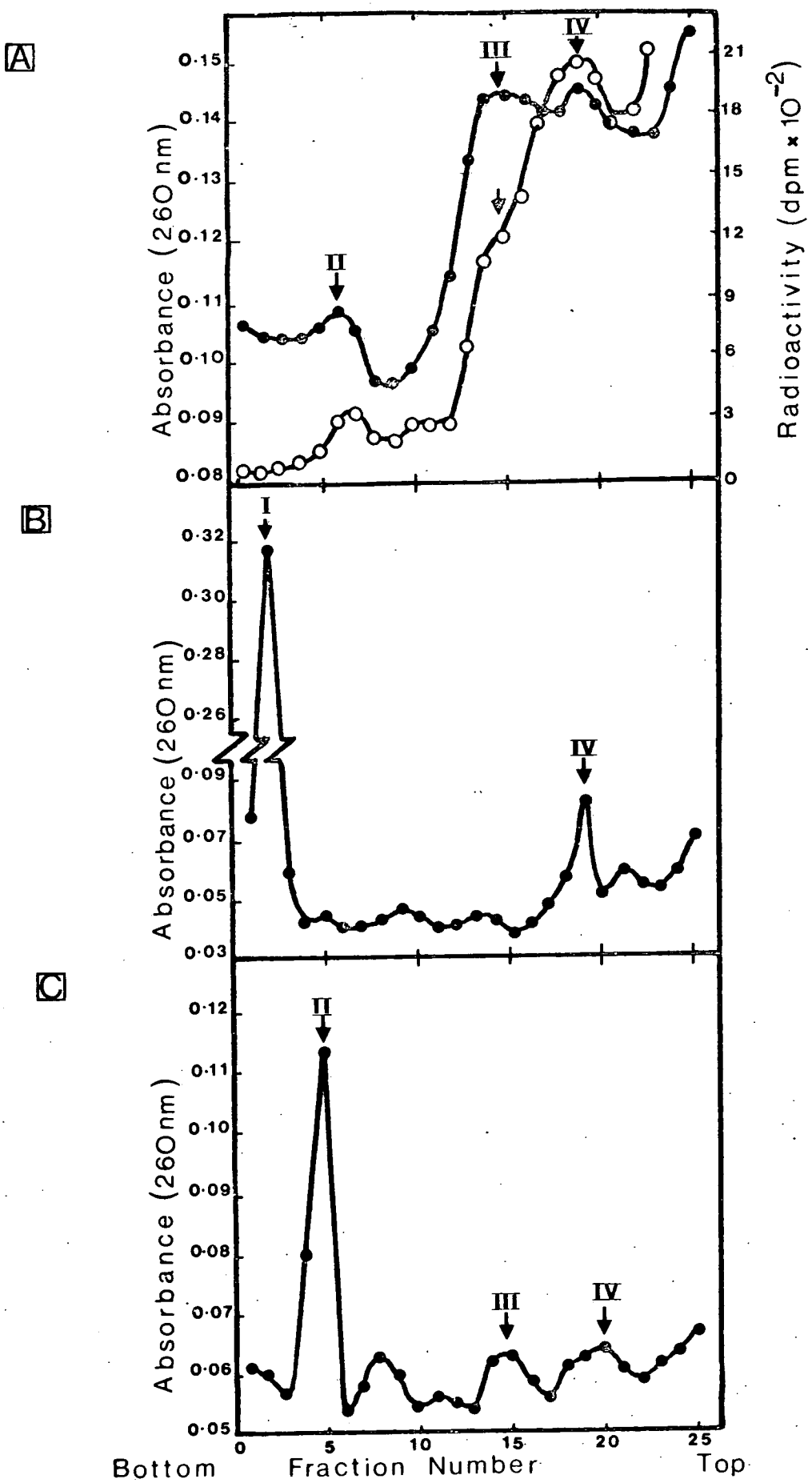
gradient corresponding to densities of bacterial or nuclear DNA no significant radioactivity was detected.

2.3 Replication of mt DNA *in vitro*

To demonstrate that the DNA synthesis by the membrane-DNA complex *in vitro* accurately reflects the mode of replication observed *in vivo*, it is necessary to show that the known replicative intermediates, i.e. dimers and lariats are present in the mt DNA synthesized by the complex. Labelled DNA from the membrane-DNA complex was isolated and analysed by sedimentation velocity in sucrose gradients. Previous work (Goddard and Cummings, 1975) showed that it is possible to separate, by this technique, the main classes of replicative intermediates (monomers, lariats and dimers) of *Paramecium* mt DNA. The sucrose gradient profile from a typical experiment with labelled DNA isolated from the membrane--DNA complex is presented in Figure 12A. The curve of absorbance at 260nm is in good agreement with the distribution of radioactive label, allowing three major peaks to be resolved, namely peaks II, III and IV. This indicates the presence in the membrane-DNA complex of several classes of labelled DNA molecules having different sedimentation velocities. In order to calibrate the gradient, i.e. to find the correlation between the peaks and the replicative intermediates they represent, a series of additional experiments was carried out.

It has been shown (Goddard and Cummings, 1975) that the percentage of replicative intermediates in *Paramecium* mitochondria can be increased by addition of certain drugs to growing cultures. When cells are grown in the presence of ethidium bromide accumulation of lariats is observed; while the addition of chloramphenicol to the culture results in accumulation of dimers. Therefore, by preparing the mitochondrial membrane-DNA

Figure 12. Analysis of DNA from the membrane-DNA complex by sucrose sedimentation velocity centrifugation: *in vitro* labelled DNA from the membrane-DNA complex (A); DNA from the membrane-DNA complex of chloramphenicol (B) and ethidium bromide (C) treated cells. The labelling conditions were as described under Methods; (the incubation was carried out with shaking for 30 minutes). The labelled DNA was extracted from the complex and analysed by 8-25% linear sucrose gradient as described under Methods. The gradient was fractionated and the fractions were processed for determination of radioactivity. The DNA in B and C was prepared in the same manner except for the labelling procedure which was omitted. ●——● absorbance; ○——○ radioactivity.


A
B
C
Absorbance (260 nm)
Radioactivity (dpm × 10⁻²)
I
II
III
IV
Bottom
Fraction Number
Top

complex from cultures treated with these compounds it should be possible to observe the enrichment of particular replicative intermediates when DNA extracted from such complexes is analysed on sucrose gradients. Thus, the exact position of lariats and dimers can be identified on the gradient. The sucrose gradient absorbence profiles from experiments where cells were treated with chloramphenicol and ethidium bromide are presented in Figure 12B and C. It was concluded that peak I corresponds to dimers as this additional peak was predominant after chloramphenicol treatment. Peak II represents the position of lariats as it was significantly increased by treatment with ethidium bromide. It is thought that peak II represents late lariats (molecules with large circles and very short tales) and their sedimentation velocity is close to that of dimers. Peak III is deduced to correspond to other forms of lariats less advanced in their replication and finally, peak IV may represent very early lariats (very small circles with very long tails) possibly cosedimenting with monomers. These results when combined with the incorporation of (^{3}H)dTTP into mt DNA (Figure 11) show that incorporation of the label occurs into the various intermediates of mt DNA replication, however, they do not indicate whether this incorporation is merely a repair function or genuinely reflects the in vivo replication process.

In order to examine which of these two forms of DNA synthesis was being observed the incorporation of (^{3}H)dTTP by the membrane-DNA complex was further examined in pulse-chase experiments. Figure 13 presents a comparison of sucrose sedimentation velocity gradient profiles of labelled DNA in a typical pulse-chase experiment. As the synthesis progresses the pulse-labelled DNA can be seen to flow from small fragments (peak IV corresponding to early lariats) to larger molecules. After a 50 minute chase the amount of radioactive material decreases

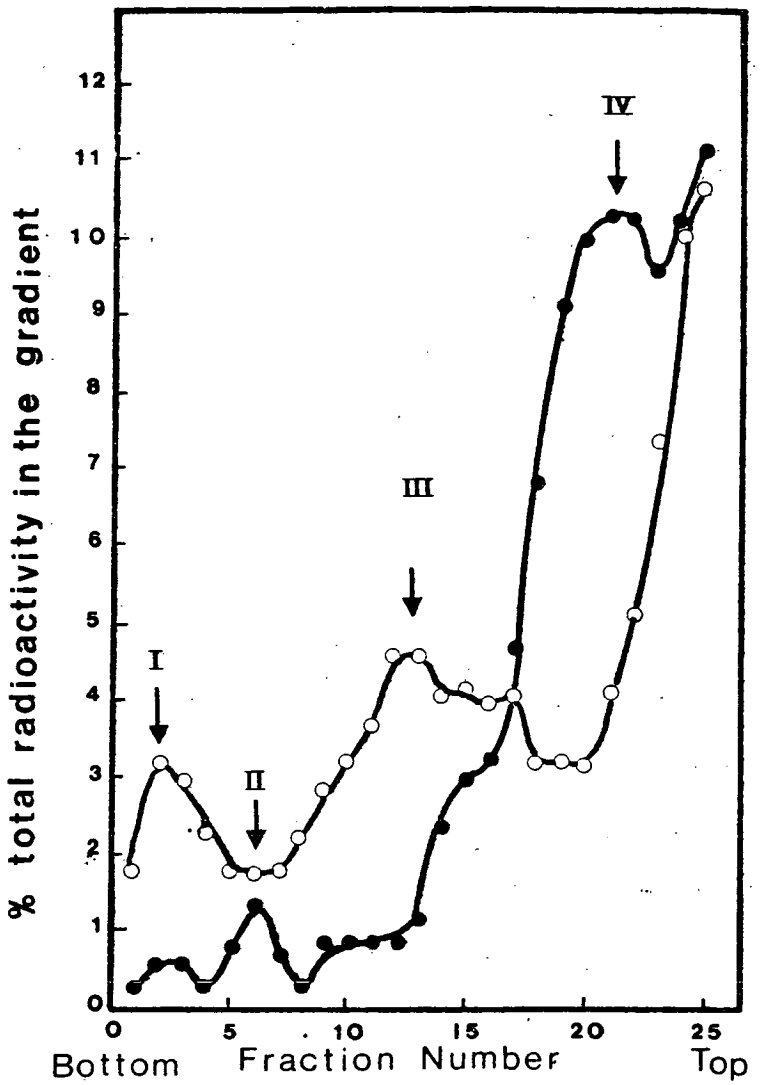


Figure 13. Analysis by sucrose sedimentation velocity centrifugation of DNA labelled by the membrane-DNA complex in vitro in a pulse-chase experiment. The labelling conditions were as described under Methods; incubation with (^{3}H)dTTP was carried out with shaking for 10 minutes. Unlabelled dTTP was added to a final concentration of 400μM and incubation continued for 50 minutes. The DNA was extracted from the complex and analysed by 8-25% linear sucrose gradient as described in Methods. The gradient was fractionated and the fractions were processed for determination of radioactivity. The total radioactivity added to each gradient ranged from 9250 to 9930dpm. —— , pulse; o——o , chase.

considerably in peak IV, while new peaks corresponding to the more advanced molecules in replication appeared (broad peak of lariats - peak III and a peak of late lariats together with dimers - peak I).

These data establish that the DNA synthesis by the membrane-DNA complex *in vitro* does occur through the same replicative intermediates as observed in mitochondria *in vivo*. Therefore, by these criteria the membrane-DNA complex can be referred to as a replication complex.

2.4 Proteins in the replication complex

A further characterisation of the replication complex involves studies of its protein components by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. The complex was prepared from purified mitochondria using the procedure described in Section 1.2 of Results. Samples were taken at various stages of the preparation of the complex: (i) purified mitochondria, (ii) the top fraction from the discontinuous sucrose gradients, (iii) the interface fraction from the gradient representing membrane-DNA complex and (iv) the pellet fraction from the same gradient. These samples were subsequently subjected to SDS polyacrylamide gel electrophoresis (Figure 14). All these fractions exhibit characteristic and highly reproducible banding patterns, the pattern from whole mitochondria being clearly distinct from that obtained from the replication complex. It can be seen that the complex contains fewer proteins than whole mitochondria, particularly of lower molecular weights (below 70,000 daltons) where the bands missing from the complex can be found either in the pellet or in the top fraction of the discontinuous sucrose gradient. The band array representing the proteins of the replication complex consists of a total of 34 bands corresponding to polypeptides ranging in molecular weight between 210,000 and about 17,000 daltons.

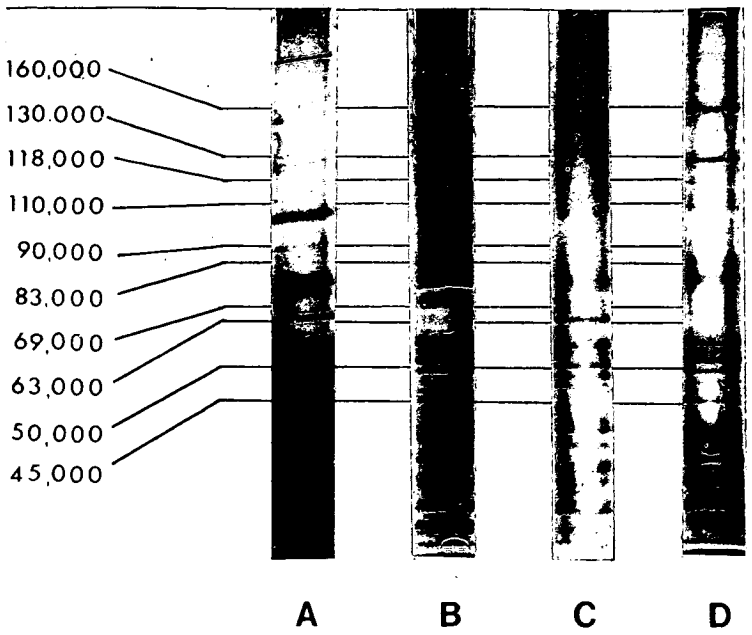


Figure 14. 7.5-15% SDS polyacrylamide gel electrophoresis of proteins from various stages of preparation of the replication complex: purified mitochondria (A); replication complex (interface fraction between 15-40% sucrose on the sucrose cushion) (B); top fraction from the sucrose cushion (8.5% sucrose) (C) and pellet from the sucrose cushion (D).

TABLE 5. Comparison of proteins from whole mitochondria and the replication complex which differ in their relative staining intensities (data from Figure 14).

Molec. weight	Mitochondria	Replication complex
160,000	-	+++
130,000	+	+++
118,000	-	++
110,000	-	++
90,000	-	+
83,000	-	+++
69,000	-	+++
63,000	+++	+
50,000	+	+++
45,000	+	+++

- : band absent or present in trace amounts

+; ++; +++ : increasing intensities of staining.

An interesting feature is that several polypeptides, present in trace amounts in the mitochondrial sample, are accumulated specifically in the replication complex (160,000; 130,000; 83,000 and 69,000 daltons - see Table 5). The selective association of these proteins with the replication complex could suggest their possible involvement in the replication of mt DNA.

As mentioned previously, the growth of cells in the presence of ethidium bromide and chloramphenicol results in accumulation of replicative intermediates (lariats and dimers respectively) in mitochondria of Paramecium (Goddard and Cummings, 1975). One can expect that these alterations in mt DNA replication would be reflected in changes in mitochondrial proteins and on this basis one should be able to detect differences in protein pattern between mitochondrial replication complexes isolated from normal and drug treated cultures. The proteins of replication complexes isolated from normally grown cultures (control) and from cells exposed either to ethidium bromide or chloramphenicol are compared by SDS polyacrylamide gel electrophoresis in Figure 15. Several differences can be detected among the samples presented, the most striking of which is the appearance of a new polypeptide with an estimated molecular weight of about 100,000 daltons in the complex from cells grown in the presence of ethidium bromide. In Tetrahymena pyriformis the drug has been shown to induce increased levels of mitochondrial DNA polymerase (Westergaard et al., 1970) leading to an accumulation of mt DNA replicative intermediates (Upholt and Borst, 1974). From Figure 15 it is impossible to determine whether the additional band is in fact a new protein induced by ethidium bromide or an elevated level of a protein undetectable in the complex from normal cultures. As the molecular weight of this protein, at about 100,000 daltons, is close to that of

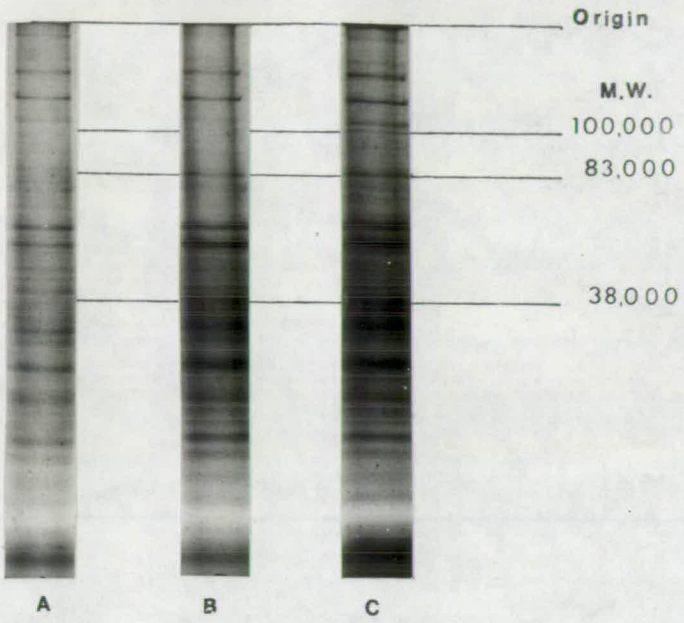


Figure 15. 12-20% polyacrylamide slab gel electrophoresis of proteins from replication complexes isolated from normally grown cells (B), chloramphenicol (A) and ethidium bromide (C) treated cells.

the ethidium bromide induced mitochondrial DNA polymerase of Tetrahymena (85,000 daltons; Westergaard and Lindberg, 1972) and similar to the molecular weight of mitochondrial DNA polymerases isolated from other organisms, this band may represent the Paramecium mitochondrial DNA polymerase.

Comparison of replication complexes isolated from control cultures and from cells grown in the presence of chloramphenicol reveals two major differences. A polypeptide of estimated molecular weight 38,000 daltons, which gives a prominent band in the control and ethidium bromide samples, is absent in the complex derived from chloramphenicol treated cells. Similarly, the distinct 83,000 dalton band in normal and ethidium bromide cultures is present in only trace amounts in the chloramphenicol sample. One of these variations in the protein content of the replication complex from the chloramphenicol treated cells might be responsible for the lack of separation of the dimers into the monomer daughter duplexes, which is observed in mitochondria of Paramecium cultures exposed to this drug.

As shown previously (Figure 14) the replication complex contains some 34 polypeptides distinguishable by SDS polyacrylamide gel electrophoresis. It was suspected that not all of these proteins are involved in the process of replication of mt DNA as some of them might represent components of the inner membrane. Therefore, an attempt was made to remove some non-replication proteins from the complex. The method chosen involved partial solubilization of the replication complex by increasing salt concentrations with the aim of finding a concentration which would remove a maximum amount of proteins while still maintaining the DNA polymerase activity.

The procedure involved treatment of the replication complex with

varying amounts of NaCl, separation of solubilized proteins by centrifugation and subsequent determination of protein amounts remaining in the pellets with simultaneous monitoring of the DNA polymerase activity. In addition, the proteins from the pellets and the supernatants were subjected to SDS polyacrylamide gel electrophoresis. Figure 16 presents the effect of various salt concentrations on the activity of the complex. It can be seen that the maximal salt concentration, which still preserves full DNA polymerase activity, is 0.2M; further increase in salt results in diminished activity while the concentration of 4M NaCl totally abolishes the incorporation of the label. The electrophoretic comparison of proteins from the replication complex treated with various salt concentrations (Figure 17) revealed that even low concentrations of NaCl (0.1M) selectively removed several polypeptides such as those of molecular weight 83,000; 63,000; 53,000 daltons and also one can observe a selective disappearance of several bands of low molecular weight (below 35,000). All of the proteins removed were recovered in the supernatant fraction. However, a further increase in salt concentration did not lead to additional specific removal of polypeptides but only caused progressive solubilisation of all proteins and, after exceeding a concentration of 3M, the bands became too faint for a meaningful interpretation.

To summarise, it was observed that with reduction of the number of polypeptides from 34 (Figure 14) to 22 (Figure 17) the replication complex was still capable of (^{3}H)dTTP incorporation. An interesting observation is the loss of the 83,000 dalton polypeptide. As this protein was also absent from the replication complex isolated from chloramphenicol treated cells (Figure 15), it might represent a protein involved in cleavage of the dimer molecules during the process of replication.

These studies provide a preliminary characterisation of the replication complex in terms of its requirements for activity and its protein

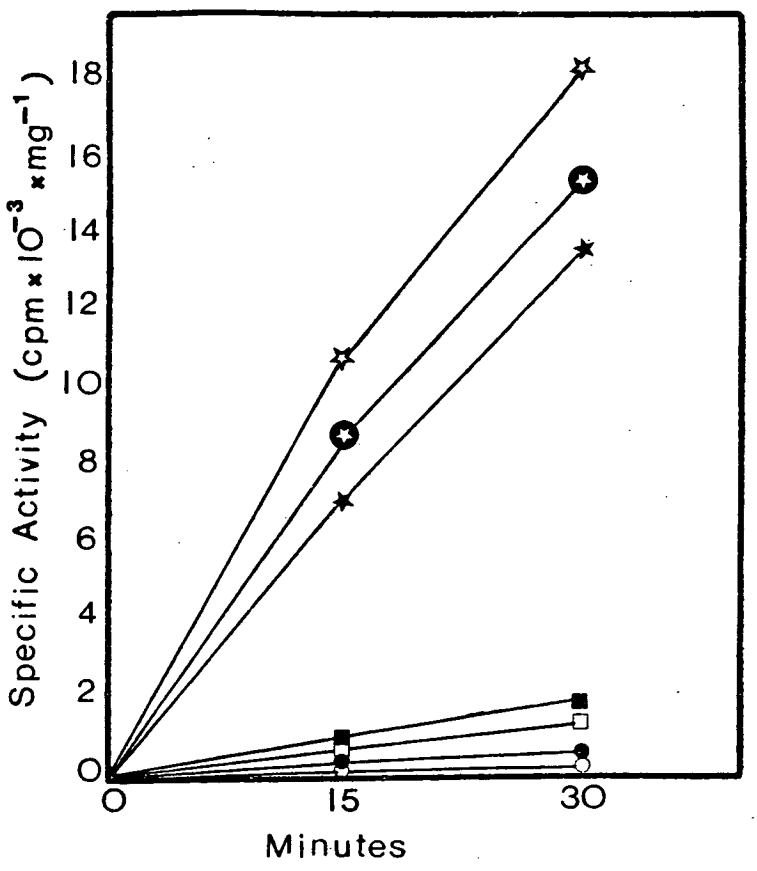


Figure 16. Specific activities of the replication complex solubilised with various salt concentrations. The complex was treated with different amounts of NaCl for 2 hours at 0°C with gentle stirring. The samples were centrifuged at 150,000xg for 90 minutes and the obtained pellets were incubated with (^{3}H)dTTP using the standard procedure described in Methods. The amount of radioactivity and protein was determined in each pellet from which the specific activity (cpm/mg protein) was calculated for each sample. ★——★ , control (0.0M NaCl); ✪—✪ 0.1M NaCl; ☆—☆ 0.2M NaCl; ■—■ 2.0M NaCl; □—□ 3.0M NaCl; ●—● 3.5M NaCl and ○—○ 4.0M NaCl.

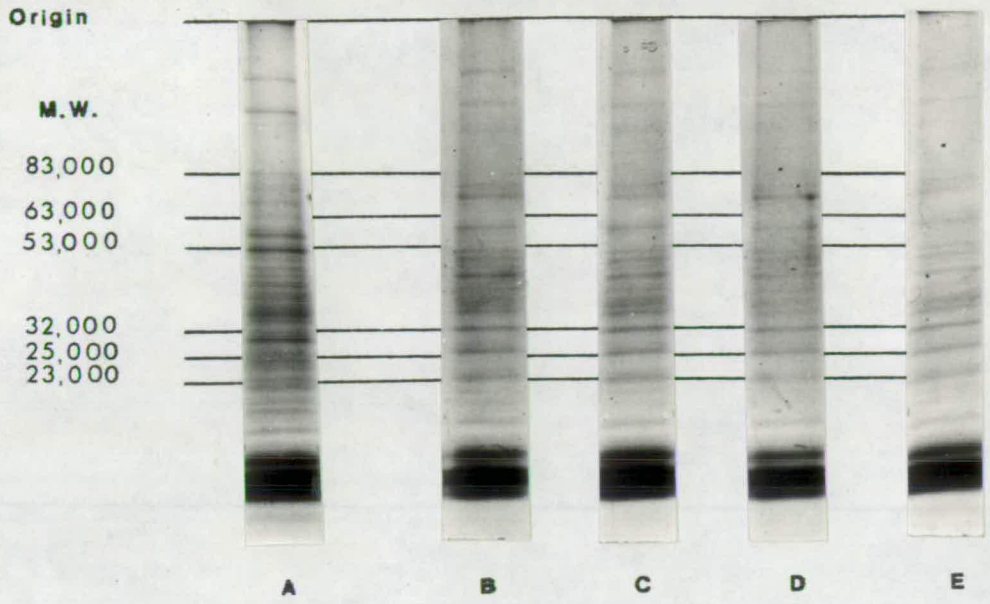


Figure 17. Electrophoretic comparison of proteins from the replication complex solubilised with various concentrations of salt. The complex was treated as described in the legend of Figure 16. After centrifugation at 150,000xg for 90 minutes, the proteins from the pellet and supernatant fractions were run on 12-20% SDS polyacrylamide slab gels. Control (untreated complex) (A); complex treated with 0.1M (B), 0.2M (C), 2.0M (D) and 3.0M (E) salt.

components, and establish that it is able to catalyze the complete replication of the mitochondrial genome through the previously characterized intermediates. Preliminary attempts were made to extract proteins (by various salt treatments) with a view to determine the minimum protein requirements for activity. It was shown that several of the proteins could be removed from the complex without loss of activity, but due to lack of specificity (in terms of specific protein removal), this approach was not pursued further. Nonetheless, the isolation and characterization of this complex provides the initial and crucial step for further work aimed at identifying and characterizing the proteins involved in mt DNA replication; the scope and possibilities now open are discussed later.

3. Mitochondrial chromatin

It was originally planned to use the electron microscope in studies on the mitochondrial replication complex with the aim of examining the purity of the complex, i.e. to show whether it was contaminated by any other cell structures. These electron microscopy studies revealed that the complex consisted of structures resembling nucleosomes characteristic of nuclear chromatin. This observation led to a series of experiments aimed at examining these mitochondrial structures in greater detail.

As in this part of the results a concept of mitochondrial chromatin will be introduced it seems appropriate at this point to provide a brief description of typical nuclear chromatin, which will be often referred to in the later sections of this study.

Despite the great variety and complexity of eukaryotic nuclear chromosomes they are at one level remarkably uniform. The chromosomal material, or chromatin, contains five histones, that are complexed with DNA in a repeating pattern. The histones, and the pattern are nearly invariant among organisms, cell types and stages of the cell cycle. The repeat unit of the pattern is the nucleosome (Kornberg, 1977). The nucleosome comprises a set of eight histone molecules complexed with about 200 base pairs of DNA. The set of eight histones consists of two each of four types; H2A, H2B, H3 and H4. The role of the fifth histone, H1, is probably concerned with a higher level of nucleosome organisation (Klug, 1978). The DNA component of the nucleosome is made up of a "core" of 146 base pairs (Prunell et al., 1979), which is highly conserved, and a "linker", which varies from 15 to 100 base pairs depending on cell type. The DNA is wrapped round an octamer of the histones forming a roughly spherical particle approximately 10nm

in diameter. Nucleosomes lie in close apposition along the length of a chromatin fibre, producing a characteristic "beads on a string" appearance in the electron microscope.

The experiments described in this section include an investigation by electron microscopy of the organisation of the mitochondrial genome in the replication complex and an attempt to detect the presence of organelle histone-like proteins. In addition, the possible association of basic proteins with the mitochondrial genome has been studied by examining the nuclease digestion of mt DNA within the intact organelle.

3.1 Electron microscope studies

The replication complex was prepared from mitochondria according to procedure presented in Figure 4. When the complex was examined by electron microscopy the only structures observed were those shown in Figures 18 and 19, namely a series of bead-like structures linked by strands of DNA. In the structure shown in Figure 18 three different regions can be distinguished. The first region (A) contains a relatively regular distribution of nucleosome-like structures similar to the "beads" observed in preparations of nuclear chromatin. The second region (B) contains stretches of a thin filament lacking any nucleosome--like structures, and in the third region (C) more condensed, larger particles are seen. The size of various particles was measured and found to be 130Å for the small "beads" which is similar to the size of SV 40 nucleosomes (Oudet et al., 1975), while large particles (about 300Å) could correspond to a condensed globular form of native SV 40 chromatin (Müller et al., 1978). It is thought that these irregularities in the state of condensation of chromatin-like structures as well as irregular distribution of beads are a result of the preparative procedure, which is not only lengthy but involves exposure of the

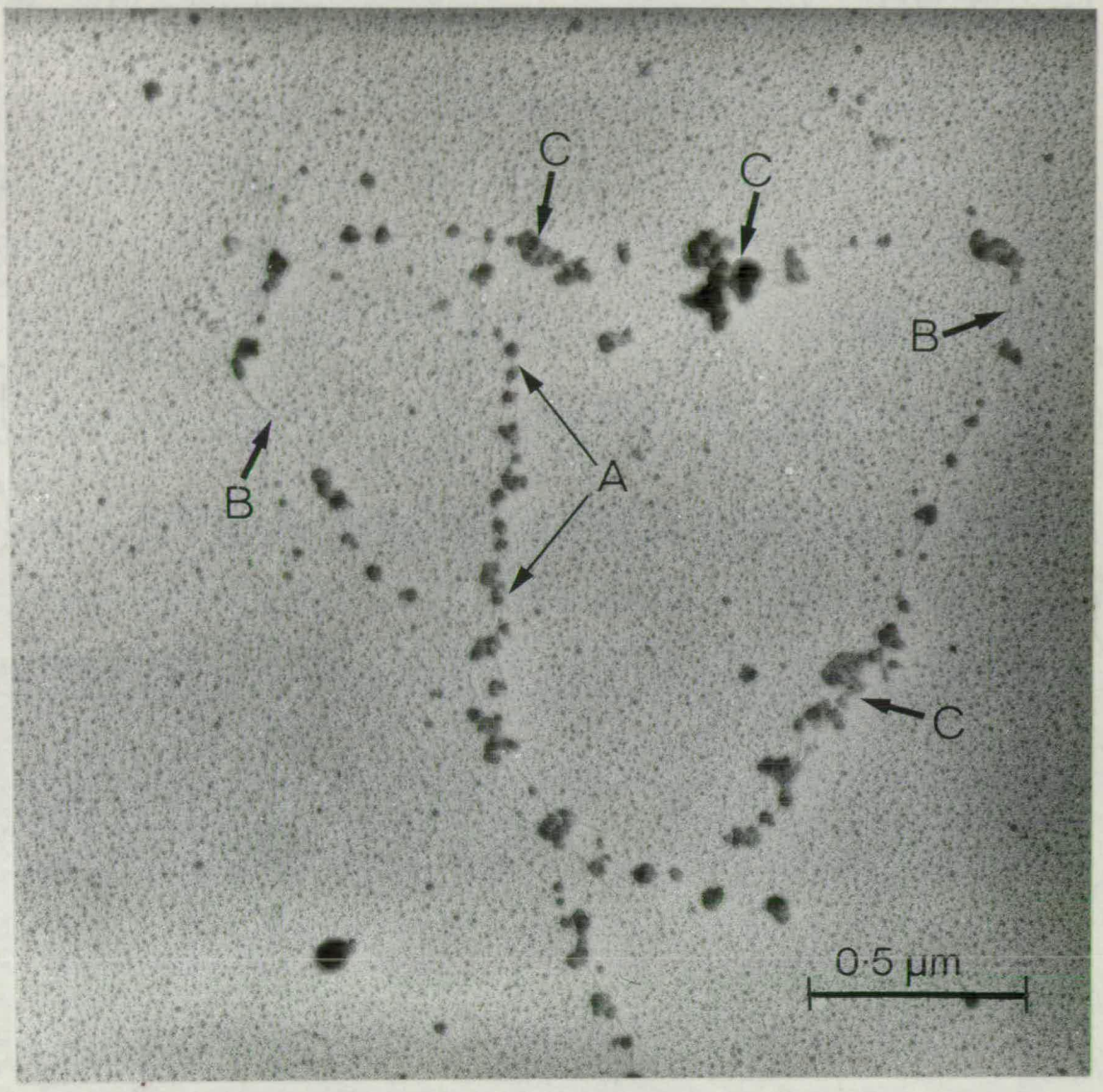


Figure 18. Electron micrograph of the mitochondrial chromatin-like structure showing regions with three different types of structure: relatively regular distribution of nucleosome-like structures (A); filaments lacking any particles (B) and regions with more condensed, larger particles (C).

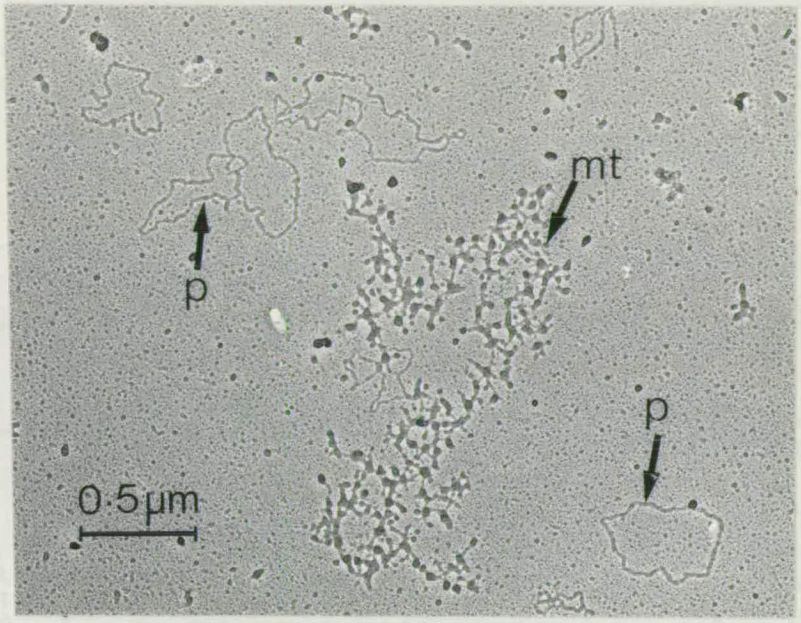


Figure 19. Electron micrograph illustrating chromatin-like structure (mt) and protein-free plasmid DNA (p).

nucleoprotein complex to low salt concentration. Müller et al. (1978) have shown that the degree of condensation of chromatin depends on the ionic strength of the buffer used for the preparation of nucleoprotein complexes. By varying the ionic strength they obtained a spectrum of forms similar to those observed in this study, ranging from fully condensed to completely extended. In order to prevent damage of the chromatin-like structure, the preparative procedure used in this study was modified by fixing the material with glutaraldehyde prior to exposure to low salt concentration and further purification. With this procedure it was possible to isolate beaded structures of more uniform size and more regular distribution (Figure 19). Strands of naked DNA could only be seen when the beaded structures were deproteinised by the standard technique (see Methods) and subsequently visualised by electron microscopy (Figure 20).

Measurement of several randomly picked molecules revealed an average length of 13.7 μm (± 1.44 μm) and 26.4 μm (± 1.0 μm) which corresponds to the size of monomers and dimers respectively of mt DNA in Paramecium as reported by Goddard and Cummings (1975).

It is necessary to demonstrate that the nucleosome-like structures do not arise as a result of the preparative procedure and also that they are indeed of mitochondrial origin, i.e. they are not the result of contamination by nuclear or bacterial material. The first possibility was eliminated by adding protein-free circular DNA (plasmid pCM-21 restricted with EcoRI; Bishop, 1979) to the purified replication complex and then preparing grids for electron microscopy. Figure 19 shows the result of this experiment and it can be seen that the plasmid DNA does not acquire any beaded structures during the preparative procedure.

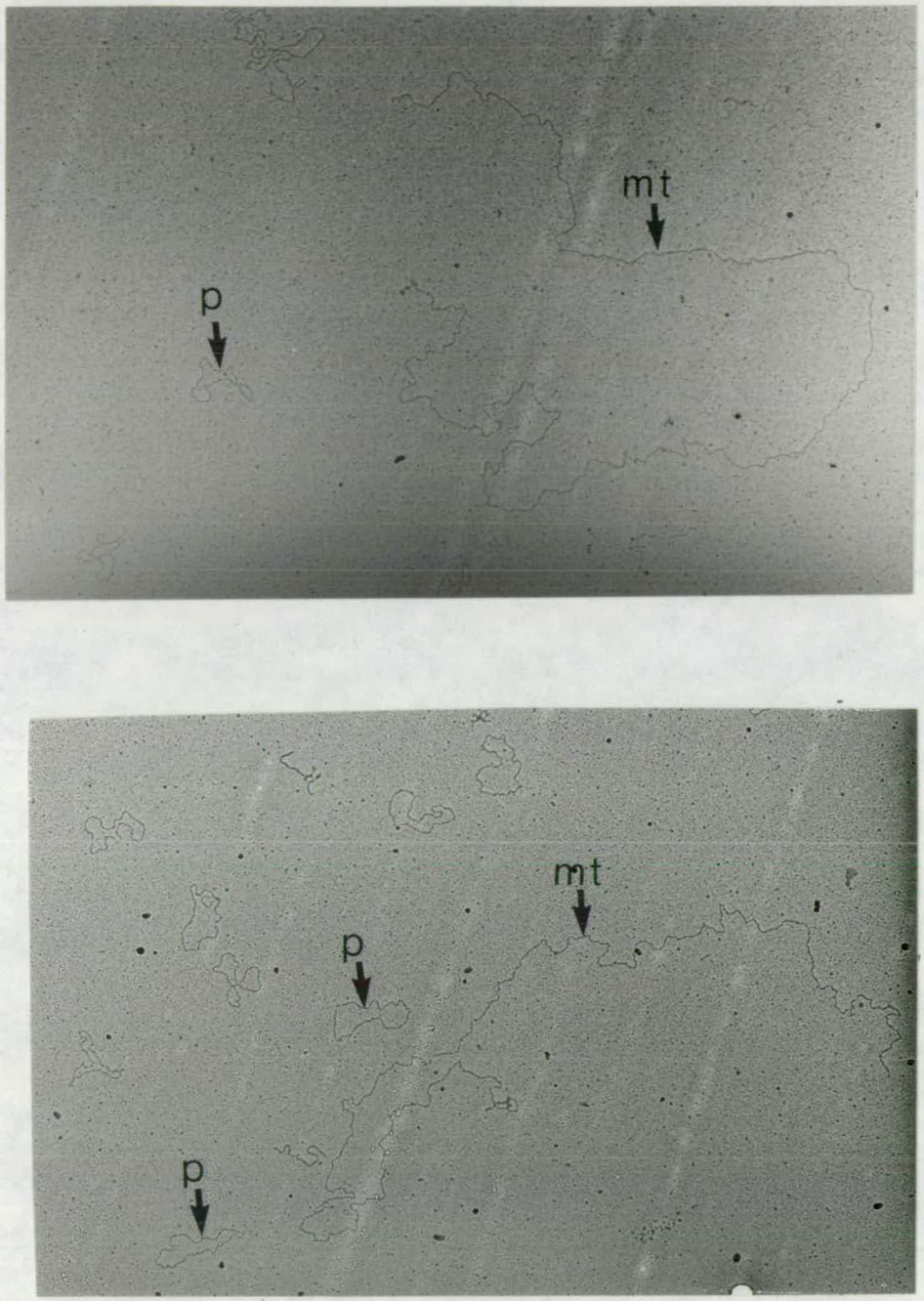


Figure 20. Electron micrographs illustrating linear "naked" mt DNA obtained after deproteinization of replication complex; linear mt DNA of monomer size (mt); marker DNA-plasmid pCM21 restricted with EcoRI (p).

The second possibility, namely that these structures were of nuclear or bacterial origin, can be excluded on the basis of the several lines of evidence presented in previous section of the Results (see section concerning the origin of the membrane-DNA complex). Further evidence against a bacterial origin of the observed chromatin--like structure came from experiments in which Paramecium was grown in axenic culture. Electron microscopic examination of the replication complex isolated from these cultures revealed the presence of chromatin--like structures identical to those obtained from bacterised cultures.

Thus it can be concluded that the chromatin-like structures seen in the electron microscopy definitely comprise mitochondrial and not nuclear or bacterial elements.

3.2 Basic proteins in the replication complex

The chromatin-like structures observed in the electron microscope were further examined in regard to the existence and identity of basic proteins associated with DNA. The proteins from the beaded structures were isolated by acid extraction of the replication complex and examined by acetic acid/urea acrylamide gel electrophoresis. In order to compare mitochondrial basic proteins with known histone proteins, calf thymus histones were used as a standard. In addition, histones were isolated from Paramecium nuclei to provide a further basis for comparison. An electrophoretic comparison of calf thymus histones, nuclear histones of Paramecium and basic proteins from the mitochondrial replication complex is presented in Figure 21. Paramecium nuclear histones show mobilities lower than those from calf thymus. A similar observation has been reported by Isaacks and Santos (1973). The basic proteins from the mitochondrial replication complex show 9 bands, five of which

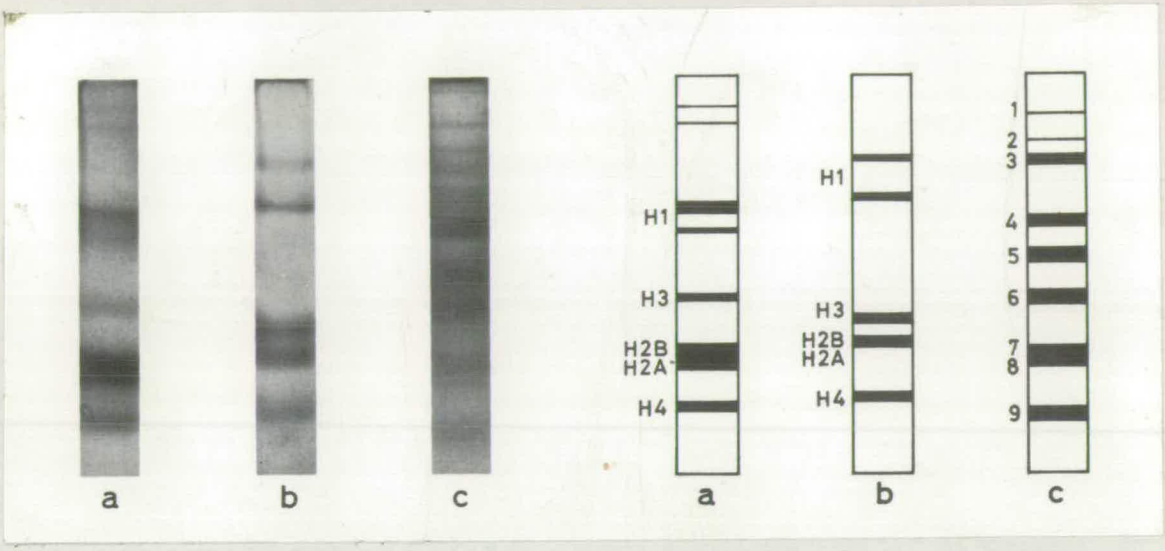


Figure 21. Electrophoretic comparison of calf thymus histones (a); Paramecium nuclear histones (b) and basic proteins from the mitochondrial replication complex (c). Histones were extracted and run on urea-polyacrylamide slab gel as described in Methods.

(bands 4, 6, 7, 8 and 9) have similar mobilities to the histone bands of calf thymus.

The results presented in Figure 21 suggest that there are basic proteins associated with the mitochondrial replication complex. These proteins have properties similar to histones in their solubility in acid and their electrophoretic mobility. The histone-like bands from the complex differ considerably in their electrophoretic mobilities from the histones extracted from Paramecium nuclei. Thus, contamination of mitochondrial material by nuclear histones may be disregarded.

In addition to P. primaurelia (routinely used in all experiments described so far), mitochondrial histone-like proteins were extracted also from P. septaurelia and a hybrid containing nuclei from P. septaurelia and mitochondria from P. primaurelia. A comparison of densitometer tracings of urea-acrylamide gels of basic proteins extracted from the replication complexes derived from these strains is presented in Figure 22. For comparative purposes calf thymus histones are also included. It can be observed that the major difference between syngen 1 and syngen 7 is observed in the peak which, on the basis of its electrophoretic mobilities, can be correlated with histone H4 from calf thymus. As this peak in the hybrid is in an identical position to that in P. septaurelia and not as in P. primaurelia, it can be concluded that the genetic control of one histone-like protein in mitochondria can be attributed to the nucleus. As far as the other mitochondrial basic proteins are concerned one can observe similarities between species in the position of the peaks except for some minor quantitative differences. In this respect the organelle basic proteins resemble the nuclear histones which, in other organisms, are conserved to a great degree.

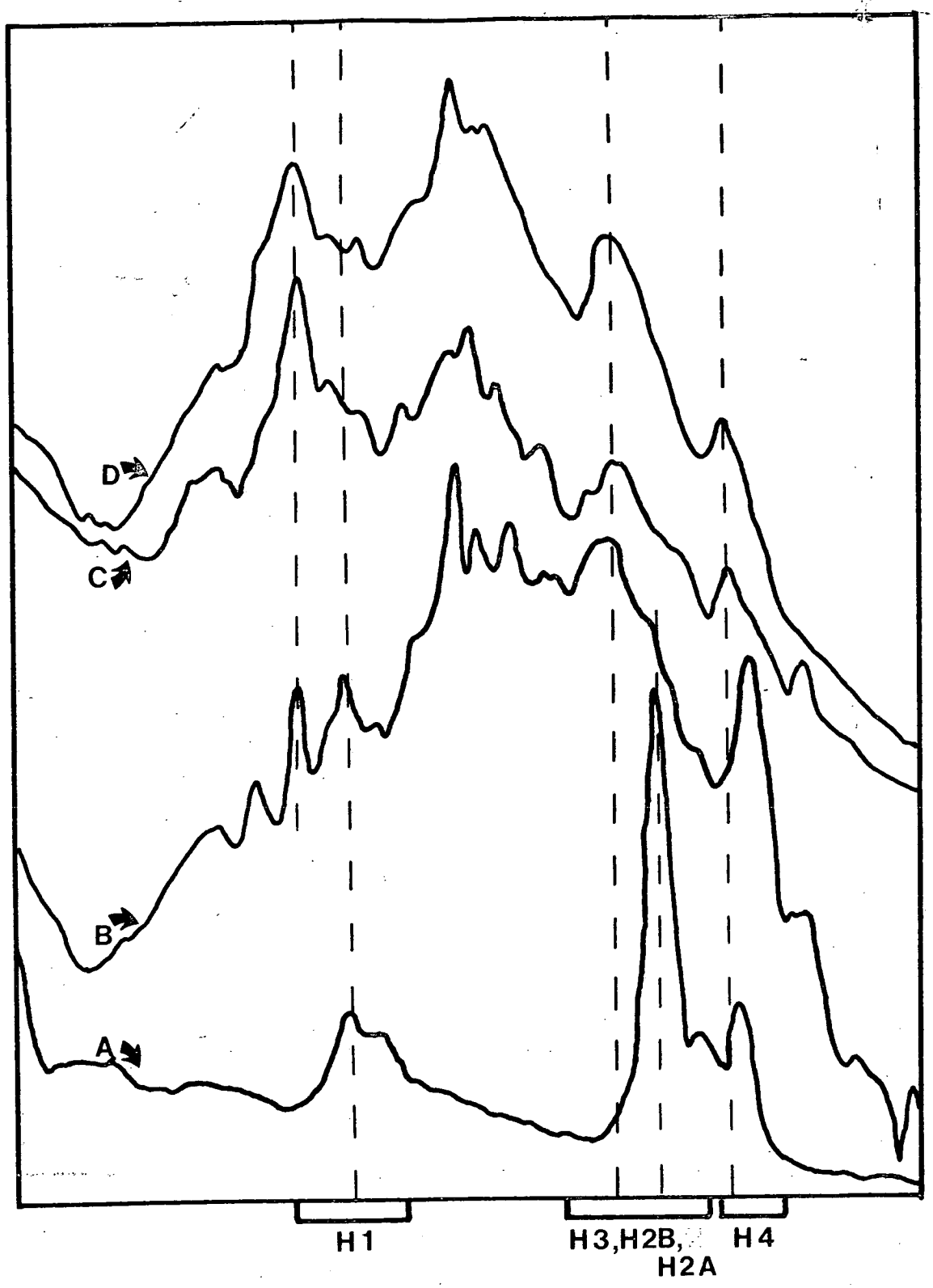


Figure 22. Densitometer tracings of urea-acrylamide slab gels of calf thymus histones (A), histone-like proteins extracted from the mitochondrial replication complex of P. primaurelia (B), P. septaurelia (C) and the hybrid containing P. septaurelia nucleus and P. primaurelia mitochondria(D).

3.3 Protection of mt DNA from nuclease digestion

In order to determine if the histone-like proteins are directly associated with mt DNA, a series of experiments involving nuclease digestion of organelle DNA was performed.

The digestion of eukaryotic chromatin with endonucleases has been routinely used in the study of nuclear nucleoprotein complexes and this technique provides the strongest evidence for the existence of nucleosomes as repeating units of chromatin. The accessibility of the chromatin to nuclease action is severely limited due to the association of nuclear DNA with histones. Therefore, digestion of native chromatin by a number of nucleases does not result in random cuts of the DNA but in a series of discrete fragments (Noll, 1976). Micrococcal nuclease, an enzyme that exhibits a marked preference for making double-strand cuts in the connecting DNA strand between nucleosomes, generates a series of multiples of an average unit length of 200 base pairs. Digestion with other nucleases (e.g. pancreatic DNase 1) or extensive digestion with micrococcal nuclease results in patterns of DNA fragments shorter than 200 base pairs as cleavage occurs within the nucleosome. In the case of DNase 1 the spacing between the cuts has been shown to be 10.4 nucleotides (Prunell *et al.*, 1979).

Digestion of mitochondria followed by separation of the DNA on acrylamide gels could lead to one of the following: (i) If mt DNA is not associated *in vivo* with histone-like proteins, the digestion with nuclease would be random, resulting in continuous range of DNA fragment sizes. This type of random degradation by nucleases has been demonstrated for deproteinized calf thymus and rat liver DNAs by Hewish and Burgoyne,(1973). (ii) If mt DNA is protected by histone-like proteins from the digestion with nucleases, one would expect a series of discrete bands corresponding to multiples of chromatin subunits.

In all experiments involving nuclease digestion of organelle DNA, whole mitochondria were used in preference to the replication complex, for the following reason. The evidence obtained from electron microscopy revealed that the preparative procedure for the isolation of the complex had caused partial removal of the nucleosome-like structures, resulting in an irregular distribution of these particles along the DNA strand (see Section 3.1 of Results). These stretches of naked DNA could distort any evidence for protection, making interpretation difficult and possibly leading to erroneous conclusions.

The first experiment involved digestion of whole mitochondria with micrococcal nuclease. As mentioned earlier (Section 1.2 of the Results) omission of BSA from the buffer used during the isolation of mitochondria results in preparation of "leaky" organelles. The same procedure (i.e. omission of BSA) was applied for the isolation of mitochondria used for the micrococcal nuclease digests in order to make the organelles accessible to the enzyme. After digestion, mt DNA was extracted from the organelle and analysed by polyacrylamide gel electrophoresis. The result of a typical experiment is presented in Figure 23A. It can be observed that after a 4 minute digestion with micrococcal nuclease a series of distinct bands were produced, which became more pronounced after 8 minutes of incubation. Although the banding pattern is well defined, it is not identical with the characteristic regular pattern of banding observed for nuclear chromatin, since no regular repeat of DNA fragments corresponding to multiples of a unit size can be found. However, some regularity can be observed among DNA fragments of molecular weight below 100 base pairs, as they represent about 10, 20, 30, 40, 60, 80 and 100 base pairs. It should be noted that this series of low molecular weight bands is already present, albeit faintly, at zero time, i.e. prior to

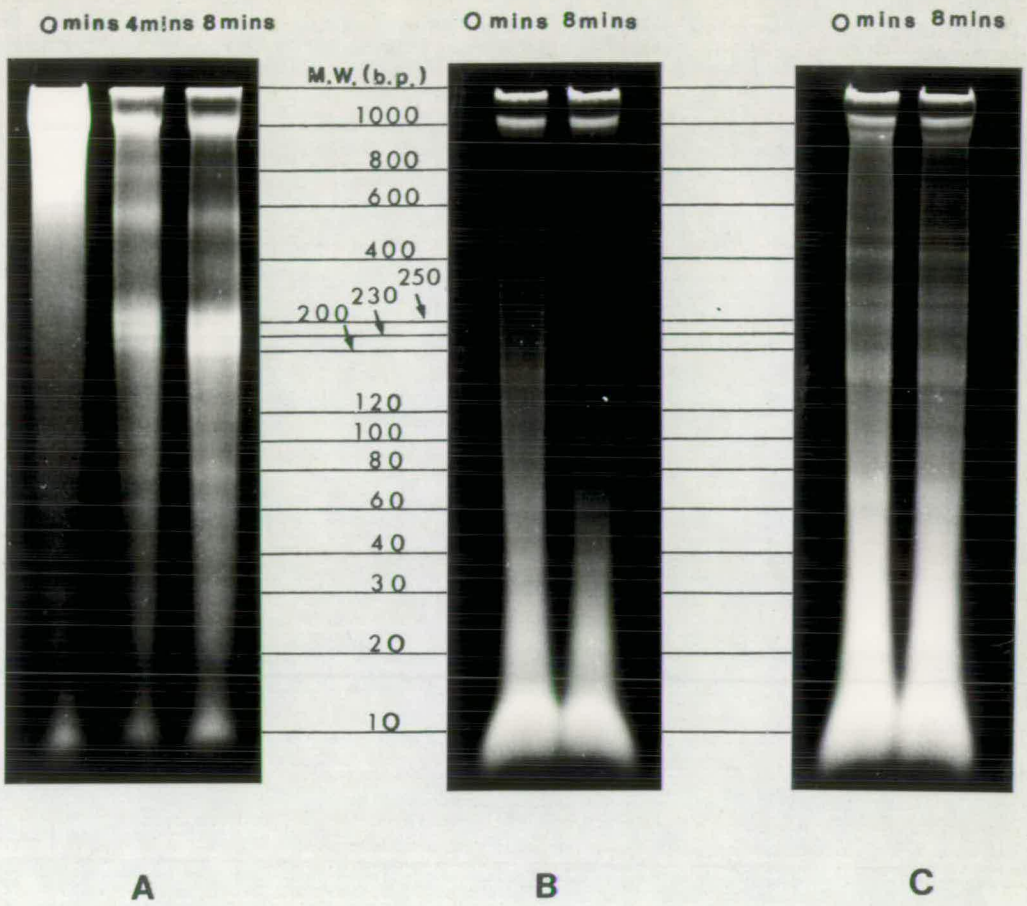


Figure 23. Micrococcal nuclease digests of mitochondria. Mitochondria were digested with 0.1 unit of micrococcal nuclease per $10A_{260}$ of mitochondrial suspension (measured in 1% SDS). Samples were processed as described in Methods and the extracted DNA was electrophoresed on 6% polyacrylamide slab gels containing 6M urea, for 3 hours at 24mA. Gels were stained with ethidium bromide (10µg/ml) for 20 minutes and photographed under UV light. Molecular weights were calculated using plasmid DNA (pMB9 x HaeIII) as a standard. (A) mitochondria not treated with DNase 1; (B) mitochondria treated with DNase 1 (10µg/ml for 20 minutes) prior to the micrococcal nuclease digestion and, (C) the same as B, but BSA (0.25%) was included in mannitol/MOPS buffer used throughout preparation of mitochondria and treatment with DNase I.

micrococcal nuclease action. A possible explanation could be that this is a product of partial digestion by mitochondrial endogenous nuclease(s), however, the majority of the DNA remains undigested at the origin of the gel. To summarize, the result of this experiment strongly suggests that mt DNA is protected in some way as the digest does not show the continuous smear characteristic for naked DNA.

An interesting feature of the nuclease digest gel patterns presented in Figure 23A is the variation in intensity of the bands. The three bands of (molecular weights about) 200, 230, 250 base pairs were reproducibly the most prominent. One possible explanation could be that these bands represent the most susceptible sites of the mitochondrial chromatin-like structure, or alternatively, that these bands represent a product of digestion of nuclear chromatin contaminating the mitochondria. It was necessary, therefore, to prove unambiguously that nuclear chromatin was not contaminating the mitochondrial preparation. To accomplish this, mitochondria were treated with DNase 1 prior to digestion with micrococcal nuclease. This treatment has been shown to be effective in removing nuclear DNA from isolated mitochondria (Goddard and Cummings, 1975). It was expected that if the digest obtained under such conditions was identical to that observed in the mitochondria not treated with DNase 1 (Figure 23A), it would provide evidence supporting the mitochondrial origin of the bands. The results presented in Figure 23B revealed the disappearance of high molecular weight DNA fragments leaving only bands below 100bp and these were digested further as incubation with micrococcal nuclease continued. Initially this would tend to suggest that the series of bands obtained in Figure 23A were attributable to nuclear chromatin which was now removed by DNase 1 treatment. The low molecular weight bands of 10 and 20bp repeat observed in Figure 23B can be explained as a product of combined digestion of nuclear chromatin

by DNase 1 and micrococcal nuclease. However, this explanation may be refuted when one considers that the mitochondria used for the digest were prepared in buffer without BSA which is normally used to protect the organelles during the standard preparative procedure. The omission of BSA may result in mitochondria being permeable to DNase 1, thus causing the digestion of mitochondrial chromatin-like structures into the fragments observed in Figure 23B.

Therefore, BSA was included in the mitochondrial preparation in the subsequent series of experiments to protect mitochondria from the action of DNase 1, although this might also result in the micrococcal nuclease being unable to penetrate the mitochondrial membrane. When mitochondria were isolated in the presence of BSA, then treated with DNase 1 to remove contaminating nuclear material and subsequently incubated with micrococcal nuclease, the digest obtained (Figure 23C) revealed a series of distinct bands virtually identical to the pattern observed in Figure 23A; the only difference being the disappearance of the intense bands of 200, 230 and 250bp. This would imply that these bands were of nuclear origin. The occurrence of DNA fragments at zero time suggests the presence of endogenous nuclease(s) in mitochondria. It can be seen that the zero time samples in Figures 23A and 23C differ markedly in the degree of digestion. This is almost certainly due to the zero time sample being taken when the mitochondria were at $4^{o}C$ in the case of A and $30^{o}C$ in the case of C. This implies that raising the temperature activates the mitochondrial endogenous nuclease(s). In order to confirm this assumption a further series of experiments were conducted, which involved preparation of mitochondria in the presence of BSA, followed by DNase 1 treatment, but no addition of micrococcal nuclease. The action of endogenous nuclease(s) was monitored by taking samples at various stages of the preparation of the mitochondria (Figure 24). No digest is observed at the early stages

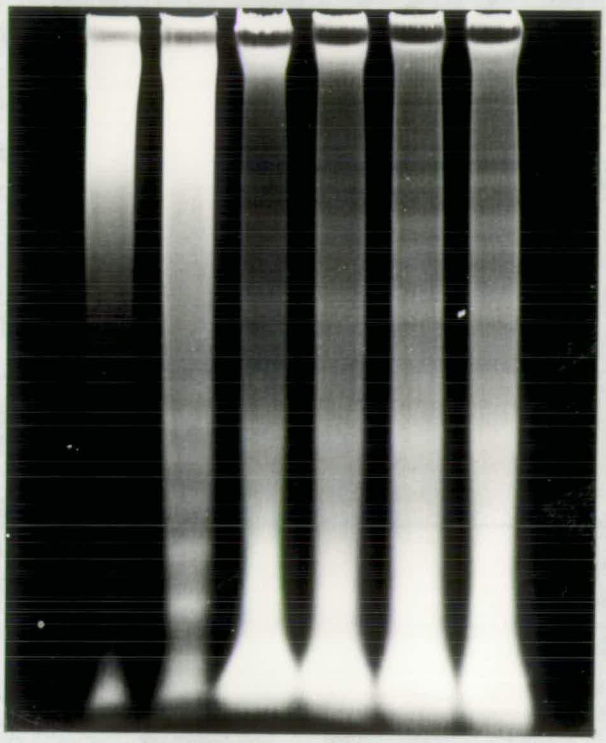


Figure 24. Polyacrylamide gel electrophoresis of DNA after digestion of mitochondria with endogenous nuclease. Mitochondria were isolated in mannitol/MOPS buffer containing 0.25% BSA. Samples were processed and electrophoresed as described for Figure 23. (A) undigested mt DNA prior to DNase 1 treatment (sample taken at $0^{o}C$); (B) partially digested mt DNA after DNase 1 treatment (sample taken at $0^{o}C$); (C-F) incubation of mitochondria at $30^{o}C$ for 2, 4, 8 and 12 minutes.

of the preparative procedure (A), but after treatment with DNase 1 some bands of low molecular weight appeared, while the majority of material remained as high molecular weight DNA. The low molecular weight DNA fragments may represent either a product of digestion by DNase 1 of contaminating nuclear chromatin, or they could be due to a partial digest of the mitochondrial chromatin-like structure from damaged organelles, thus rendered accessible to DNase 1. The typical digest starts as soon as the temperature is raised from $4^{o}C$ to $30^{o}C$ and progresses with time (C-F) as shown by the increase in intensity of lower molecular weight bands and the decrease in intensity of the high molecular weight DNA at the origin.

The experiments presented in this section establish that mt DNA of Paramecium is protected from random digestion by nucleases. The digestion products show a characteristic reproducible pattern of bands when analysed by gel electrophoresis, different from the typical "ladder pattern" obtained with nuclear chromatin. This finding, when combined with the electron microscope studies and with the evidence supporting the presence of histone-like proteins in the mitochondria, strongly implies that the organelle genome is organised in a chromatin-like structure.

DISCUSSION

It has been shown to be possible to isolate from the mitochondria of *Paramecium aurelia* a membrane-DNA complex, essentially free of contaminating bacterial and nuclear DNA. This complex was found to be capable of incorporating (^{3}H)dTTP into replicative intermediates *in vitro* using its endogenous mt DNA as template.

The evidence for the mitochondrial origin of the replication complex.

In all the work described, both on the replication complex itself and the chromatin-like structures isolated from it, it is crucial to establish that one is dealing with a structure of mitochondrial origin rather than of nuclear or bacterial origin. Thus, it is important to critically consider the evidence supporting the conclusion that those structures are mitochondrial.

Several technical approaches were used to verify that the DNA found in the complex was indeed of mitochondrial origin. An initial indication that bacteria were an unlikely source of the complex came from the failure to obtain a membrane-DNA complex from *Klebsiella* cultures using an identical procedure as for mitochondria. Nuclear DNA contamination can be ruled out on the basis of the buoyant density of the DNA extracted from the complex (Figure 5). The observed value of 1.700g x cm^{-3} is in good agreement with densities reported for mt DNA in *P. primaurelia* (1.699g x cm^{-3} (Goddard and Cummings, 1975) and 1.701g x cm^{-3} (Anott, personal communication)) and does not correspond to the value of 1.686g x cm^{-3} expected for nuclear DNA (Goddard and Cummings, 1975). However, in view of the data indicating that in *P. primaurelia* the density of mt DNA coincides with that obtained for ribosomal DNA (Cummings, 1975), the density criterion can no longer be considered sufficient evidence for the presence of mitochondrial and absence of nuclear

DNA in the membrane-DNA complex. Therefore, restriction analysis of DNA extracted from the membrane-DNA complex was undertaken with the expectation of obtaining conclusive evidence. Although the result of these experiments (Figure 6) did not provide an unambiguous answer due to the failure to obtain restricted DNA, it still provided some evidence in favour of the mitochondrial origin of the complex. Firstly, the molecular weight of the undigested DNA on the gel was estimated to be about 27 x 10^6 daltons which is in good agreement with the size of Paramecium mt DNA (27.1 x 10^6 daltons - Goddard and Cummings, 1977). Secondly, if bacterial or nuclear DNA were present in the preparation in significant amounts, it would appear on the gel as a smear rather than as one discrete band. Thirdly, electron microscopic analysis of ribosomal DNA in P. primaurelia revealed that the majority of the molecules consisted of 1.5 - 2μm linear duplexes (Cummings, 1975). Whether the length of these molecules represents the actual length of the ribosomal DNA genes, remains to be proven as the author could not exclude the possibility of degradation during the isolation procedure. However, in P. tetraurelia analysis of the satellite ribosomal DNA on agarose gel electrophoresis showed a broad distribution of sizes, with some clustering around molecular weights of 5-7, 9-11, 20-23 and 29-30 x 10^6 daltons (Findly and Gall, 1978). Although the sizes of ribosomal DNA in P. primaurelia might be different from those of P. tetraurelia, a similar heterogeneity in the length would be expected. If this is the case either a smear or several bands would be observed on the gel and not a single distinct band.

The fact that no radioactivity was detected in the membrane-DNA complex obtained from a mixture of unlabelled cells and purified nuclei labelled with (methyl-^{3}H) thymidine provides additional evidence for

the absence of nuclear DNA in the complex (Table 3). Moreover, one could argue that since the sucrose gradient profiles of DNA extracted from the replication complex showed only replicative intermediates characteristic of mt DNA (Figures 12 and 13), this precludes any major contamination of nuclear or bacterial origin.

Taken together, the results of these experiments, using a variety of technical approaches, all fail to provide any evidence for the presence of bacterial or nuclear DNA in the membrane-DNA complex and consistently point to the DNA being mitochondrial. It was, therefore, concluded that DNA present in the membrane-DNA complex isolated from mitochondria is indeed of mitochondrial origin. Obviously, the final proof for the presence of mt DNA in the complex would be achieved from successful restriction digests of the DNA extracted from the complex, or hybridization of the DNA with labelled mt DNA.

Incorporation of (^{3}H)dTTP by the membrane-DNA complex.

Unequivocal proof that mt DNA replication occurs on the membrane-DNA complex requires the demonstration that the complex is capable of DNA synthesis *in vitro* and that the known replicative intermediates are present in the mt DNA synthesized by the complex.

The membrane-DNA complex is capable of incorporating (^{3}H)dTTP *in vitro* (Figure 7). The optimal conditions for this incorporation in terms of pH and magnesium ions requirements were found to be 7.0 and 10mM respectively (Figures 8 and 9), which differ from the reported optimal conditions for the membrane-DNA complex isolated from rat liver mitochondria (pH 8.4 and 30mM Mg^{2+}; Shearman and Kalf, 1975). Differences in optimal magnesium requirement are commonly found in purified mitochondrial DNA polymerases from various organisms, e.g. 50mM for yeast

mitochondrial DNA polymerase (Wintersberger and Wintersberger, 1970) and 8-10mM for rat liver (Shearman and Kalf, 1975). The 10mM magnesium concentration required for optimal (^3H)dTTP incorporation by the membrane--DNA complex from Paramecium is significantly higher than that reported for all three DNA polymerases extracted from nuclei of Paramecium (1-2mM; Tait and Cummings, 1975). These differences in magnesium requirement could be due to variation between mitochondrial and nuclear polymerases. However, as the membrane-DNA complex represents a multi-enzyme system for the synthesis of DNA, the optimal concentration of Mg^{2+} required by the complex may reflect an overall requirement for this cation by all enzymes present in the complex. It has been reported that the addition of manganese ions to the standard assay conditions including magnesium caused a marked reduction of mitochondrial DNA polymerase activity in yeast with 5mM Mn^{2+} leading to total inhibition (Wintersberger and Blutsch, 1976). A similar effect, although less pronounced, was observed in the studies with the mitochondrial membrane-DNA complex from Paramecium; the incorporation of (^3H)dTTP was reduced about 50% by the addition of 5mM Mn^{2+}. This finding is of interest with regard to mutations in the mitochondrial genome induced in yeast cells grown in the presence of manganese ions (Putrament et al., 1973; 1975; 1977). An explanation of the mechanism of interference by Mn^{2+} with the mitochondrial DNA polymerase activity, and of the induction of cytoplasmic mutants is not yet known. However, it is suspected that the mutagenic action of manganese is produced through its effect on DNA polymerase, possibly by an alteration in the fidelity of the enzyme.

All four deoxyribonucleoside triphosphates were required for maximal incorporation of (^3H)dTTP by the membrane-DNA complex (Figure 7). However, the incorporation of the label was not totally dependent on the

presence of all four triphosphates and, in fact, showed about 30% of optimal activity with only one of them present. This activity could be partially attributed to a limited repair synthesis in which thymidine monophosphate was incorporated at many single strand breaks. A similar lack of total dependence on four triphosphates has also been observed for nuclear DNA polymerases isolated from Paramecium (Tait and Cummings, 1975) and from other eukaryotic sources, where 66% of optimal DNA polymerase activity in the absence of three triphosphates has been reported for rat ascites hepatoma cells (Tsuruo et al., 1972). An alternative, but highly speculative, explanation for the incorporation of (^{3}H)dTTP by the membrane-DNA complex in the absence of three triphosphates could be that terminal nucleotydyltransferase is present in the complex. This enzyme behaves like a DNA polymerase in synthesizing a DNA chain, but unlike a DNA polymerase the enzyme neither requires nor responds to a template and may even dispense with a primer, thus interfering with and complicating assays of DNA polymerase (Kornberg, 1974). In addition, actinomycin D inhibits DNA polymerase but has no effect on the activity of terminal nucleotydyl transferase (Keir et al., 1963). If terminal transferase is in fact present in the mitochondrial membrane--DNA complex it has to be questioned to what extent the observed incorporation with all four triphosphates represents DNA polymerase activity and what part of it is due to terminal nucleotide addition. On the basis of the 70% inhibition of (^{3}H)dTTP incorporation by the membrane-DNA complex in the presence of actinomycin D, it was concluded that the major enzyme activity in the complex was that of DNA polymerase. This conclusion is in agreement with the reported observation that the terminal transferase incorporates one deoxyribonucleoside triphosphate at a maximal rate in the absence of the other three triphosphates

(Goltesman and Canellalis, 1966). Since ethidium bromide at low concentrations preferentially inhibits mitochondrial DNA polymerases (Meyer and Simpson, 1969, 1970: Hecht, 1975; Radsak and Seidel, 1976; Fujisawa et al., 1977), the observation that the incorporation of (^{3}H)dTTP by the membrane-DNA complex is inhibited by this compound provides strong evidence for the DNA polymerase in the complex being of mitochondrial origin. By comparison ethidium bromide had no effect on the activity of any of the three nuclear DNA polymerases isolated from Paramecium (Tait and Cummings, 1975).

That the DNA synthesis observed is actually being carried out by the membrane-DNA complex per se rather than contaminating mitochondria is evidenced by the following facts. The concentration of Sarkosyl employed is sufficient to lyse virtually all mitochondria present. Furthermore, any unlysed mitochondria would pass through the discontinuous sucrose gradient and pellet on the bottom of the tube during the preparation of the membrane-DNA complex.

Replication of mt DNA in vitro

The results presented in this study indicate that replicative synthesis, i.e. DNA synthesis involving formation of known replicative intermediates (lariats and dimers) does indeed occur on the membrane-DNA complex in vitro. Preliminary evidence was obtained from sucrose gradient analysis of mt DNA labelled by the complex which shows the incorporation of label into molecules with sedimentation characteristics identical to those of replicative intermediates formed during mt DNA synthesis in vivo (Figure 12). Proof for the occurrence of replicative synthesis and not merely repair activity in the membrane-DNA complex was obtained from pulse-chase experiments. Such experiments (Figure 13) showed that the labelled DNA could be chased from small molecular

weight species (interpreted as monomers or very early lariats) into more advanced lariats and dimers. The results of these studies also imply that the membrane-DNA complex contains some if not all of the enzymes necessary for replication of mitochondrial DNA.

Taken together, the demonstration that:

(i) the membrane-DNA complex contains mt DNA;

(ii) the complex is capable of DNA synthesis *in vitro*; and

(iii) this synthesis is not due to a repair function but involves the formation of known replicative intermediates, permits the conclusion that the membrane-DNA complex isolated in this study represents a mitochondrial replication complex.

An important aspect of the results obtained with the replication complex is the implication that at least at one stage of the replication cycle *Paramecium* mt DNA is attached to the inner membrane of mitochondria. The presence of mt DNA in the complex must be a consequence of an original attachment of the DNA to the membrane, since it has been shown that nucleoproteins or nucleic acids alone do not interact with the magnesium--Sarkosyl crystals (Tremblay *et al.*, 1969). An important question is whether the attachment of DNA to the inner mitochondrial membrane has any functional significance in the replication and/or segregation of mt DNA during the biogenesis of the organelle. Since this problem was not investigated in this study, further work is required before any answer to this question can be provided. However, evidence supporting a functional significance for the attachment of mt DNA to the inner membrane has been obtained for rat liver mitochondria. Membrane-DNA complexes obtained from this material contained only the replicating portion and not the bulk of non-replicating DNA (Shearman and Kalf, 1975, 1977). If this functional significance should prove to be true for *Paramecium*

mitochondria, it raises the interesting point that, regardless of the structure of the DNA (circular or linear) and its mode of replication, membrane attachment seems to be a prerequisite for replication of organelle DNA. The association of replicating DNA with a membrane has been reported in several other systems: (i) bacterial DNA attached to the cell membrane (Firshein, 1972); (ii) eukaryotic chromosomal DNA attached to the nuclear membrane (Infante et al., 1973); (iii) bacterial plasmid (Dowman and Meynell, 1973; Hershfield et al., 1973) and phage DNA (Siegel and Schaechter, 1973) to the host cell membrane and (iv) adenovirus and simian virus 40 DNA attached to the host nuclear membrane (Smith and Vinograd, 1972; Le Blanc and Singer, 1974).

Proteins in the replication complex

The above proof that the replication complex is capable of replicating mt DNA in vitro implies that apart from membrane proteins the complex must contain the multienzyme component of DNA synthesizing machinery, its presence in the complex resulting from its association with mt DNA. There is therefore an expectation that the procedure for the isolation of the complex will result in the specific selection of functionally related proteins and not simply a random pool of mitochondrial proteins. The comparison of proteins obtained from whole mitochondria and the replication complex did indeed show highly significant differences in protein content. Relative differences observed by SDS polyacrylamide gel electrophoresis included not only the absence in the complex of polypeptides present in the mitochondria, but also the specific accumulation in the complex of several proteins either undetectable or present in trace amounts in the intact organelle (Figure 14 and Table 4). The experiments involving solubilisation of the replication complex by salt showed that not all 34 proteins detected by SDS

polyacrylamide gel are necessary for the incorporation of (^{3}H)dTTP. The complex was still active in terms of DNA synthesis when the number of proteins was reduced to 22 (Figure 16 and 17). Interestingly, none of the proteins (except 83,000 daltons) which were selectively accumulated through the isolation of the replication complex were removed by the salt concentrations which still maintained the incorporation of the label (compare Table 4 with Figure 17). This observation can be considered as an additional indication of the involvement of the replication complex proteins in mt DNA synthesis. Although no attempt has been made in this study to separate protein components of the complex, a similar mitochondrial membrane-DNA complex from mouse cell cultures has been successfully used as a partially purified source of mitochondrial DNA polymerase (Radsak and Seidel, 1976).

It has been observed that the drugs ethidium bromide and chloramphenicol both interfere with the process of mt DNA replication, leading to the accumulation of replicative intermediates. In Tetrahymena pyriformis, treatment with ethidium bromide results in about a 35-fold increase in the level of mitochondrial DNA polymerase activity (Westergaard et al., 1970; Westergaard and Lindberg, 1972) and also in an accumulation of DNA molecules in early stages of replication (Upholt and Borst, 1974). A similar effect of ethidium bromide has been observed in Paramecium where an increased proportion of lariats was found while treatment with chloramphenicol led to the accumulation of dimers (Goddard and Cummings, 1975). It was considered likely that changes induced by these drugs in the replication of mt DNA should be reflected in an alteration of some of the proteins in the replication complex. Analysis of mitochondrial replication complexes obtained from normal and drug-treated cultures did indeed reveal differences in the

polypeptides detected by SDS-polyacrylamide gel electrophoresis (Figure 15). If the mechanism of action of ethidium bromide in Paramecium is similar to that in Tetrahymena, i.e. leading to accumulation of replicative intermediates coupled with induced levels of mitochondrial DNA polymerase, then the 100,000 dalton polypeptide appearing in the complex from drug-treated cells may represent the induced Paramecium mitochondrial DNA polymerase. The induced polymerase may play a role in the increase of replicative intermediates, but the connection is not obvious. Westergaard and co-workers (1970) suggested that ethidium bromide treatment of Tetrahymena results in a damage of mt DNA and that the induced polymerase is a repair enzyme. However, this interpretation was not confirmed since results obtained by Upholt and Borst (1974) showed that no repairable damage (breaks or gaps) was induced, but the drug led only to an inhibition of mt DNA synthesis. The induction of mitochondrial DNA polymerase must, therefore, have another explanation. One of them is a hypothesis presented by Uphold and Borst who assume that the treatment with ethidium bromide also causes an inhibition of mt RNA/or protein synthesis. They speculate, therefore, that the block in transcription leads to a general overproduction of nuclear gene products involved in mt DNA replication because these genes are controlled by a mitochondrial repressor that cannot be synthesized in the presence of the drug. However, if all the proteins concerned with mt DNA replication are coded by nuclear genes and controlled by the putative repressor, one would expect ethidium bromide to merely increase the rate of mt DNA synthesis. In order to explain the accumulation of replicative intermediates, one would have to assume that either not all replication proteins are controlled by a mitochondrial repressor or that there is at least one mitochondrially synthesized

replication protein.

Inhibitory drugs have proved an invaluable tool with which to investigate a central problem of mitochondrial genetics: the genetic control of mitochondrial functions. Chloramphenicol and ethidium bromide have, in particular, contributed to the study of the genetic control of proteins involved in replication. Since the coding capacity of the mitochondrial genome is limited, due to its small size, many, if not the majority of mitochondrial functions must be controlled by the nucleus. Consequently, the mitochondrial and nuclear genomes must interact extensively during the formation and function of the organelle. Only limited information is available concerning the genetic control of the proteins involved in the replication of mt DNA, and consists largely of data on the DNA polymerase. Yeast mitochondrial DNA polymerase is most probably coded by the yeast nuclear genome as synthesis of the enzyme has been shown to occur on cytoplasmic ribosomes (Wintersberger and Wintersberger, 1970). In addition, the fact that many "petite" strains of yeast with highly distorted genetic information continue to replicate mt DNA (Linnane et al., 1972), implies that the proteins required for the replication are coded by the nucleus and synthesized on cytoplasmic ribosomes.

To date, the only data suggesting mitochondrial control of any of the proteins involved in mt DNA replication comes from studies with Paramecium. As mentioned previously, chloramphenicol, a specific inhibitor of protein synthesis on mitochondrial ribosomes, blocks the separation of the dimers into two monomeric units (Goddard and Cummings, 1975). It appears, therefore, that one or more proteins synthesized on mitochondrial ribosomes are necessary for at least one step in the replication of mt DNA. As the evidence presented so far indicates that mRNA

import into mitochondria can be practically ruled out (Borst, 1972; Gillham, 1978), one can hypothesize further that at least one protein involved in the final stage of replication is coded by the mitochondrial genome. In this study some variation was observed in the proteins from the mitochondrial replication complex after exposure of cells to chloramphenicol, the most striking being the loss of one polypeptide readily detectable in the control (Figure 15). In addition, a further protein was diminished to trace amounts. The disappearance of proteins from chloramphenicol treated cells could readily be explained by the failure of drug-treated mitochondria to synthesize membrane proteins. Nevertheless, since the replication complex is specifically enriched with proteins involved in DNA replication, the loss could equally be attributed to the absence of one of these replicative proteins, in particular that responsible for cleaving dimers into monomers. However, in order to obtain unambiguous evidence that the missing protein is indeed involved in the separation of dimers, further investigation of this problem is necessary. Future research would concern the isolation of this protein from the normal replication complex and its characterisation using the ability to cleave the dimer molecule as the assay system. It should be stressed, however, that conclusions based upon drug-inhibition experiments may have several limitations. Synthesis of mitochondrial proteins involves cooperation between the protein synthesizing systems of organelle and cytoplasm. Inhibition of the synthesis of any one component can easily lead to an array of secondary pleiotropic effects on membrane structure and function as a whole. For example, apart from the possibility that a protein may be missing from chloramphenicol treated mitochodnria because its synthesis occurs on mitochondrial ribosomes, it can be also true that the protein is supplied by cytoplasmic ribosomes

but requires for its activity an additional "carrier or binding" factor which is mitochondrially synthesized (Schatz and Mason, 1974).

The isolation of the replication complex from mitochondria of Paramecium opens promising possibilities for future research. Future work would involve (i) fractionation of the proteins present in the complex, (ii) identification of their specific functions in the process of DNA replication and (iii) determination of their genetic control. The first step would involve a separation of replication proteins from those not involved in the process of replication. This could be done by a variety of technical approaches, e.g. gradual extraction of proteins by various solubilising agents with simultaneous assay of DNA synthetic activity in the complex, or DNA affinity chromatography which would allow isolation of DNA binding proteins from the complex. The proteins could be characterised according to their functional involvement in the process of replication by using assay systems containing one class of replicative intermediates and purified protein(s) isolated from the replication complex. A relatively simple assay would be sufficient for the protein(s) involved in the cleavage of dimers (as mentioned previously) and for the protein(s) specific for the linkage of monomers at the unique initiation site. Assay systems which include purified protein(s) from complexes isolated from ethidium bromide and chloramphenicol treated cells could serve as an additional aid in determining the replicative functions of the proteins. Another approach could be visualised as an assay of DNA synthesis in protein depleted replication complexes with simultaneous monitoring of the manner in which the removal of particular protein(s) affects the progression of replication. The determination of genetic control of proteins involved in replication could be facilitated by use of hybrid cells produced by

microinjection. In this study comparisons of replication complexes isolated from P. primaurelia and P. septaurelia were made and revealed some protein variation. Furthermore, an assay of DNA polymerase activity in the complex from mitochondria of P. septaurelia showed a different optimal magnesium requirement from that of P. primaurelia. As the experiments were preliminary these results were treated only as provisional evidence and have not been presented. However, it is possible that the variations observed might affect the replication proteins, and if this supposition is proved to be correct, a replication complex isolated from the hybrid cells would provide a means for determining genetic control of such proteins.

In comparison with replicative proteins, considerable information is now available concerning the genetic control of the components of the mitochondrial protein synthesizing machinery. The existence of specific tRNAs and corresponding aminoacyl tRNA synthetases in mitochondria has been known for some time. The available evidence indicates that most, if not all, mitochondrial tRNAs are coded by the mitochondrial genome, while in contrast, the corresponding aminoacyl tRNA synthetases are most likely coded by nuclear genes whose messages are translated in the cytoplasm (Gillham, 1978). DNA-RNA hybridisation experiments and DNA sequencing studies offer the most direct approach to determine what is coded by the organelle DNA. From such experiments it has been shown that mtDNA contains sequences complementary to rRNAs present in the organelle and a large number of tRNAs. In Saccharomyces cerevisiae mtDNA has been shown to code for about 26 tRNA species (Rabinowitz et al., 1976; Borst and Grivell, 1978). Approximately 25-30 tRNAs have been found in Neurospora crassa (Terpstra et al., 1977), while sequencing studies of human mtDNA have revealed 22 tRNA

genes (Anderson et al., 1981). In the case of Tetrahymena, Suyama and Hamada (1976) reported finding only 7 tRNA species transcribed from mtDNA and suggested that 23 tRNAs were imported into the mitochondria. On the other hand, detailed studies of mitochondrial tRNAs in yeast strongly argue against the import of tRNA into mitochondria (Borst and Grivell, 1978). More recently, using a combination of two dimensional electrophoresis and DNA-RNA hybridisation, Suyama (1981) has provided convincing evidence for the import of at least 26 tRNA species into Tetrahymena mitochondria, a further 10 tRNAs being mitochondrially coded.

A search has been made in various organisms for the presence of mitochondrial mRNA, and 18 species of polyA-containing mRNA have been found in the mitochondria of HeLa cells (Attardi et al., 1976). Messenger-like polyA-containing RNA has also been associated with hamster, Drosophila and mosquito mitochondria (Hirsh et al., 1974). In yeast a mitochondrial mRNA component has been successfully translated in vitro and the product identified as three subunits of cytochrome oxidase (Padmanaban et al., 1975; Rabinowitz et al., 1976). Other respiratory components have also been found to be synthesized within the mitochondrion, including the cytochrome b apoprotein and ATPase subunit 6. Interestingly, ATPase subunit 9 while mitochondrially coded in yeast is coded by the nucleus in both Neurospora and man (Borst and Grivell, 1978; Anderson et al., 1981). In general, excluding tRNAs and rRNAs, the contribution of mitochondrial gene products to the biogenesis of the organelle is restricted to the respiratory components of the mitochondrion. However, a notable exception is provided by Paramecium, in which several ribosomal proteins may be coded by the mitochondrial genome (Tait et al., 1976a). The most recent data obtained from sequencing of the human mitochondrial

genome has revealed 13 reading frames in mtDNA sequence, five of which have been assigned to the known mitochondrially synthesized proteins mentioned above (Anderson et al., 1981). The remaining eight reading frames are as yet unidentified (URFs) and the function of proteins they code for is at present a matter of speculation.

Recapitulating, the mitochondrion is a functional organelle which can be considered as a phenotypic hybrid, resulting from the co-ordinate expression of two distinct gene pools. It is well established that the nuclear gene pool bears the major responsibility for determining organelle proteins, while the mitochondrial contribution, though minor, is still crucial to the function of the organelle.

Mitochondrial chromatin

The most interesting finding which emerged during the course of this work with the Paramecium replication complex concerned not the mitochondrial replicative proteins but the presence of basic proteins associated with mtDNA, resulting in a chromatin-like organisation of the genome. It is generally believed that, in contrast to nuclear DNA in eukaryotes, mtDNA is not associated with histones but occurs as "naked" DNA. The results of this study do not agree with this established view as examination of the replication complex isolated from Paramecium mitochondria revealed the existence of structures bearing a high resemblance to typical nucleosomes. The size of the spherical particles, found to be 13nm, is very similar to the size of SV40 nucleosomes isolated from nuclei of cells infected with this virus. As the length of mtDNA in Paramecium aurelia is reported to be 13.8μm (Goddard and Cummings, 1975) which is approximately 43,000 base pairs, one would predict between 210 and 220 nucleosomes per genome, assuming

an average of a 200 base pair repeat as observed in nuclear chromatin. However, due to the technical difficulties discussed in the Results section, it was not possible to estimate the exact number of "beads" per mitochondrial genome in Paramecium.

If these chromatin-like structures observed by electron microscopy are indeed similar to nucleosomes, they must contain histones, and these should be easily extracted from the replication complex. Using the standard techniques for extracting eukaryotic nuclear histones it proved possible to obtain histone-like proteins from the mitochondrial replication complex. When these proteins were analysed on acetic acid/urea polyacrylamide gels nine bands were observed, five of which had similar electrophoretic mobilities to calf thymus histone standards.

One of the mitochondrial basic proteins had no electrophoretic counterpart among either calf thymus or nuclear histones from Paramecium. As yet there is not enough evidence to state whether this band represents a histone-like protein which is unique to mitochondria, or whether it is a non-histone basic protein contaminating the preparation. Further studies involving purification and fractionation of mitochondrial histone-like proteins are necessary to answer this question. A striking feature is the apparent lack of homology between the nuclear histones and mitochondrial histone-like proteins isolated from Paramecium. Although at present one can merely conclude that the electrophoretic characteristics of organelle and nuclear histones are distinct, future work on the degree of homology in amino-acid composition and sequence may reveal more profound differences.

The presence of basic proteins has also been reported in the mitochondria of other organisms. Kuroiwa et al. (1976) presented evidence for at least one species of basic protein in the mitochondrial nucleoid

of the slime mold Physarum polycephalum. This nucleoid protein had an electrophoretic mobility on acid/urea gels similar to, but not exactly the same as, histone H1 from calf thymus. Although no evidence was available for any association of the protein with nucleoidal DNA, the authors considered it likely that this protein was a structural component of the DNA complex in the nucleoid on the basis of its resemblance to nuclear histones. An analogous protein, migrating on acid/urea gels slower than H1, has been isolated together with other basic proteins from the mitochondrial nucleoid of bovine heart tissue (Hillar and Schwartz, 1972; Hillar et al., 1979). It remains to be explained why there is a disparity in the number of histone-like proteins found in mitochondria of various organisms (at least five in the case of Paramecium and only one in the organisms mentioned above). A possible interpretation of this discrepancy may be either the marked differences among mitochondria of diverse organisms, or the possibility that there is more than one histone-like protein present in the mitochondria of these organisms, but that only one has so far been isolated.

The strongest suggestion that the histone-like proteins isolated from Paramecium mitochondria might be in some way functionally analogous to eukaryote nuclear histones came from experiments involving nuclease digestion. It was clearly demonstrated that a nuclease activity exists within isolated mitochondria. This endodeoxyribonuclease(s) under appropriate conditions (temperature and divalent cation concentration) is capable of cleaving the mitochondrial genome into a series of discrete fragments, forming a highly reproducible banding pattern when analysed by electrophoresis. This strongly implies that the mitochondrial genome in situ must be associated with basic proteins,

this association resulting in partial protection against nuclease attack. Although the banding pattern is not identical to that of a typical nuclear digest, i.e. a series of bands being multiples of a unit size, nevertheless a series of discrete intermediates can be distinguished.

Two possible interpretations of this result appear plausible. First, the banding pattern obtained after endogenous nuclease digestion could result from the combined effect of more than one endonuclease having different potential cleavage sites in the nucleoprotein complex. The second interpretation, perhaps more appealing, assumes that mitochondrial chromatin-like structures are unique for the organelle and represent a lower level of organisation than the well advanced nuclear chromatin.

To summarise, on the basis of several lines of evidence, i.e. (i) the observation of nucleosome-like structures by electron microscopy, (ii) the presence of histone-like proteins in the replication complex, and (iii) the association of basic proteins with mtDNA, it was tentatively concluded that the mitochondrial genome from Paramecium aurelia is organised in a chromatin-like structure. In contrast, Caron et al. (1979) did not find evidence favouring a chromatin-like organisation for the yeast mitochondrial genome. They did however demonstrate the presence of a single basic protein having some of the characteristics of histones. The authors claimed no evidence for protection of a 140-165 base pair repeat. However, since the results of micrococcal nuclease digestion were not presented it is impossible to determine whether this statement implies that no protection of any kind was obtained. Possible explanations for these apparently conflicting results may lie in the different techniques

used in extraction of mitochondrial basic proteins (DNA affinity chromatography - Caron et al., 1979) and mineral acid in studies in Paramecium. Alternatively, significant differences may exist between mitochondria of Paramecium and Saccharomyces.

Albring et al. (1977) 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 microscope between a 10-20nm knob and a 100-500nm membrane-like patch. This structure was found to be bound near the origin of replication.

More recently evidence has been reported for the partial association of proteins with mtDNA from Drosophila melanogaster embryos (Potter et al. 1980). The protein protected region (about 10% of the genome) was mapped within the A+T rich fragment and contained five closely spaced segments; four of which were 394 base pairs in length, while the fifth measured about 200 base pairs and appeared to be located at the origin of replication. This suggested that the protection may be due to proteins involved in membrane attachment of the mitochondrial genome or its replication. The data presented in the study support the conclusion that the major part of the mtDNA in Drosophila embryo is not interacting with histone proteins to form the same type of nucleosome structure as in nuclear chromatin. However, the authors could not exclude the possibility that the bulk of mtDNA is protein-free in vivo. Although the technique used (trimethylpsoralen photoreaction) was shown to have no disruptive effect on nucleosomes, nevertheless, it is only 50% efficient when applied to nuclear chromatin of a typical 200bp repeat. Moreover, if mitochondrial chromatin had a repeat of

less than 100bp it would not be detected.

Van Tuyle and McPherson (1979) have reported the isolation of a compact form of rat liver mtDNA associated with tightly bound proteins. This nucleoprotein complex, visualised by electron microscopy, appeared as a compact rosette that was constrained at the centre in what seemed to be a rather dense core. This folded form of rat liver mtDNA contrasts markedly with the chromosome-like structure isolated by Pinon *et al.* (1978), from *Xenopus laevis* oocyte mitochondria, although in both cases the same lysing detergents were used in the respective extraction procedures. The DNA-protein complex obtained from *Xenopus* oocyte mitochondria appeared in the electron microscope as a relaxed circular molecule with the typical "beads on a string" structure of chromatin. This nucleoprotein complex shows a remarkable resemblance to the chromatin-like structure of the mitochondrial replication complex of *Paramecium aurelia* as seen in the electron microscope.

Further studies on the state of mtDNA *in vivo* are necessary in order to establish whether the discrepancies in the various reports are due to inadequacies in the techniques applied, or whether they represent true variations in the organisation of mitochondrial genomes of various organisms. The detailed elucidation of the organisation of mtDNA is important for the understanding of the mechanisms and the regulation of replication and transcription of the mitochondrial genome.

The discovery that the mitochondrial genome of *Paramecium aurelia* is organised in a chromatin-like structure has further interesting implications on the evolutionary origin of the organelle. The question of the evolution of mitochondria has not yet been unambiguously answered and at present there are two main types of hypothesis as well as a

number of variations of each. According to the endosymbiont hypothesis mitochondria have evolved from invading bacteria (Margulis, 1970; 1975), while the non-symbiotic hypothesis argues that mtDNA originated as an episome which enclosed itself in a membrane containing the respiratory chain (Raff and Mahler, 1972; 1975). Another interpretation of the evolutionary origin of mitochondria was suggested by Reijnders (1975). Nuclear and mtDNAs underwent "compartmentalisation", which led to separation from the rest of the cell and each other, by surrounding themselves with membranes, inside which these two genomes evolved in different directions. It can be seen that for such a hypothesis postulation of either a symbiont or an episome is not required. As yet there is no direct experimental evidence supporting unequivocally any of these theories, but the hypothesis favouring the prokaryotic origin of mitochondria was formerly predominant as it explains the striking similarities between the mitochondrial and bacterial protein-synthesizing systems. These similarities concern sensitivity of organelle ribosomes to antibiotics (e.g. chloramphenicol) which inhibit also ribosomes of bacteria but not of higher organisms. Another prokaryotic feature of mitochondria which was used to support the endosymbiont hypothesis refers to the mitochondrial genome as "naked" DNA, lacking histones and chromatin structure.

Currently the hypothesis postulating eukaryotic origin of mitochondria is more favoured, as new evidence indicates certain eukaryotic characteristics attributable to these organelles, e.g. "split" genes in organelle DNA and a segment of polyadenylic acid (poly-A) covalently linked to mitochondrial messenger RNA. Furthermore, the evidence reported here concerning the organisation of the mitochondrial genome as a form of chromatin-like structure supports a eukaryotic

rather than a prokaryotic origin of the organelle.

However, during the last few years the old concept of organisation of the bacterial chromosome requires reconsidering. Griffith (1976) presented evidence that prokaryotic DNA is condensed in a chromatin-like structure and more recent data showed the association of one (Rouviere-Yanive, 1975) or two (Varshavsky et al., 1977) bacterial histones with E. coli chromosome. However, it has been suggested that the histone-like proteins of E. coli are present in too small amounts to complex bacterial DNA into structures analogous to nucleosomes (Griffith, 1976). Since in the case of Paramecium mitochondria evidence has been presented for five histone-like proteins (as opposed to one or two in bacteria), this still implies nuclear similarities in organisation of the mitochondrial genome rather than bacterial.

It is of consequence in the discussion concerning the evolutionary history of mitochondria to refer to the point of view presented by Beale (1979), who questions in the first place, the relevance of discussing the origin of mitochondria. The author implies that any new structure appearing in a eukaryotic cell is liable to show certain eukaryotic features, the extent of which is limited by the genetic capabilities of the host cell. For example, even some viruses (adenovirus and SV40), when infecting animal cells associate themselves with host histones forming a viral chromatin structure.

In my view, features displayed by mitochondria should be mainly considered as a direct consequence of metabolic functions of this organelle rather than being attributed to its evolutionary origin. The fact that natural selection maintains chloramphenicol-sensitive ribosomes in mitochondria may imply that this type of protein-synthesizing machinery is vital for their metabolic functions and the

resemblance of the mitochondrial ribosomes to the prokaryotic ribosomes in respect of drug sensitivity might be purely coincidental. Similarly, the fact that the cell exhibits dualism in terms of various proteins (e.g. DNA polymerase, RNA polymerase, histones, ribosomal proteins, isocitrate dehydrogenase, etc.) one set for mitochondria and another for cytoplasm, may indicate that the organelle would not be able to perform its functions using the cytoplasmic counterparts of these proteins. From the recent reports concerning sequencing, organisation and expression of mammalian mitochondrial genomes (Anderson et al., 1981; Montoya et al., 1981) it appears that they have some unique individual properties not found in any other non-mitochondrial genome and therefore, the classification of the mammalian mitochondrial genetic system simply as either pro- or eukaryotic-like has little significance. The most striking evidence of evolutionary individuality of mitochondria is provided by the finding that the mitochondrial genetic code is different from the "universal" genetic code, e.g. UGA is not used as a stop codon but as a tryptophan codon and this has been shown not only for the mitochondria of mammalian cells (Barrell et al., 1979), but also for those of yeast (Macino et al., 1979) and Neurospora crassa (Heckman et al., 1980). Furthermore, the mitochondrial genetic code is read in a fashion unique to mitochondrial systems (Heckman et al., 1980; Barrell et al., 1980; Bonitz et al., 1980) and by a minimal set of mitochondrial tRNAs: 22 tRNAs in the case of human and bovine mtDNA (Anderson et al., 1981) while, as postulated previously 32 tRNAs are required to read the "universal" genetic code (Crick, 1966). Another example of mitochondrial individuality are the mitochondrial tRNAs which lack some of the common features of tRNAs from non-mitochondrial genetic systems,

as observed in yeast (Berlani et al., 1980; Newman et al., 1980; Canaday et al., 1980) and even to a greater extent in human mitochondria (Anderson et al., 1981). Moreover, most of mitochondrial mRNAs in mammals either starts directly at the initiator codon or have only a few nucleotides preceding this codon (Montoya et al., 1981), while in both eukaryotic and prokaryotic mRNAs there is a leader sequence of variable length preceding the initiation codon. In view of the evidence obtained for E. coli mRNAs that this sequence contains a ribosome-binding site one can assume that in the case of mitochondria at least from human cells (and most probably from all animal cells) the ribosomes must have special adaptive features for binding directly to the initiator codon of mRNA. This implies that the mitochondrial ribosomes are unique in this respect, resembling neither pro- nor eukaryotic ribosomes.

Summing up, one could suggest that because mitochondrial characteristics resemble in some aspects those of prokaryotic, in other eukaryotic cells and yet in other aspects they are unique to mitochondria, these organelles might have evolved from some biological entities not related to any of the then existing prokaryotic or eukaryotic organisms. Obviously these biological entities underwent evolution under selective pressures different from those which were operating during the evolution of pro- and eukaryotic organisms. In any case, such discussion is purely speculative and at the moment cannot lead to any final conclusions. It is rather hazardous to reconstruct the course of the evolutionary events on the basis of evidence obtained from contemporary organisms and therefore due to the lack of experimental verification the evolutionary origin of mitochondria will probably remain an open question.

I would like to close this thesis with the general conclusion that mitochondria of one organism should not be considered as a representative system for mitochondrial genetics in general. Although mitochondria are by enlarge uniform in terms of the functions they code for, they show remarkable differences in respect of structure of their genome, its organisation, replication and expression. These differences can be observed not only between highly diverged organisms like yeast and mammals (see Borst and Grivell, 1981) but even among related species like Tetrahymena and Paramecium. The work on Paramecium mitochondria reveals that this system displays several features not found in other organisms so far studied: (i) the absence of complementation and recombination in contrast to its frequent occurrence in yeast; (ii) the linearity of the genome as opposed to circular DNAs of all other organisms except Tetrahymena; (iii) unusual mode of replication of mtDNA through lariats and dimers in head-to-head configuration; (iv) presence of several histone-like proteins and a chromatin-like structure organisation of the genome; (v) the determination of some mitochondrial ribosomal proteins by the mitochondrial genome.

It is vital, therefore, for the meaningful development of mitochondrial genetics to include in the study a variety of different groups of organisms.

ACKNOWLEDGEMENTS

I would like to thank Professor G.H. Beale and Dr A. Tait for their constant help and useful advice throughout this work.

I am specially indebted to Dr A. Tait, whose stimulating suggestions and discussions, continuous interest and enthusiastic encouragement were, to me, essential components for the completion of this work.

Thanks are also due to Dr J.O. Bishop for performing restriction enzyme digestions and providing me with two plasmid DNAs, to Dr J. Jacob for an introduction to electron microscopy, Mr I.F. Purdom for carrying out the DNA density determinations, and Mr A. Sanderson for preparing the drawings.

I would also like to acknowledge the expert photographic work of Mr F.M. Johnston and Mr A. McEwan.

I certify that, apart from the assistance mentioned above, all of the experiments presented in this thesis were planned and carried out by myself.

E. Olszewska
August, 1981

PUBLICATION

OLSZEWSKA, E. and Tait, A. (1980). Mitochondrial chromatin in *Paramecium aurelia*. Molec. Gen. Genet. 178: 453-457.

Molec. gen. Genet. 178, 453–457 (1980)

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Mitochondrial Chromatin in *Paramecium aurelia*

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Summary. Chromatin-like structures have been observed in material extracted from the mitochondria of *Paramecium aurelia* and evidence is presented which establishes that these structures do not originate from nuclear contamination of mitochondrial preparations but are exclusively of mitochondrial origin. Extraction of these chromatin-like structures with dilute acid followed by gel electrophoresis of the extract shows the presence of five major basic proteins which are of similar electrophoretic mobility to histones. Digestion of mitochondrial extracts with endogenous nuclease, followed by electrophoresis of the extracted DNA, demonstrated that the mitochondrial genome is protected from nuclease digestion by regular repeating units very similar to those observed in the nuclease digestion of chromatin.

Introduction

The structure of chromatin has been extensively studied over the last few years and Kornberg's model of the nucleosome structure of chromatin (Kornberg, 1974; Oudet et al., 1975) has been shown to occur in a wide range of eukaryotic organisms. It is generally accepted that prokaryotic and organelle genomes do not have a nucleosome type structure, but occur as "naked" DNA, although the recent finding of histone-like proteins associated with the genome of *E. coli* (Varshavsky et al., 1977) raises some doubts about the established view. In view of the widespread occurrence of DNA condensed with basic protein and the fact that most techniques used to isolate organelle DNA would necessarily remove any bound proteins, it seemed possible that organelle genomes might have a chromatin-like structure.

In order to investigate this question we have examined the mitochondrial genome of *Paramecium aurelia*, an organism extensively used for the study of mitochondrial biogenesis (Beale and Knowles, 1978), to see whether this organelle genome has any of the characteristic features of chromatin. We have isolated a replication complex from the mitochondria which was shown to contain membrane bound mitochondrial DNA and the enzymes involved in DNA replication. This complex was then used to study the organisation of the mitochondrial genome in terms of the existence of nucleosomes, the association of the DNA with basic proteins and the protection of the DNA from nuclease digestion.

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Materials and Methods

Isolation of Cell Fractions

All experiments were carried out using stock 513, species 1 of *Paramecium aurelia*. Purified mitochondria were prepared by standard techniques (Preer and Preer, 1959) from cells grown on either a bacterised grass infusion or on an axenic medium (Soldo et al., 1966). A replication complex was prepared from mitochondria using a technique based on the procedure described by Shearman and Kalf (1975): a mixture containing 15 mg of mitochondrial protein, 0.5% Sarkosyl and 8.5% sucrose-TMK (10 mM tris-HCl, pH 7.4, 10 mM magnesium acetate, 100 mM KCl) was layered on a discontinuous gradient consisting of 15% and 35% sucrose-TMK. The gradient was centrifuged at 23,000 g for 15 min and the fraction containing the replication complex collected from the interface of the 15% and 35% sucrose. This fraction was concentrated by centrifugation and dialysed overnight against 20 mM tris-HCl pH = 8.5, 20% glycerol, 10 mM EDTA. The complex was subsequently purified by differential centrifugation and dialysed overnight against 20 mM tris-HCl pH = 7.5, 20% glycerol.

Nuclei were labelled and isolated by previously described techniques (Cummings et al., 1974; Cummings and Tait, 1975).

Extraction and Gel Electrophoresis of Basic Proteins

Mitochondrial basic proteins were prepared from the purified replication complex by extraction with 0.25 N HCl for 1 h at 4° C. After centrifugation basic proteins were precipitated from the supernatant with 10 volumes of acetone for 24 h at −20° C. The

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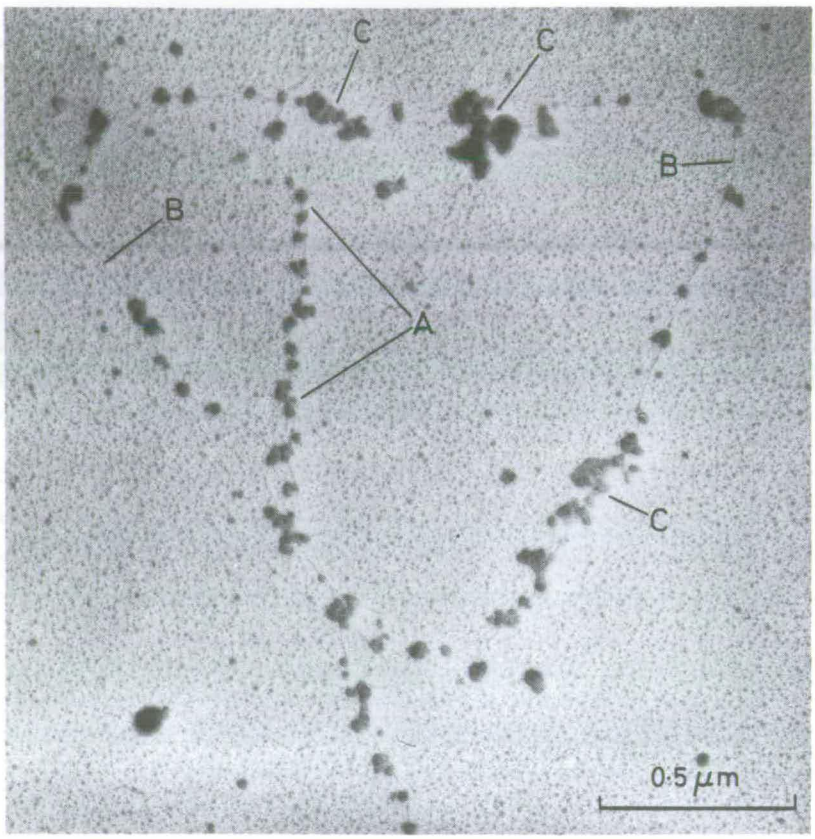

Fig. 1. Electron micrograph of the mitochondrial chromatin-like structure showing regions with three different types of structure (*A*, *B* and *C*). See text for details

precipitate was washed twice with acid acetone, twice with acetone and dried in vacuo. Histones from nuclei were prepared in the same manner except for mechanical homogenisation of whole nuclei prior to extraction with acid. All preparations of basic proteins were carried out in the presence of 0.05 M sodium bisulfite.

Electrophoresis of histones was performed on 15% acrylamide slab gels containing 6.25 M urea and 0.9 N acetic acid according to Panyim and Chalkley (1969). The electrophoresis was carried out at 4 mA for 14 h. The gels were stained for 1 h in 0.25% Coomassie Brilliant Blue (w/v), 45% methyl alcohol, 9% acetic acid in H_2O (w/v) and destained by diffusion in a solution of 5% methyl alcohol and 7.5% acetic acid in H_2O (w/v).

Electron Microscopy

The purified replication complex was fixed in 1% glutaraldehyde for 15 min at 37° C, dialysed against 10 mH Tris-HCl, pH 7.5 and spread by the cytochrome monolayer technique (Davis, 1971). The spread material was picked up on nickel grids coated with formvar film and carbon. They were stained in uranyl acetate and rotary shadowed with platinum. The grids were examined in an AE1 EH6 electron microscope.

Extraction and Gel Electrophoresis of DNA after Endonuclease Digestion

Prior to incubation with endogeneous nuclease, mitochondria were treated with pancreatic DNase 1 (10 μg/ml) for 20 min in ice. The conditions of the digest with endogenous nuclease were basically as described by Seale (1976). Mitochondria were incubated for 10 min at 37° C and the reaction was terminated by the addition of 3% SDS, 20 mM EDTA. DNA was prepared according to Gause et al. (1973) and extracted with iso-amylalcohol/chloroform (24:1). Products of digestion were analysed on 6% acrylamide slab gels containing 6 M urea as described by Peacock (1967). Heat denatured (5 min at 100° C) DNA samples were subjected to electrophoresis at 24 mA for 3 h. Gels were stained with 10 μg/ml ethidium bromide for 20 min and photographed under ultra-violet light.

Results and Discussion

As a starting point for studies of the proteins associated with the mitochondrial genome, we isolated a mitochondrial replication complex which will be described in detail elsewhere. When the complex was examined by electron microscopy the only structures observed were those shown in Figs. 1 and 2, namely a series of bead-like structures linked by strands of DNA. In the structure shown in Fig. 1 three different regions can be distinguished. The first region (A) contains a relatively regular distribution of nucleosome-like structures similar to the "beads" observed in preparations of nuclear chromatin. The second region (B) contains stretches of a thin filament lacking any nu-

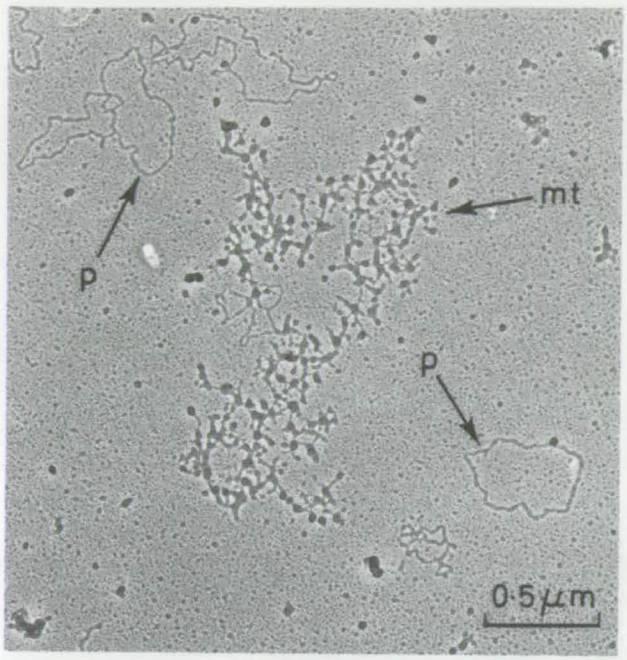

Fig. 2. Electron micrograph of mitochondrial chromatin-like structure (*mt*) and protein-free plasmid DNA (*p*)

cleosome-like structures, and in the third region (C) more condensed, larger particles are seen. The size of various particles was measured and found to be 130 Å for the small "beads" which is similar to the size of SV 40 nucleosomes (Oudet et al., 1975), while large particles (about 300 Å) could correspond to a condensed globular form of native SV 40 chromatin (Müller et al., 1978). We think that these irregularities in the state of condensation of chromatin-like structures as well as the irregular distribution of beads are a result of the preparative procedure, which is not only lengthy but involves exposure of the nucleoprotein complex to low salt concentration. Müller et al. (1978) have shown that the degree of condensation of chromatin depends on the ionic strength of the buffer used for the preparation of nucleoprotein complexes. By varying the ionic strength, they obtained a spectrum of forms similar to ours, ranging from fully condensed to completely extended. In order to prevent damage to the chromatin-like structures we modified our preparative procedure by fixing the material with glutaraldehyde prior to exposure to low salt concentration and further purification. With this procedure we were able to isolate beaded structures of more uniform size and more regular distribution (Fig. 2). Thus, the electron microscope pictures lead us to conclude that chromatin-like structures can be observed reproducibly.

It is necessary to show that these structures do not arise as a result of the preparative procedure

Table 1. The distribution of ^{3}H-labelled nuclear DNA during preparation of the mitochondrial replication complex

Fraction	dpm ($\times 10^3$)	dpm/mg DNA ($\times 10^3$)	µg DNA
Homogenate[a]	222	32	7×10^3
Mitochondria	7.2	7	1×10^3
Replication complex	0	0	9

[a] labelled nuclei (404×10^3 dpm/mg DNA) mixed with unlabelled cell homogenate

and that they are indeed of mitochondrial origin i.e. they are not the result of contamination by nuclear or bacterial material. The first possibility was eliminated by adding protein-free circular DNA (plasmid pCM-21 restricted with EcoR1) to the purified replication complex and then preparing grids for electron microscopy. Figure 2 shows the result of this experiment and it can be seen that the plasmid DNA does not acquire any beaded structures.

The second possibility, namely that these structures were of nuclear origin, was tested by preparing the replication complex from a mixture of unlabelled cells and purified, ^{3}H-thymidine labelled nuclei. Samples were taken at various stages of the purification and the amount of DNA and TCA precipitable radioactivity was determined. The results obtained (Table 1) show that there was no detectable radioactivity in the replication complex. Furthermore, knowing the specific activity of the nuclear DNA added (404×10^3 dpm/mg) and the ratio of unlabelled to labelled nuclei (7:1), it can be calculated that if a significant fraction of the replication complex DNA were nuclear it would have been detected. Thus it can be concluded that the replication complex is free of significant nuclear contamination and that the chromatin-like structure seen in the electron microscope are of mitochondrial origin. (The possible bacterial origin of the mitochondrial replication complex was eliminated by preparing an identical complex from axenically grown Paramecium.)

Having established that the chromatin-like structures were of mitochondrial origin, two further properties of this chromatin were examined, namely, the existence and identity of the basic proteins associated with it and the protection of the DNA from nuclease digestion.

The proteins from the beaded structures observed in the electron microscope were isolated by acid extraction of the replication complex and examined by acetic acid/urea acrylamide gel electrophoresis. An electrophoretic comparison of calf thymus histones, nuclear histones of *Paramecium* and the basic proteins

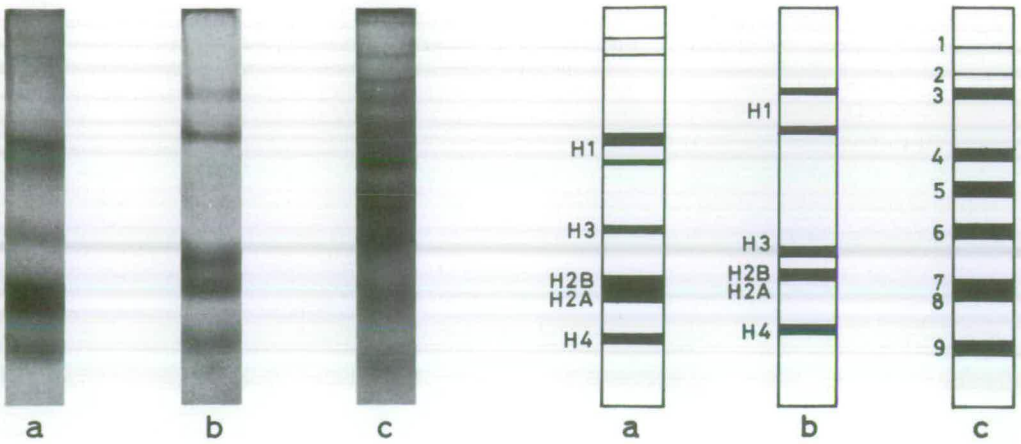

Fig. 3a–c. Electrophoretic comparison of calf thymus histones (**a**), *Paramecium* nuclear histones (**b**) and mitochondrial basic proteins (**c**)

from the mitochondrial replication complex is presented in Fig. 3. *Paramecium* nuclear histones show mobilities lower than those from calf thymus as has been previously reported (Isaacks and Santos, 1973). The basic proteins from the mitochondrial replication complex show 9 bands, five of which (bands 4, 6, 7, 8 and 9) having similar mobilities to the histone bands of calf thymus of *Paramecium* nuclei. This result suggests that there are histone-like proteins of mitochondrial origin associated with mitochondrial DNA. As the histone bands from the replication complex differ slightly in electrophoretic mobility from those of the nucleus the possibility of contamination of mitochondrial material by nuclear histones is excluded.

On the basis of these results one can only say that nuclear and mitochondrial histones have slightly different electrophoretic mobilities. Future work on the degree of homology in amino acid composition and sequence may reveal more profound differences.

Further evidence that a chromatin-like structure is present in mitochondria was obtained when the organelles were incubated with endogenous nuclease. If DNA is protected by histones from digestion with nucleases, one would expect to observe a series of discrete bands corresponding to multiples of chromatin subunits. On the other hand, if DNA is protein-free the digestion with nuclease will be random, resulting in a smear when DNA is separated by electrophoresis (Hewish and Burgoyne, 1973). The result obtained from digestion of *Paramecium* mitochondria with endogenous nuclease (Fig. 4) reveals a series of distinct bands indicating the existence of mitochondrial chromatin rather than "naked" DNA. The possible contamination of the sample by nuclear DNA or chromatin was eliminated by treating mitochondria with pancreatic DNase 1 prior to digestion with nuclease. This treatment with pancreatic DNase 1 has been shown to be effective in removing nuclear DNA from mitochondrial preparations (Goddard and Cummings, 1975).

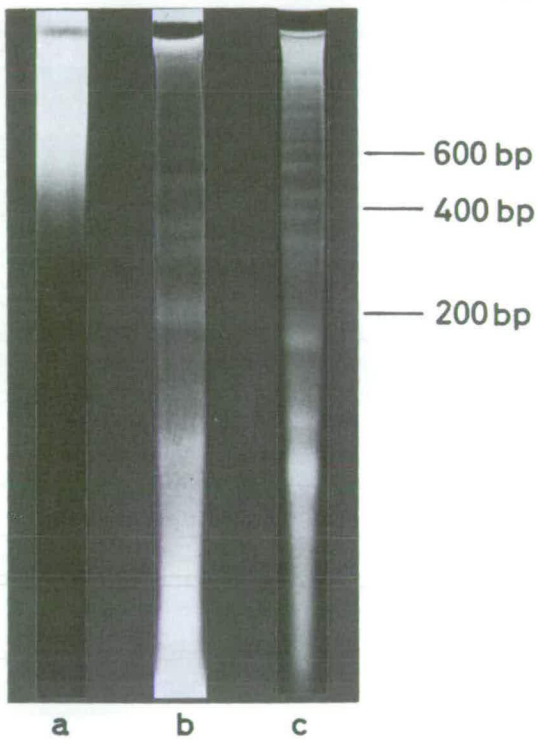

Fig. 4a–c Polyacrylamide gel electrophoresis of DNA after digestion of mitochondria with endogeneous nuclease. **a** 0 min incubation, **b** 10 min incubation, **c** the same as b but from another experiment

On the basis of our results we tentatively conclude that the mitochondrial genome from *Paramecium aurelia* is organised in a chromatin-like structure, which is not necessarily identical with eukaryotic chromatin. This finding is relevant to the question of the evolutionary origin of mitochondria. Griffith (1976) presented evidence that prokaryotic DNA is condensed in a chromatin-like structure and more recent data

showed the association of two bacterial histones with the *E. coli* chromosome (Varshavsky et al., 1977). Our results suggest the presence of five histone-like proteins which are organised in association with mitochondrial DNA into a chromatin-like structure which implies a eukaryotic rather than prokaryotic organisation of the DNA in mitochondria.

The presence of basic proteins has been reported in mitochondria of other organisms. Kuroiwa et al. (1976) presented evidence for at least one species of basic protein isolated from the mitochondrial nucleoid of the slime mold *Physarum polycephalum* and Hillar et al. (1979) reported basic proteins in the mitochondrial nucleoid of bovine heart tissue.

Recently, Caron et al. (1979) have demonstrated the presence in yeast mitochondria of a single basic protein having some of the characteristics of histones but in addition some non-histone features. Although the results of digestion of mitochondria with micrococcal nuclease are not presented, the authors found no evidence for protection of a 140–165 bp repeat. This, therefore, together with the absence of true histones led these authors to conclude a prokaryotic structural organisation for the yeast mitochondrial genome. Our evidence supporting the presence of 5 histone-like proteins indicates a eukaryotic organisation in *P. aurelia* mitochondria. Possible explanations for these apparently conflicting results may lie in the different techniques used in extraction of mitochondrial basic proteins (DNA affinity chromatography, Caron et al., 1979 and mineral acid in our case). Alternatively, significant differences may exist between mitochondria of *Paramecium* and *Saccharomyces*.

Our electron microscopy studies are in agreement with the evidence for a mitochondrial chromosome in *Xenopus laevis* oocytes (Pinon et al., 1978). Our results presented here are the first report of combined electron microscopy and biochemical evidence supporting the existence of mitochondrial chromatin.

This research was supported by a postgraduate studentship from University of Edinburgh to E. Olszewska and by the Science Research Council. We would like to thank Professor G.H. Beale for helpful discussions, Dr J. Bishop for providing plasmid DNA, Frank Johnson for photography and Janis Wallace for technical assistance.

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Communicated by G. Melchers

Received December 14, 1979/February 11, 1980

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