

MITOCHONDRIAL DNA FROM
REPTILIA AND CNIDARIA

MITOCHONDRIAL DNA FROM
REPRESENTATIVE SPECIES OF REPTILIA AND CNIDARIA

By

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SCOPE AND CONTENTS:

Radioactive thymidine was used to label DNA in turtle fibroblasts grown in tissue culture. The circular mDNA was separated from the linear nDNA on EB-CsCl gradients. The buoyant densities of both DNAs were determined on CsCl gradients by comparison with Micrococcus lysodecticus DNA. The buoyant densities of the two DNAs were found to be less than 2 mg cm^{-3} apart and were $1.704 \text{ gm cm}^{-3} \pm 2 \text{ mg cm}^{-3}$.

Mitochondrial DNA was isolated from turtle livers and subjected to electron microscopy. Electron micrographs showed that turtle mDNA was of a similar size to mDNA isolated from rat and hamster liver.

Hydra littoralis were cultured and the mitochondrial fraction of a homogenate of hydra polyps identified. The DNA from this fraction was extracted and electron microscopy indicated that the molecules may have been circular in vivo and have a length slightly longer than $5 \mu\text{m}$.

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ABBREVIATIONS

DNA	deoxyribonucleate
mDNA	mitochondrial DNA
nDNA	nuclear DNA
EB	ethidium bromide
EDTA	ethylenediaminetetraacetate
RNA	ribonucleate
rRNA	ribosomal RNA
tRNA	transfer RNA
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TRIS	tris (hydroxymethyl) aminomethane

CHAPTER I

Introduction

The presence of DNA-like filaments in mitochondria of chick embryos was first reported by Nass and Nass in 1963. This observation was extended by Nass et al (1965) to include a wide range of invertebrate and vertebrate mitochondria. Others demonstrated the presence of these filaments in plant mitochondria and chloroplasts. By 1966 mitochondrial DNA had been isolated and characterized from a wide range of species, by several groups. Reviews by Borst & Kroon (1969) and Rabinowitz & Swift (1970) cover the work done in detail. Borst (1972) has reviewed the more recent work on the structure and function of mDNA. In the following, the properties of mDNA will be discussed, as they pertain to the experiments reported in this thesis.

Mitochondrial DNA

Mitochondrial DNA is usually characterized by its length, and by its buoyant density in CsCl gradients which is a measure of its base composition. The base composition (mole fraction of guanine + cytosine) is related to the buoyant density by the equation: density in $\text{gm cm}^{-3} = 0.098 (\text{mole fraction G+C}) + 1.660 \text{ gm cm}^{-3}$ (Borst & Kroon, 1969). These measurements (length and density) can be used to distinguish mDNA from other DNA molecules present in eukaryotes, and are the initial steps in a more comprehensive description of its structure and function.

Characterization of mDNA from a wide variety of species has revealed some interesting phylogenetic relationships. In Table 1,

TABLE 1 - Buoyant densities of mDNA and nDNA and the lengths of mDNA from various species

	Buoyant density in gm cm ⁻³	Length in μ m	Circularity Observed	Reference
<u>PROTISTA</u>				
<u>MITOCHONDRIA NUCLEI</u>				
<u>Euglena gracilis</u>	1.691*	0.6-0.9	-	Nass & Ben Shaul (1972)
<u>Tetrahymena</u>	1.683*-6	17.6-14.5	-	Arnberg et al (1972)
<u>Paramecium</u>	1.699	17 -20**	-	Flavell & Jones (1971)
<u>FUNGI</u>				
<u>Neurasporea crassa</u>	1.698	20	+	Clayton & Brambl (1972)
<u>Saccharomyces</u>	1.683-4*	25	+	Hollenberg et al (1970)
<u>ANIMALIA</u>				
<u>HELMINTHS</u>				
<u>Hymenolepis diminuta</u>	1.691	4.76	+	Carter et al (1972)
<u>Ascaris lumbricoides</u>	1.690	4.79	+	" "
<u>ECHINODERMS</u>				
<u>Sea Urchin</u>	1.704	4.45	+	Borst & Kroon (1969)
<u>CHORDATES</u>				
<u>FISH</u>				
<u>Carp</u>	1.703	5.4	+	" "
<u>AMPHIBIANS</u>				
<u>Xenopus laevis</u>	1.704	5.4	+	" "
<u>Rana pipiens</u>	1.702	5.6	+	" "
<u>BIRDS</u>				
<u>Chick</u>	1.708	5.1-5.5	+	" "
<u>MAMMALS</u>				
<u>Man</u>	1.705	4.8	+	" "
<u>Rat</u>	1.701	4.9-5.4	+	" "
<u>Mouse</u>	1.698	4.7-5.2	+	" "
<u>Hamster</u>	-	5.1-	+	Oda, T. (1968)

* - density values from Borst & Kroon (1969)

** - estimated from renaturation kinetics 3.5 x 10⁷ daltons for Paramecium
3.0 x 10⁷ " "Tetrahymena

values of the buoyant density and length of mDNA of some protists, animals and fungi are given. These figures are representative of those found for these kingdoms as demonstrated in more extensive tables (Borst & Kroon, 1969; Rabinowitz & Swift, 1970). A range of values indicates either differences in the strains studied or in the laboratory, and/or technique.

Several interesting generalizations can be made. If the overall base composition of an organism's DNA is under evolutionary control (Singer & Ames, 1970) one might expect some relationship between the density of mDNA and nDNA, but as Rabinowitz & Smith (1970) have pointed out... "The values show precisely the pattern that might be expected of two distinct genetic systems, each undergoing gradual but essentially independent changes in composition with evolution". On the other hand, there does seem to be a similarity in the density of mDNA from related species. Thus in animals, while the range of buoyant densities is from 1.695 to 1.712 gm cm⁻³, the range within the classes is less. For example, in mammals the range is 1.698 - 1.705 gm cm⁻³, birds 1.706 - 1.712 gm cm⁻³, and amphibians 1.695 - 1.702 gm cm⁻³. In fungi the range is from 1.685 gm cm⁻³ (yeast) to 1.700 gm cm⁻³ (Neurospora) and the mDNA of plants show a very narrow range of 1.705 - 1.708 gm cm⁻³. These values are from Rabinowitz & Swift (1970). There is no trend in buoyant density of mDNA going from 'lower' to 'higher' organisms, and although the buoyant density is similar in related species, no conclusion can be drawn about the evolution of the organism from the actual density value. The buoyant density of the mDNA can be used to differentiate it from other DNAs in the cell; for example, in yeast the buoyant density of

nDNA and mDNA differ by 13 mg cm^{-3} .

Of perhaps greater evolutionary significance is the length of mDNA. All species of Metazoa investigated thus far have circular mDNA that is about 5 μm in circumference. Some small differences in size might reflect technical variations, but Nass (1969) has shown by mixing experiments that chicken mDNA is longer than rat or mouse mDNA. In contrast to metazoan mDNA, the mDNA of protists (except for Euglena), fungi and plants is longer than 5 μm . Although in earlier studies these mDNAs appeared to be linear, the mDNA of yeast and Neurospora have recently been observed as circles as indicated in Table 1. Plant mDNA, although not listed in the table, appears to be linear and longer than 25 μm . An anomaly seems to exist in Euglena, in that only small linear pieces have been observed (Nass & Ben Shaul, 1972; Borst, 1972).

In higher animals, mDNA longer than 5 μm has also been observed. These molecules have, however, been demonstrated to be multimers of the 5 μm genome. The molecules can either be linked together like a chain or they can be single circles whose length is a multiple of 5 μm . This latter form was shown by Clayton et al (1970) to be a head to tail arrangement of the 5 μm genome. The long molecules found in protists do not appear to be multimers of 5 μm molecules. Renaturation kinetics of mDNA of yeast and Tetrahymena indicate a homogeneous population of molecules, having an informational content consistent with their length. Moreover, there is only one cistron for each of the mitochondrial rRNAs on 5 μm mDNA and on 25 μm mDNA of yeast. This is consistent with the idea that the longer mDNA is not a multimer of the 5 μm circle seen in animals (Borst, 1972).

In contrast to the mDNA of fungi, the mDNA of Tetrahymena has only been observed as linear molecules. Attempts have been made to see if these linear molecules are circular in vivo. It was claimed that, since renatured mDNA showd only linear molecules of the original length under electron microscopy, they must contain a unique sequence of genes. This implied that, if they were circular in vivo, random breakage did not occur and the possibility of the presence of a specific nuclease was suggested (Borst, 1972; Arnberg et al, 1972). More recent work, however, from Borst's laboratory, indicates that this may not be the case after all (Arnberg et al, 1972) for reasons described below. They now suggest that circularity might exist in vivo and that random breakage during isolation occurs.

A new form of mDNA has been observed both by Vinograd (Kasamatsu et al, 1972) and Borst (Ter Schegget & Borst, 1972). By making use of a spreading technique that allows single stranded DNA to be visualized under electron microscopy, they described what they called "D-loop" DNA, as shown in Fig. 1. This form consists of duplex mDNA in which a short piece (0.2 μm) of DNA (E-strand) is hydrogen bonded to one of the strands, displacing a piece of the other strand (D-strand). When mDNA strands are separated, one strand bands below the other in alkaline CsCl gradients (heavy and light strands respectively). Kasamatsu et al (1972) showed that the E-strand hybridized with the light strand mDNA, indicating the D-loop is part of the heavy strand. Since in 10 μm dimers there are sometimes two D-loops, 5 μm apart, it was suggested by both laboratories that these D-loops could represent a unique position on the mDNA genome for initiation of replication. Kasamatsu & Vinograd (1973, by using

FIGURE 1

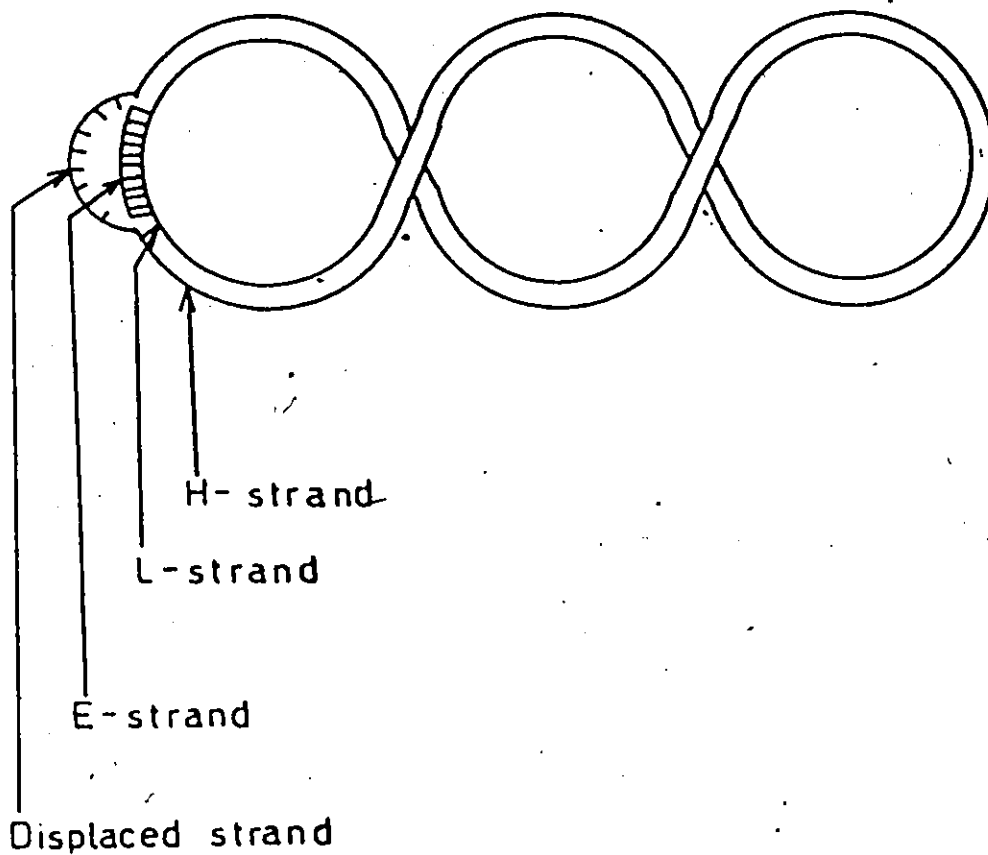


DIAGRAM OF mDNA WITH A

D-LOOP

(after Kasamatsu et al 1971)

dimer molecules and one D-loop as a fixed marker, have described a model where replication of mDNA proceeds unidirectionally from a stationary fork located in the area of the second D-loop. Borst's laboratory investigated D-loops in mDNA of Tetrahymena (Arnberg et al, 1972). Since it was thought that Tetrahymena mDNA was a unique sequence of genes (see above) on a linear molecule, they wanted to examine the location of the D-loops in these molecules. They found up to six loops per molecule, at random locations with respect to the ends. Rearranging the molecules as if there was a circularly permuted gene sequence, they found that up to eight unique sites on the molecule could contain a D-loop. If this is the case, and if D-loops are sites where replication is initiated, then there are at least eight such sites in Tetrahymena mDNA and only one in vertebrate mDNA. In establishing these eight sites, the existence is implied of an in vivo circular molecule that became randomly broken during isolation. At the moment, the circularity of Tetrahymena mDNA has not been definitively demonstrated and there is no clear reason for the necessity of eight sites for the initiation of replication.

It is known (Borst, 1972) that mDNA codes for rRNA, tRNA, and a few proteins (for example protein components of cytochrome oxidase and ATPase). Recently Wu et al (1972) have mapped the relative positions of the genes for rRNA and some tRNAs on the mDNA from HeLa cells. The rRNA and most of the tRNAs tested are complementary to the heavy mDNA strand. Barath and Küntzel (1972) found that in Neurospora, when transcription and translation of mDNA are blocked, there was an increased level of the enzymes which are involved in the expression of the mitochondrial genome but coded for by the nDNA. They, therefore, postulated that as well as

a few inner membrane proteins, rRNA and tRNA, this mDNA codes for a repressor molecule that co-ordinates nuclear and mitochondrial division cycles.

Other Cytoplasmic DNAs

At present there is considerable knowledge of the structure and function of mDNA and attention has been directed to the possibility of other DNAs in the cytoplasm of various species. Astergibbe et al (1972) working with two strains of Neurospora have noted a population of small circular DNA molecules ranging in size from 0.5 to 7 μ m. These molecules were found in the deoxyribonuclease treated and osmotically shocked mitochondria from one of these strains; the other strain showed only a few of these molecules. Both this laboratory and that of Clayton and Brambl (1972) have found 20 μ m DNA circles in Neurospora mitochondria. Wood and Luck (1969) have calculated a minimal molecular weight of 6.6×10^7 daltons or about 33 μ m for mDNA from Neurospora. It is unclear at this time, particularly since there is some doubt as to the validity of the renaturation kinetic data, if there is a heterogeneous population of molecules with a combined genetic complexity of 33 μ m, or whether these small circles arise from some other organelle. There is also no apparent reason for the abundance of small circles in one strain and very few in the other.

In yeast there have been reports of small circles in preparations of mDNA (Borst, 1972). Recently, Clark-Walker (1972) has found circular DNA in both ρ^+ and ρ^- strains of yeast. This DNA has a nuclear density and is found in a particulate band that is slightly lighter than mitochondria in sucrose gradients. He called this oDNA (omicron) and

suggested that it might be located in the peroxisomes. The size of the DNA was not determined. Koch (1972) demonstrated, in deoxyribonuclease-treated mitochondrial fractions of Chang liver cells, a linear DNA of lower density than mDNA or nDNA. The origin of this linear DNA remains unexplained at present. In Euglena gracilis, as mentioned before, the mDNA has only been observed (Nass & Ben Shaul, 1972) as small linear pieces even in osmotically shocked mitochondrial preparations. In addition, Nass & Ben Shaul (1972) have found a closed circular species of DNA of 3.13 μm circumference, that is of a density different from that of mDNA, nDNA or chloroplast DNA. It is present in both green and bleached strains of E. gracilis and is associated with most organelle fractions. They suggest that this might be a DNA under nuclear control and associated with chloroplast function. Although all these studies have not clearly demonstrated novel cytoplasmic DNAs with genetic roles, they tend to open the possibility that such DNAs exist.

Relationship and Phylogenetic Origin of mDNA

Living organisms can be divided into five kingdoms according to Whitaker (1969). These are the Monera (prokaryotes), Protista (unicellular eukaryotes), Plantae, Fungi and Animalia. As indicated in Table 1, in the animal kingdom the length of mDNA is 5 μm for the Metazoa examined thus far. The question arises as to when in evolutionary time this length was established. Two theories of the evolution of mitochondria are prevalent today. The classic, or symbiotic theory, was first propounded in the late 19th century. Basically, this theory states that eukaryote organelles were generated by symbiotic events. In this model, (Margulis, 1970), mitochondria arose from aerobic bacteria which

were phagocytized by anaerobic protoeukaryotes. At some point in time, an event (or events) took place in which the aerobic bacterium became an endosymbiont giving rise to a new type of cell with a higher probability of survival. This theory also postulates the endosymbiosis of blue-green algae as the origin of chloroplasts and the incorporation of spirochaetes to become flagella and the mitotic apparatus.

A consequence of this theory is that much of the original bacterial genome must have been incorporated into the nucleus of the host, since most of the mitochondrial proteins are coded by nuclear genes and synthesized on cytosolic ribosomes. In evolution, plants and fungi have lost less mDNA than animals. Few protist mDNA have been examined, but for some of these there has been less lost than animals but somewhat more than plants or fungi. It is unclear why different kingdoms have different sizes of mDNA. This could reflect multiple initial symbiotic events, followed by differential evolution, or a single symbiotic event followed by differential evolution. In either case the loss of information could have happened as a single event, or as multiple events (Raven, 1970).

In contrast to the symbiotic theory is the episomal theory of Raff and Mahler (1972). In this model aerobic bacteria, slightly larger than the others, required a larger respiratory surface. This was provided by invaginations of the cell membrane which later evolved into membrane bound vesicles, topologically closed and with selective permeability properties. It became an advantage to the cell to then have a protein synthesizing system inside this respiratory particle for membrane maintenance. This was accomplished by the incorporation

of a stable episome having the required genetic material. A consequence of this theory is that the genes for mitochondrial proteins have always resided in the nucleus. The difference in size of mDNA in different kingdoms might reflect different initial events or in evolution that mDNA could have increased or decreased in length.

When in evolution was the 5 μ m mitochondrial genome of the metazoans established? Was it established by episomal or symbiotic events at the pre-eukaryote level or was a symbiotic event followed in time by the loss of mDNA? If this latter is the case, when in time did this loss occur? If it happened early in time, one would expect some protists in the line of animal evolution as well as all animals to have a 5 μ m genome. If the establishment was later in time or occurred in stages, one would expect to find intermediate lengths at some pre-metazoan level. It is interesting to note at this point that there has been some sequence homology found between the mDNA of various metazoans, but not between mDNA of yeast and metazoans, or between Paramecium and Tetrahymena (Borst, 1972).

All metazoans analyzed thus far, have three germ layers. There are two groups of animals that have only two germ layers, the Porifera (sponges) and the Cnidaria. If these animals could be shown to have 5 μ m circular mDNA, one could conclude that the length had been established at some "lower" level. If the mDNA was of an intermediate length, one could conclude that at this level the 5 μ m genome had not been established. This would mean that the episomal model in its simplest form would not be correct but one could not distinguish between multiple or single symbiotic events.

The purpose of part of this thesis was to try to determine if at the level of Cnidaria the 5 μ m genome had already been established, or whether this group of animals have a mDNA of length intermediate to that of protists and to that of animals with three germ layers.

It can be seen from Table 1 that although the length and buoyant density of mDNA from many amphibians, birds and mammals have been determined, there are no studies of mDNA of reptiles. The first part of this work was to partially characterize mDNA from turtles.

CHAPTER II

Methods and Materials

Solutions

Phosphate Buffered Saline (PBS)

0.137 M NaCl

0.027 M KCl

0.015 M KH_2PO_4 0.081 M Na_2HPO_4

pH 7.4

Reticulocyte Standard Buffer (RSB)

0.01 M NaCl

0.0015 M MgCl_2

0.01 M Tris-HCl

pH 7.4

Tris-EDTA (TE)

10 mM Tris Cl

1 mM EDTA

pH 7.5 or pH 8.0 as made up at ten times concentration (10 x TE)

Medium B

0.3 M sucrose

0.002 M Tris Cl

0.002 M EDTA

pH 7.4

SDS solution for rat and turtle mitochondria

0.8% SDS

0.01 M EDTA

0.15 M NaCl

0.01 M Tris

pH 8.0

Sucrose - EDTA (SE)

(a) Rat

0.25 M sucrose

0.002 M EDTA

pH 7.2

(b) Turtle

0.3 M sucrose

0.002 M EDTA

pH 7.2

Turtle DNA

"Red Eared Turtles" (Pseudemys ornata) obtained from a pet store were used. Fibroblasts from heart muscle were cultured at 30°C in minimal essential medium containing 10% fetal calf serum, penicillin, streptomycin, fungizone and anti-PPLO (Clarke et al., 1970). DNA from these cells was used for buoyant density determinations. The livers of 4-6 small turtles were used for electron microscopy of mDNA.

Buoyant Density of mDNA and nDNA

Labelling:

Approximately 30 μCi [$^3\text{H} - \text{CH}_3$] thymidine per plate or 5 μCi [$^{14}\text{C} - \text{CH}_3$] thymidine per plate were used to label DNA for 48 hr prior to

confluency of cells.

Isolation of Mitochondria:

Two methods were used in the preparation of the mitochondria. The first (i) used 13 plates (80, sq cm) of cells and the second (ii) used 30 plates of cells and unlabelled carrier mitochondria. The results of both methods were similar. The first method had the advantage of speed, since a number of steps were omitted.

(i) Plates were rinsed and the cells harvested and washed with PBS. The cell pellet, kept on ice, was resuspended in 0.5 ml RSB, and the cells ruptured with 25 strokes in a Dounce homogenizer. Monitoring of disruption was unsatisfactory because cells and nuclei did not maintain normal shape in the nigrosin stain.

The suspension, plus an additional 0.5 ml RSB which was used to wash the homogenizer, was put on a step sucrose gradient containing 1.5 ml each of 1.0 M sucrose and 1.5 M sucrose in 2 x TE pH 7.4. After centrifugation for 1 hr at 30,000 rpm (73,449 x g av) at 4°C in a Spinco SW 39 rotor, the material at the interface was collected, diluted with 5 ml Medium B and the pellet collected at 17,000 x g max for 15 min in Sorval RC2B centrifuge.

(ii) Cells were harvested as in (i) and disrupted in 1 ml Medium B with 10 strokes of Dounce homogenizer. The disruption was monitored with nigrosin stain. The suspension was mixed with 4×10^8 unlabelled L-cells which had been disrupted in 10 ml Medium B. Nuclei were removed by centrifugation at 1000 x g max for 10 min and the nuclear pellet was washed once. The crude mitochondrial pellet was collected from the supernatant by centrifugation at 6,600 g max for 12 min. The mito-

chondrial fraction was washed twice and further purified on a sucrose step gradient as in (i).

Isolation of mDNA:

The mitochondria were lysed at room temperature by adding 1.5 ml SDS solution to the mitochondrial pellet. After 10 min the solution was made 1 M in CsCl by the addition of 7 M CsCl in 1 x TE pH 8.0. The resulting suspension was chilled on ice for 15 min and the precipitate removed by centrifugation at 17,000 x g max for 15 min. The supernatant solution was adjusted with CsCl and 1 x TE pH 8.0 to a final volume of 4.5 ml and a density of 1.60 gm cm^{-3} containing 200 μg (i) or 400 μg (ii) EB per ml. The solution, layered on top with light mineral oil, was centrifuged in nitrocellulose tubes in a No. 65 Spinco rotor at 42,000 or 40,000 rpm for 44 or 48 hr respectively at 20°C. The tubes were punctured, and 10 drop fractions collected. The refractive index was immediately read for every 10th tube and 20 μl of each fraction spotted on glass fiber filters, dried and counted in toluene-omnifluor scintillation fluid.

The nuclear pellet from the bottom of the sucrose gradient in (i) was lysed and prepared for EB-CsCl gradient exactly as described for mitochondria.

^{14}C nDNA Preparation:

Three to five plates of fibroblasts, labelled with [^{14}C -CH₃] thymidine, were harvested and washed with PBS. The cells were lysed and the whole cell DNA prepared for EB-CsCl gradients as described in the preparation of mDNA.

Buoyant Density of mDNA and nDNA:

In Fig. 2 and 3 the results of the EB-CsCl gradients are shown. The bands in the middle of the gradient contain circular DNA, the linear DNA is located at the top of the gradient (Nass, 1969). The approximate density of the fractions was calculated from the refractive index by the use of conversion tables in the Handbook of Biochemistry.

Fractions containing circular or linear DNA were pooled as indicated in the figures. The EB was removed by one passage through a 250 μ l bed volume of Dowex-50 anion-exchange resin. The DNA was further eluted with two rinses of 0.5 ml of 1 x TE, 0.1 M NaCl pH 8.0 (Hudson et al, 1969). The eluates were used in the preparation of the gradients described below. Each gradient had a total volume of 4.5 ml, adjusted to an initial density of 1.71 gm cm⁻³ with CsCl, and contained as a marker 150 μ l of 1 mg Micrococcus lysodecticus DNA per ml (density = 1.731 gm cm⁻³ Hollenberg et al, 1970). Gradients from the first preparation were:

- (1) ³H mDNA
- (2) ³H mDNA + ¹⁴C nDNA
- (3) ¹⁴C nDNA

and from the second preparation (ii) were the same.

A Spinco No. 65 rotor was used, conditions are described on the figures 4 to 7. Eight drop fractions were collected and the refractive index read immediately on every 10th fraction. The A₂₆₀ of 10 μ l of the relevant fractions diluted in 300 μ l distilled water was determined. In single labelled gradients 40 μ l of each fraction was counted on glass fibre filters in toluene-ornifluor scintillation fluid. In the dual

labelled gradients a 50 μ l aliquot was applied to filter paper, washed twice in ice-cold 5% TCA, once in ethanol-ether (1:1) and ether. The papers were then oxidized in a Packard Tri-carb Sample Oxidizer and the resultant [14 C]CO $_2$ and [3 H]H $_2$ O counted separately.

The density of turtle mDNA and nDNA was determined by comparison with the density M. lysodecticus DNA (density = 1.731 gm cm $^{-3}$).

Preparation of Turtle mDNA for Electron Microscopy

For the isolation of mDNA in sufficient quantities for electron microscopy, turtle livers were used. The isolation of mDNA from hooded male rats was tried first and essentially the same method followed for turtle mDNA.

Isolation of Liver Mitochondria:

The animal(s) was sacrificed by cervical dislocation (rat) or decapitation (turtle). The livers were removed and put in SE at 0°C. The tissue was minced and rinsed with SE to remove as much blood and bile as possible and then homogenized by 3 strokes of a Potter homogenizer in 10 volumes of SE. Nuclei and unbroken cells were pelleted at 600 x g for 10 min in a swinging bucket rotor. Mitochondria, collected from the nuclear supernatant at 6,600 x g max for 10 min, were washed once in 5 volumes SE. The crude pellet was washed with 2.5 volumes 0.3 M sucrose, 50 mM Tris-HCl, 5 mM MgCl $_2$ and resuspended in 0.5 volumes of this medium. The suspension was left at room temperature for 30 min after the addition of 100 μ g deoxyribonuclease per ml. The digestion was stopped by chilling and dilution with Medium B. Mitochondria were recovered at 6,600 x g max for 10 min, washed once with 5 volumes of Medium B and then with one volume. A drop of this final

suspension was used in examination of the pellet by electron microscopy. A sample of the final pellet was put in 5 M ammonium acetate to examine the DNA by the osmotic shock procedure.

Isolation of mDNA from Liver Mitochondria:

(a) Rats: The remaining final pellet was suspended in 0.5 volumes SDS solution with 100 μ g proteinase K per ml and incubated at room temperature for 30 min. The suspension was placed on ice, brought to 1 M CsCl with 7 M CsCl, and left for 15-30 min at 0°C. The precipitate was removed at 17,000 x g max for 15 min. The supernatant was diluted to 13 ml and the DNA collected at 100,000 x g max at 25°C for 16 hr in a SW 41 rotor. The bottom 1 ml and the pellet were dialysed against 2 l of 1 x TE pH 7.5 for 18 hr, followed by dilution and centrifugation at 150,000 x g max for 16 hr at 4°C in a SW 41 rotor. The supernatant was removed to 0.5 - 0.7 ml above the pellet and the resuspended pellet was used in both the aqueous and formamide electron microscopic procedures.

(b) Turtle: Lysis was performed in 0.1 M NaCl, 10 mM Tris-HCl, 1% SDS and 300 μ g Proteinase K per ml. Incubation followed by precipitation of SDS was performed as described above. The supernatant was dialysed for 18 hr against 2 l of 1 x TE and then used for electron microscopy. Further concentration by dilution and centrifugation resulted in an apparent loss of DNA as seen on the grids.

Length of mDNA:

The DNA was spread on grids as described in sections of electron microscopy procedure. The molecules were photographed, usually at a magnification of 8,500, the negatives projected, and the molecules traced and measured as described in electron microscopy method.

Electron Microscopy of mDNA

Mitochondrial DNA was prepared for electron microscopy by the protein monolayer technique (Kleinschmidt 1959). The principle of this technique is as follows: A DNA solution containing cytochrome c and a high concentration of salt (hyperphase) is delivered slowly down a glass ramp onto the surface of a solution of low salt concentration (hypophase). A cytochrome c film starts to form on the ramp at the air:water interface and adsorbs the DNA. The protein film with the DNA attached then spreads out over the surface of the hypophase and sections of the film can be picked up by touching grids to it. After dehydration and drying of the grids, the DNA can be contrasted by shadowing and/or staining. Three variations in the content of hyper- and hypo- phases described in Table 2 were used to prepare the DNA for spreading.

Closed circular mDNA under aqueous conditions is supercoiled and, because it is so highly twisted, cannot be measured in the aqueous or Kleinschmidt technique. Some molecules have a single-strand nick, allowing relaxation of the closed circles, to give open circles, under these conditions. The addition of formamide to 40-50% produces partial denaturation and removal of super coils in the closed circles. The methods described for the above procedures are essentially those of Simon et al (1971). A third variation utilizes the change from the high salt concentration of the hyperphase to osmotically shock mitochondria, releasing DNA which then adsorbs to the cytochrome c monolayer (Nass, 1966; Van Bruggen et al, 1968). The composition of the phases and the time of spreading are presented in Table 2.

TABLE 2

Hyper- and Hypo- phase Solutions and Grid PreparationAqueous or Kleinschmidt Technique

Hyperphase: 5 μ l of 1 mg cytochrome c per ml
 5 μ l of 5.0 M ammonium acetate, pH 8.0
 40 μ l of DNA in 1 x TE pH 7.5 (1 μ g DNA per ml or less)

Hypophase: 0.25 M ammonium acetate pH 7.5

Grids: A sample of 40 μ l is spread and DNA was picked up on grids,
 up to 10 min later, stained and shadowed as described below.

Basic Formamide Technique

Hyperphase: 5 μ l of 1 mg cytochrome c per ml
 5 μ l of 0.1 M EDTA pH 8.0
 20 μ l of 99.9% formamide
 20 μ l of DNA solution in 1 x TE pH 7.5 (2 μ g DNA per ml or less)

Hypophase: 10% formamide in 1 x TE pH 8.0, prepared immediately before use.

Grids: A sample of 40 μ l is spread and picked up after 60 seconds,
 dehydrated and shadowed.

Osmotic Shock

Hyperphase: 50 μ l of stock suspension: approximately 20 μ l of the pellet
 to be used was suspended in 500 μ l of 4 M or 5 M ammonium
 acetate and allowed to stand on ice for 30 min. This may
 be used immediately or stored for several days at -20°C .
 5 μ l of 1 mg cytochrome c per ml 5 M ammonium acetate.
 These solutions are mixed and equilibrated to room temperature.

Hypophase: Distilled water (room temperature)

Grids: A sample of 50 μ l was spread, picked up on grids after 2 min,
 stained and shadowed.

Preparation of Grids

Spreading of DNA:

A glass microscope slide, previously rinsed in 0.25 M ammonium acetate and drained dry, was placed as a ramp in a plastic petri dish which contained the hypophase. The aqueous surface was dusted lightly with talcum. The hyperphase sample was applied to the ramp from a glass micropipette, using a back and forth motion. Delivery started about 1 cm above the hypophase and ended when contact was made with it. The remaining sample was then expressed slowly from the top of the wet area. The film that formed on the hypophase was visualized by the movement of the talcum. Carbon coated parlodion on 200 mesh copper grids (prepared by the electron microscope laboratory) were used to pick up the film. The best grids were usually picked up near the point of contact with the hypophase.

Staining and Dehydration of Grids:

A stock solution of 5×10^{-2} or 5×10^{-3} M uranyl acetate in 50 mM HCl was diluted with 95% ethanol to 5×10^{-5} M uranyl acetate just prior to use. The more dilute stock solution appeared to give more contrast and a finer grain. Grids to be stained were dipped in this solution for 30 seconds and then either dipped for 10 seconds in isopentane or air dried. In those preparations where staining was not required, grids were dipped for 30 seconds in 95% ethanol and dried.

Shadowing of DNA Molecules:

A piece of 0.1 mm platinum wire 3 - 5 cm long, was evaporated on carbon electrodes in a Varian Vacuum Evaporator at a distance of 7 cm and an angle of 8° . Grids (up to nine) were rotated, during evaporation,

at 80-100 rpm. It was found in formamide preparations that a slightly shallower angle gave better contrast.

Electron Microscopy and Measurement of DNA

The grids were examined with a Phillips 300 electron microscope at 80 kv accelerating voltage. Pictures were usually taken at a magnification of 9,500. Negatives were enlarged ten times in a Durst point source enlarger, the molecules traced on paper and measured with a map measuring device. Under these spreading conditions the diameter of double stranded DNA was 10 to 15 nm (Simon et al, 1971).

Note: In the first experiments (rat and turtle) the microscope was used in such a way that exact magnification could not be determined. In all later experiments, the magnification was stabilized prior to photography and the magnification used was correct to within $\pm 5\%$ as calibrated with a replica grating.

Mitochondrial Suspension

Since, in the case of the anemone, the presence of mitochondria could not be detected either by oxygen uptake or cytochrome c oxidase activity, a method was attempted to visualize the contents of the 6,600 x g and 23,400 x g pellet. A drop of pellet suspension was placed on a piece of parafilm. The grid was touched to the surface of the drop and excess suspension removed with filter paper. The grid was then touched to a drop of 3% ammonium molybdate stain. After 60 seconds the excess stain was absorbed and the grid was air dried. Since these grids were not shadowed, they were rather unstable and had to be examined immediately.

Hydra Culture

Hydra Culture Medium:

Stock Solutions

- | | | | |
|-----|---------------------------|-----|-----------------------|
| (a) | 10 gm NaHCO_3 | (b) | 1.0 M CaCl_2 |
| | 5 gm Na_2 EDTA | | |
| | in 500 ml deionized water | | |

The culture medium contained 5.2 ml (a) and 1-1.5 ml (b) per litre of deionized water.

Artemia salina (Brine shrimp) Culture Medium:

Stock Solution

- 24.7 gm NaCl
 - 0.7 gm KCl
 - 6.3 gm $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
 - 4.6 gm $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$
 - 0.15 gm anhydrous CaCl_2
 - 0.04 gm NaHCO_3
- in 200 ml solution.

The stock solution was diluted 5 times for hatching brine shrimp. Alternatively Hykro Brine Shrimp Hatching mix (obtainable at pet stores) can be used. The use of NaCl as the only salt (Loomis & Lenhoff, 1956) did not produce as high a yield of live shrimp.

Hatching Procedure for Shrimp:

Hykro brine shrimp hatcheries were used as directed, except that it was found that aerating the hatching solution increased the yield.

Culture of Hydra:

Hydra were grown in Pyrex baking dishes basically as described

by Loomis and Lenhoff (1956). For feeding, brine shrimp were harvested as described in hatchery instructions, rinsed, and released into the dish with the hydra. After 1 to 2 hr the medium was changed, and again 6 to 8 hr later if cultures became crowded. To change the medium it was poured into a round container; any loose polyps that poured off were collected from the centre of the container with a pipette after swirling the medium and allowing it to come to rest.

For harvesting, polyps were released by spraying with a jet of deionized water and were then washed into a round container where they were recovered by swirling as described above. At least once a week the culture dishes were cleaned with Ajax.

Cnidaria mDNA

Hydra mDNA

Hydra littoralis were obtained from the Carolina Biological Supply, Burlington, N.C.

Subcellular Fractionation:

Approximately 1,000 hydra polyps were placed in a clean dish and fasted for 48 hr. The culture medium was changed several times during this period. Hydra were harvested and placed in a Dounce homogenizer at 0°C. As much water as possible was removed and 10 ml of ice-cold homogenizing medium (0.3 M sucrose, 2 mM Tris, 2 mM EDTA pH 7.4) added. The polyps were disrupted by 7 strokes.

Nuclei, unbroken cells, and cell debris were removed by centrifugation at 750 x g max for 10 min and the pellet was washed once. The supernatants were pooled and the pellets collected after 15 min differential centrifugations at 6,600 x g max and 23,400 x g max. The pellets

were resuspended in 2 ml of deoxyribonuclease incubation medium (0.3 M sucrose, 10 mM Tris pH7.4, 5 mM $MgCl_2$) and incubated with 100 μ g deoxyribonuclease per ml for 15 min at room temperature. The suspension was then chilled and 8 ml homogenizing medium added. The pellets were recovered and washed three times. A 5 μ l sample of the final pellet was added to 200 μ l of 5 M ammonium acetate and prepared for Electron Microscopy by the osmotic shock procedure.

Isolation of DNA:

The final pellets were suspended in 1 ml of 0.5% SDS, 0.1 M NaCl, 10 mM Tris pH7.4 to which 500 μ g Proteinase K was added, and incubated for 1 hr at room temperature. The solution was chilled and the SDS precipitated with CsCl as described in turtle mDNA preparation. The clear supernatant was dialysed, as in the above section, and then used directly for electron microscopy of the DNA.

Anemone

Metridium, harvested from the Atlantic Ocean, were obtained from the Marine Biological Laboratory, Woods Hole, Mass., and kept in a marine aquarium until needed.

Subcellular Fractionation:

Since, when stimulated, anemones contract into a very hard compact form, homogenization seemed best accomplished by surprising a relaxed specimen. Therefore, an anemone (about 9 gm) was placed in fresh sea water and kept in the dark overnight. In the morning, 80 ml of ice-cold homogenizing medium (1.0 M sucrose (Prosser et al., 1966) 10 mM Tris, 10 mM EDTA, pH7.4) was placed in a chilled blender and the blender turned on. As quickly as possible the anemone was rinsed in deionized water and

dropped into the blender. Homogenization was allowed to proceed for 10 sec.

Preparation of the pellets was performed essentially as described for the hydra using anemone homogenizing medium and adjusting the deoxyribonuclease medium to be 1 M in sucrose. Nuclei and debris were removed at 750 x g max for 15 min and the supernatant fractionated by centrifugation at 4,300 x g max and 23,400 x g max for 15 min. The crude pellets were washed once in 10 ml deoxyribonuclease medium, followed by incubation in 10 ml of this medium with 100 µg deoxyribonuclease per ml for 15 min at room temperature. The digestion was stopped by the addition of 30 ml homogenizing medium and chilling. The pellets were washed in 10 ml medium twice.

Isolation of DNA:

This was accomplished as described for the hydra except that 3 ml of SDS solution was necessary. The DNA was concentrated after dialysis by centrifugation from 12 ml 1 x TE pH7.5 in a SW 41 rotor for 18 hr at 153,000 x g max, keeping the bottom 0.5 - 0.7 ml for electron microscopy of the DNA. A sample of the final 4,300 x g pellet was taken for osmotic shock.

Chemicals and Reagents

Deoxyribonuclease (crystalline beef pancreas) and Micrococcus lysodecticus (type X) DNA were obtained from Sigma Chemical Co.; ethidium bromide (2,7-diamino-10-ethyl-9-phenylphenanthridium bromide), B grade, from Calbiochem; Omni-Fluor from the New England Nuclear Co.; Methyl ³H and ¹⁴C thymidine from Amersham Radiochemical Centre. All other reagents were of the highest grade and all water except where noted was glass distilled deionized water.

CHAPTER III

Results and Discussion

Turtle mDNA

Buoyant Density of Turtle mDNA and nDNA

Results:

In Fig. 2 and 3 the results of the separation of mDNA and nDNA on EB-CsCl gradients are shown. The ^3H -mDNA prepared as described in method (i) is shown in Fig. 2A. The ^{14}C -nDNA prepared at the same time from a whole cell lysate is shown in Fig. 2B. Closed circular DNA intercalates less dye than does linear DNA and remains more dense, allowing the dye buoyant density separation of closed circular and linear DNA (Nass, 1969). Nicked circular DNA can intercalate as much dye as linear DNA and floats to the top of these gradients. Catenated multimers, where one chain is nicked and the other closed, have an intermediate density. It should be noted that DNA from the nuclear pellet, from the sucrose gradient, lysed and centrifuged at the same time as DNA from the mitochondrial fraction, showed a circular DNA:linear DNA peak ratio of 1:140. The peak ratio in Fig. 2A is 1:70 indicating a 2-fold enrichment of circular DNA in the mitochondrial fraction. The fractions of these gradients used in the determination of buoyant density are marked on the figures.

Fig. 3 shows EB-CsCl gradients from ^3H -DNA of the mitochondrial lysate prepared by method (ii) and the ^{14}C -DNA of a whole cell lysate prepared at the same time. The peak ratio in Fig. 3A of circular

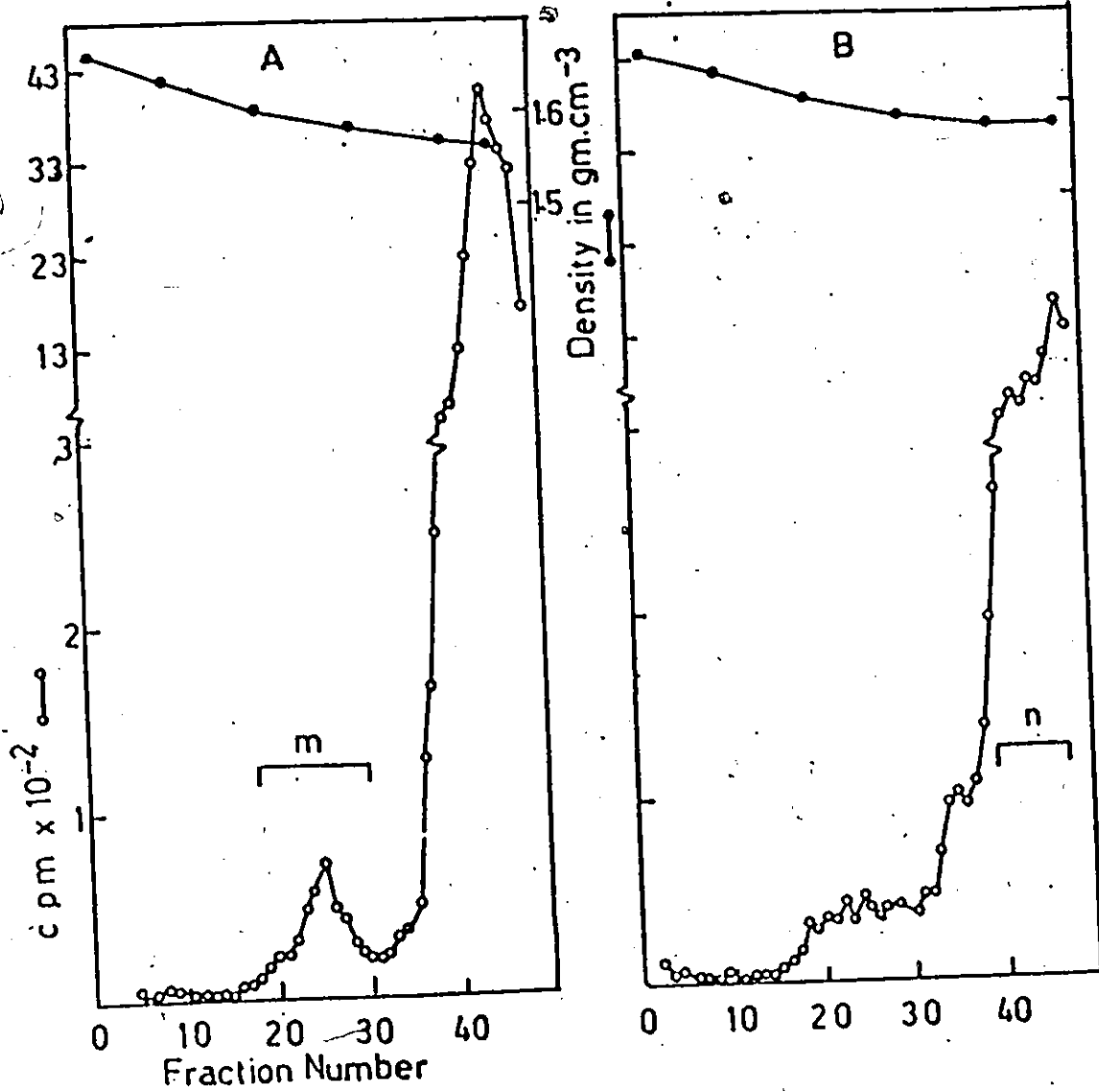


Fig. 2: EB-CsCl density gradients of turtle DNA. (A) ³H labelled turtle fibroblast mdDNA prepared as described in method (1) in text. (B) ¹⁴C labelled whole cell DNA. Centrifugations were in a Spinco No. 65 rotor for 44 hr at 44,000 rpm and 20°C (A); 43,000 rpm for 60 hr at 25-30°C (B); m - indicates fractions used for ³H mdDNA; n - indicates fractions used for ¹⁴C ndNA.

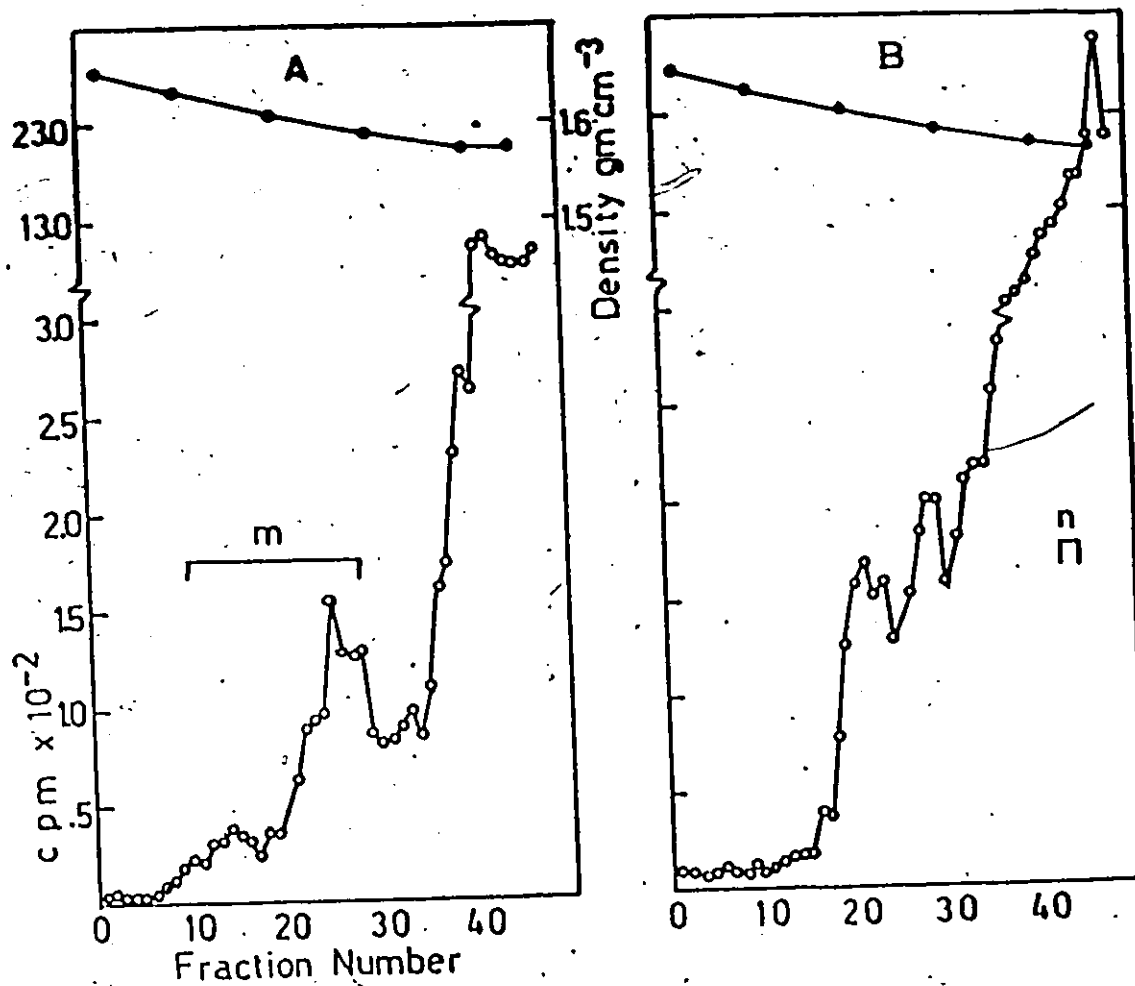


Fig. 3: EB-CsCl density gradients of turtle DNA. (A) ³H labelled turtle fibroblast mRNA prepared as described in method (11) in text. (B) ¹⁴C labelled whole cell DNA. Centrifugation was at 40,000 rpm, 44 hr at 20°C in a Spinco No. 65 rotor; m and n indicate fractions used as described in Fig. 2.

DNA:linear DNA of about 1:10 shows a 7-fold enrichment of circular DNA compared to the results shown in Fig. 2A. The mitochondria used to prepare the mDNA in Fig. 3A were more highly purified than those in Fig. 2A. The presence of linear DNA at high densities in Fig. 3B than in Fig. 2A, 2B and 3B, is characteristic of gradients overloaded with DNA. Under these conditions, the linear DNA is not saturated with EB. The fractions used for buoyant density determinations are indicated on the Figure as before.

Fig. 4 and 5 show the ^3H -mDNA and ^{14}C -nDNA from Fig. 3 run separately on CsCl density gradients with a marker of M. lysodecticus DNA having a buoyant density of 1.731 gm cm^{-3} . The density of ^3H -mDNA is 1.703 gm cm^{-3} and that of the ^{14}C -nDNA peak is 1.701 gm cm^{-3} with respect to the marker. The dual-labelled gradient obtained with the same amounts of ^3H -mDNA and ^{14}C -nDNA as in Fig. 4 and 5, is shown in Fig. 6. The peaks were less than one fraction apart and have a density of 1.702 gm cm^{-3} compared with M. lysodecticus DNA. In the centre of these gradients there was a density slope of about 2 mg cm^{-3} per fraction.

The double labelled gradient prepared from fractions indicated in Fig. 2 is shown in Fig. 7. Again, the peaks differ by less than 1 fraction but in this gradient the density with respect to M. lysodecticus is 1.706 gm cm^{-3} . The peaks in both Fig. 6 and Fig. 7 are separated by the same number of fractions. The discrepancy in absolute value between gradients is not unexpected, since this value depends heavily on three density determinations.

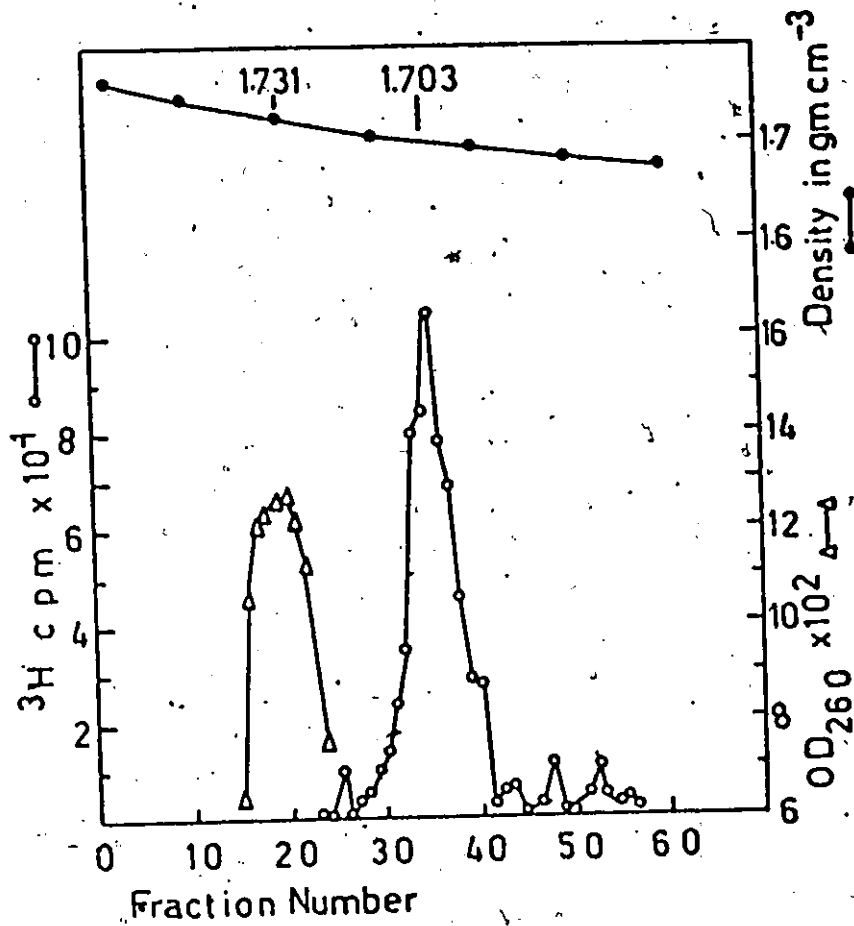


Fig. 4: Buoyant density of turtle mDNA. ³H labelled DNA prepared by method (11) was centrifuged at 40,000 rpm for 44 hr at 20°C in a Spinco No. 65 rotor. Δ — Δ , *Micrococcus lysodecticus* DNA; o—o, ³H mDNA; ●—●, density of gradient.

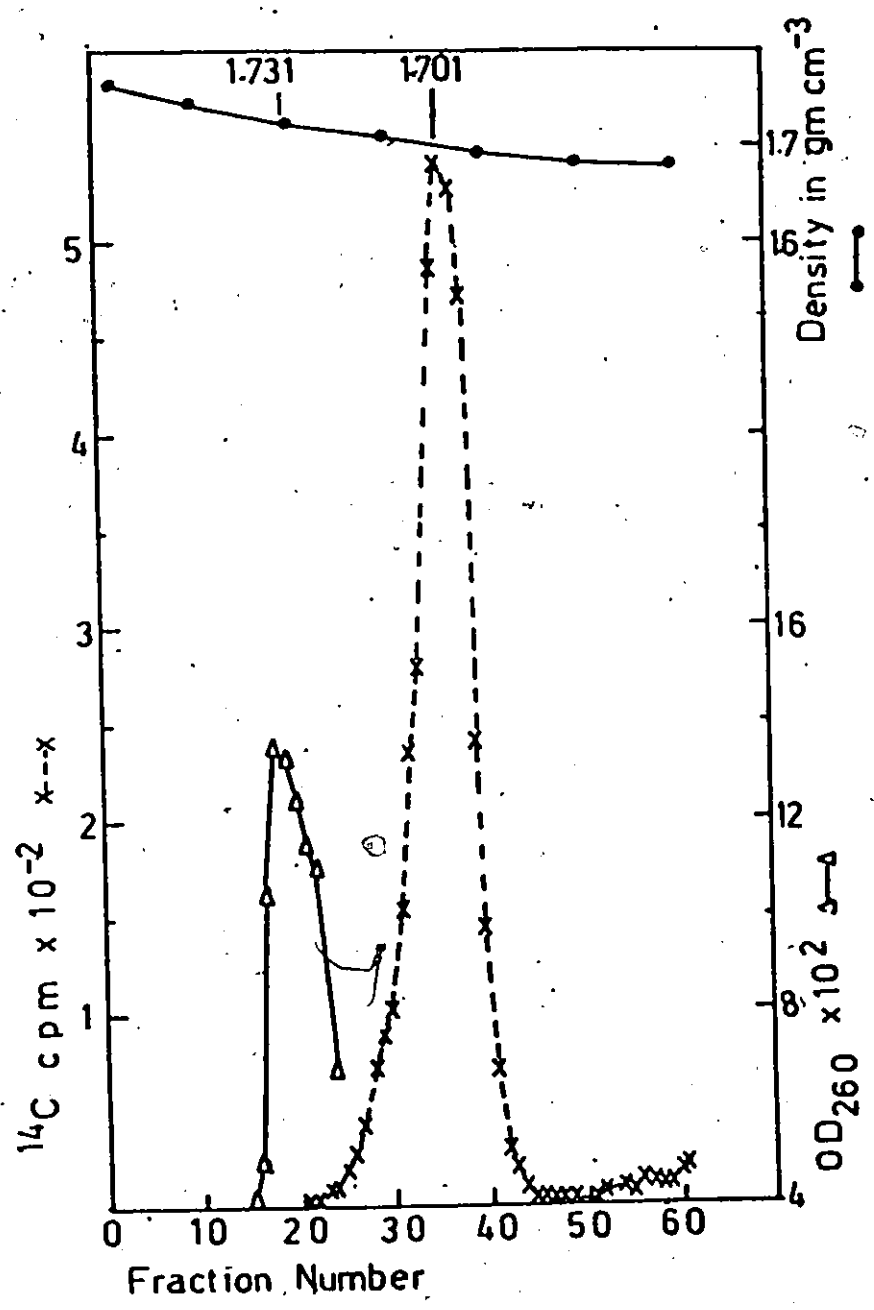


Fig. 5: Buoyant density of turtle nDNA. ^{14}C nDNA prepared by method (ii) was centrifuged exactly as in Fig. 4. Δ — Δ , *Micrococcus lysodecticus* DNA; x—x, ^{14}C nDNA; \bullet — \bullet , density of gradient.

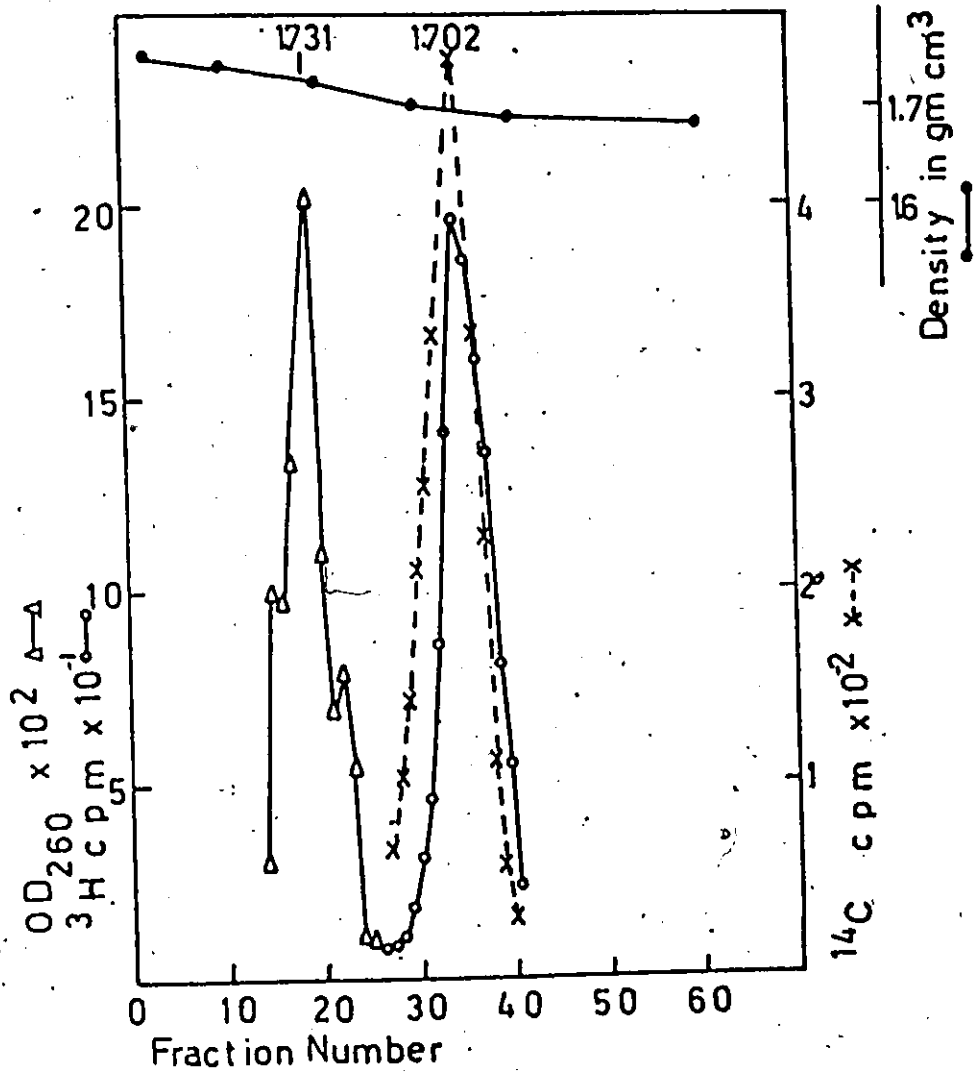


Fig. 6: Buoyant density of turtle mRNA and nDNA. ³H mRNA and ¹⁴C nDNA were prepared as described in method (ii). Centrifugation conditions were exactly as described in Fig. 4. x---x, ¹⁴C nDNA; ○---○, ³H mRNA Δ---Δ, Micrococcus lysodecticus DNA; ●---●, density of gradient.

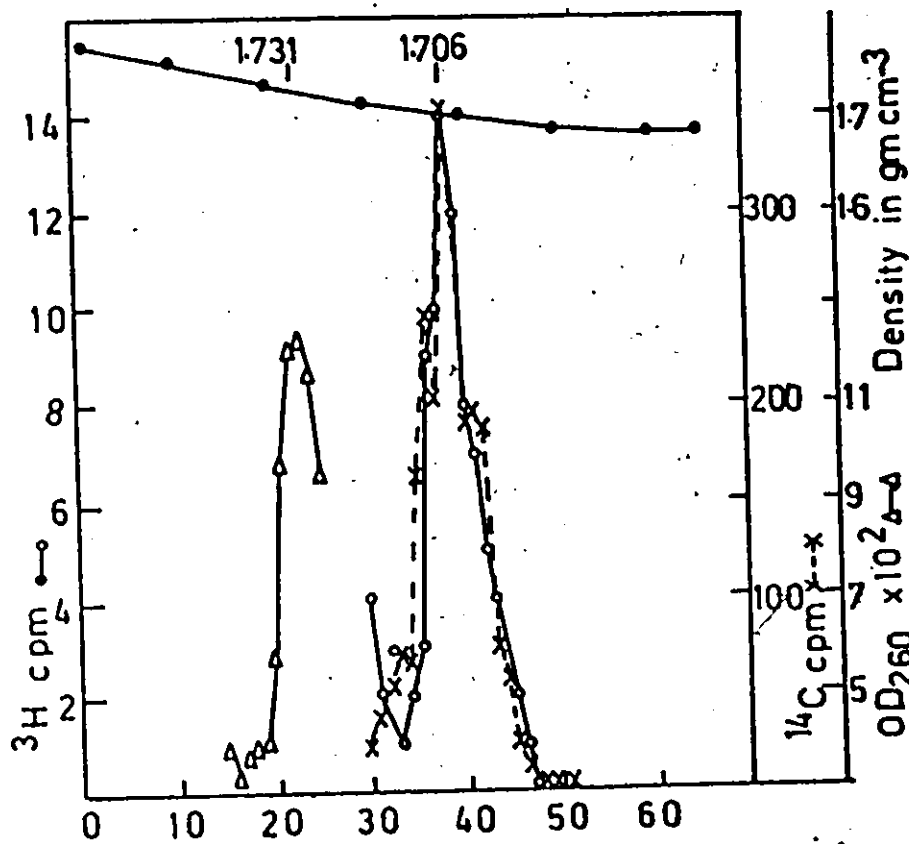


Fig. 7: Buoyant density of turtle mRNA and nDNA. ³H mRNA and ¹⁴C nDNA were prepared as described in method (1). Centrifugation was in a Spinco No. 65 rotor at 40,000 rpm and 20°C for 42 hr. The symbols are as in Fig. 6.

Discussion:

The buoyant density of mDNA and nDNA of turtle were virtually the same. One possible explanation is that mDNA is contaminated with excess nDNA. Several observations argue against this interpretation. The preparation of mDNA depended on two purification steps: the first being the preparation of purified mitochondria, and the second, the separation of circular from linear DNA. Considering the latter first, the fractions from EB-CsCl gradients used for buoyant density determinations were in the expected density range for closed circular DNA. Moreover, the peak, especially in Fig. 2, was sharp and therefore probably a unique species. The contamination with linear molecules should be minimal. It is likely, then, that circular DNA has been isolated.

Does the circular DNA come from mitochondria? The higher proportion of circular DNA in more highly purified mitochondria indicates that it does. It is possible that the DNA comes from organelles which co-sediment with mitochondria. However, other circular cytoplasmic DNAs have not been reported in vertebrates.

It is concluded that the density of mDNA and nDNA of Pseudemys is virtually the same and about 1.704 gm cm^{-3} . Both these results are reasonable in the light of observations of others. Mouse liver mDNA and nDNA have a density of 1.701 gm cm^{-3} (Borst & Kroon, 1969) and mDNA and nDNA of Rana pipiens is 1.702 gm cm^{-3} . Mitochondrial and nuclear DNAs of other vertebrates usually differ by at most $2-4 \text{ mg cm}^{-3}$ except for birds where the difference is $3-11 \text{ mg cm}^{-3}$. The buoyant density of mDNA of Mammalia is 1.698 to 1.705 gm cm^{-3} , that of Amphibia

1.702 gm cm⁻³ to 1.704 gm cm⁻³, and that of Osteichthyes 1.703 gm cm⁻³. The value found for the buoyant density of a reptilian mDNA, then, falls within the expected density range.

The establishment of an absolute density in this type of gradient is very difficult. There are minor sources of error, such as drop errors which will shift the density curve as well as the DNA peaks slightly. It was noted that by the time the third tube had been fractionated, the density gradient had flattened slightly due to diffusion. This affected the extremes of the gradient more than the middle. The DNA did not seem to diffuse in this short a time. The result is that, although the DNA peaks coincided from gradient to gradient, the density curve varied. A small shift in this curve in the region of the M. lysodecticus DNA marker increases the error, since the slope of the density gradient in this region is about 4 mg cm⁻³ per fraction. The determination of the absolute density depends on the accuracy of the density curve here and in the region of the unknown DNA. If a marker having a density in the region of the unknown had been used, the two densities could be compared within 2 mg cm⁻³. An analytical Model E centrifuge could also be used if optical amounts of DNA were available.

Adjustment of the M. lysodecticus DNA density to 1.731 was done to compensate for temperature difference between that of the centrifuge during the run and room temperature when the refractive index was read. This adjustment also compensates for refractive index errors due to other dissolved substances not contributing to the density. The actual values obtained for M. lysodecticus DNA ranged from 1.728 to 1.730 gm cm⁻³ (six gradients).

It is concluded that mDNA and nDNA of Pseudemys have a density less than 2 mg cm^{-3} apart, and their density is 1.704 gm cm^{-3} with a variation of 0.002 gm cm^{-3} .

Length of Turtle mDNA

Results:

Fig. 8 shows an electron micrograph of a rat mitochondrial fraction and Fig. 9 that of the turtle mitochondrial fraction. Damaged and intact mitochondria are apparent together with some membrane fragments. Other organelles were not seen. The osmotic shock procedure to release DNA was attempted with liver mitochondria of both rat and turtle but, although the mitochondria were ruptured, no DNA was observed. Osmotic shock was, however, successful when applied to BHK mitochondria as demonstrated in Fig. 10. Here, several DNA molecules are visible near the ghosts. This difficulty with osmotic shock of liver mitochondria has been experienced by others (L. Pinteric; personal communication).

DNA was extracted from the mitochondria and measured as described in the Methods section. Grids with rat mDNA were well populated with open and closed circular molecules. An example is shown in Fig. 11. Grids prepared from turtle mDNA were badly contaminated and, although circular molecules were present, there were not many. Fig. 12 shows a turtle mDNA molecule. An attempt was made to purify and concentrate the turtle mDNA further, but this only resulted in the complete loss of turtle mDNA as judged by its absence on the grids. To test whether this was an artefact of staining and shadowing, salmon sperm DNA was mixed with a sample of turtle mDNA before spreading. The result was grids heavily populated with short linear pieces; whereas, control grids



Fig. 8: Rat liver mitochondria. Magnification 21,000



Fig. 9: Turtle liver mitochondria. Magnification 13,000

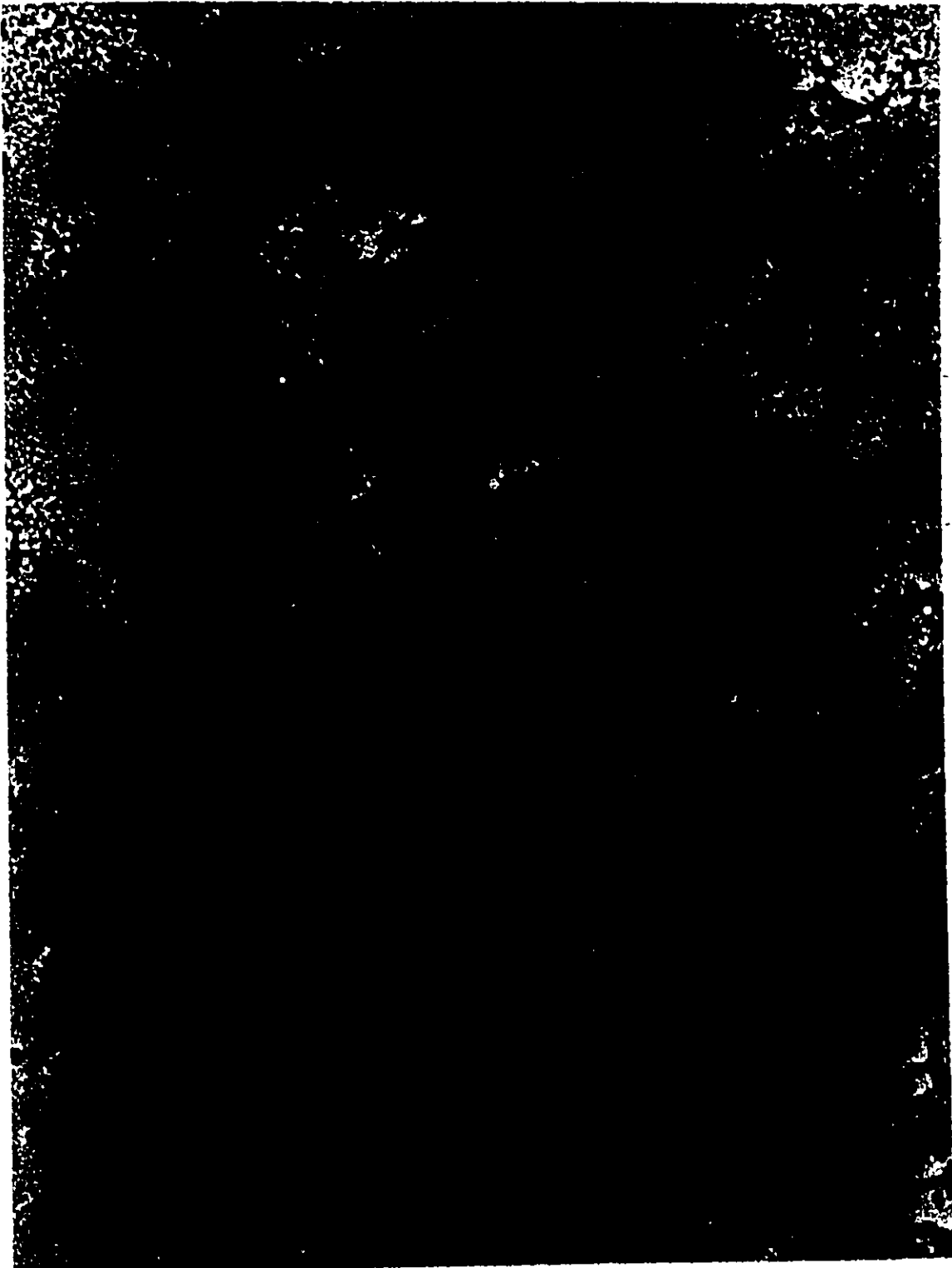


Fig. 10: Osmotically shocked mitochondrion of BHK cells.
Magnification 27,000



Fig. 11: Rat liver mRNA spread by aqueous method. Magnification 36,000

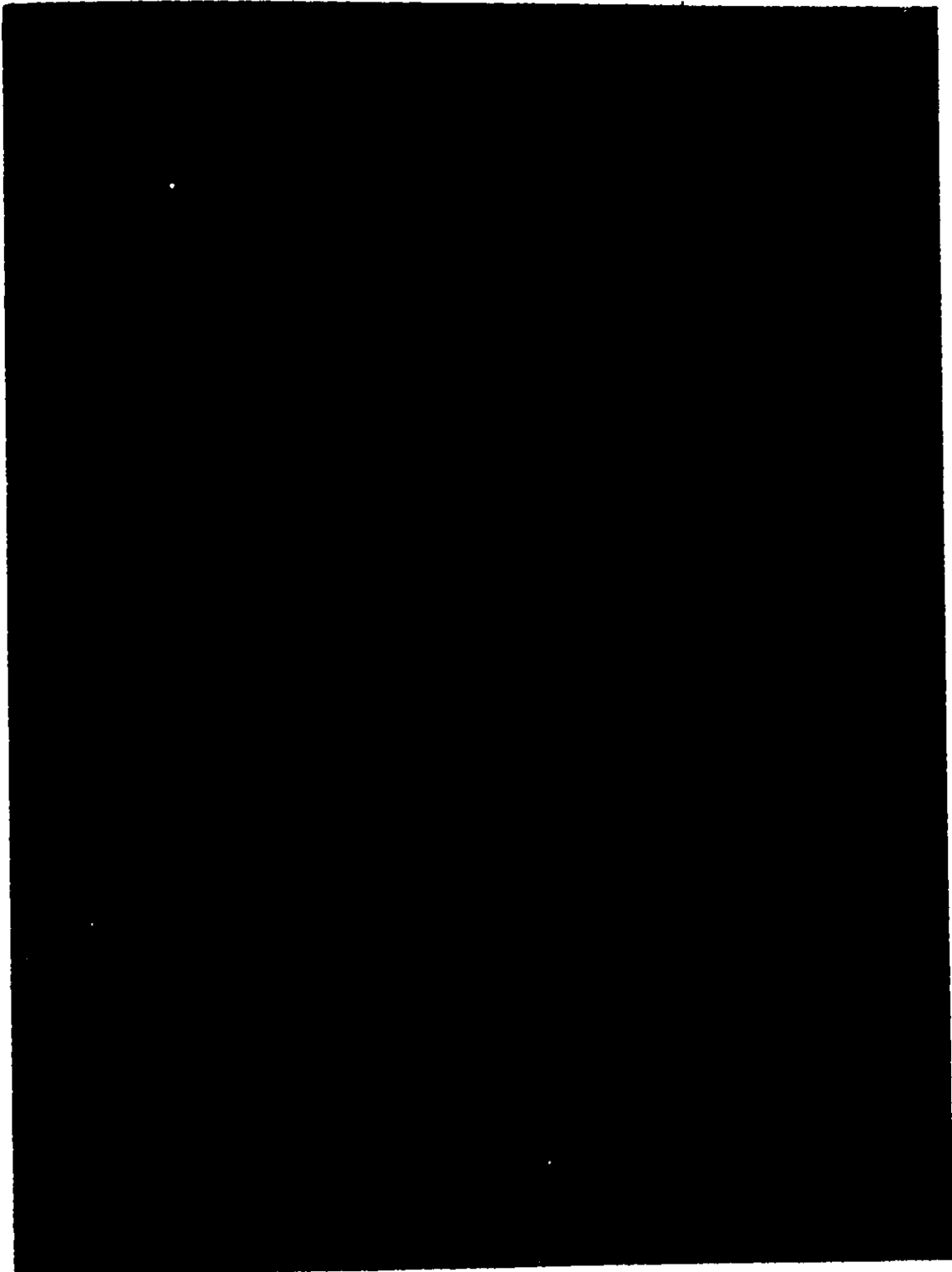


Fig. 12: Turtle liver mRNA spread by aqueous method. Magnification 36,000

without added DNA were still void of DNA. As a further control, hamster liver mDNA supplied by Dr. K.B. Freeman was also examined. Hamster mDNA is shown in Fig. 13.

Molecules were measured from the original turtle preparation and, as a reference, the rat mDNA. Fig. 14B and 14C show the results of these measurements. It was found later that the microscope was unstable when the pictures were made, and that the calibration of the magnification was incorrect. The values on the histogram have been adjusted to the correct magnification, but the instability could not be compensated for and produced a large variation in the size. Hamster liver mDNA was measured under stable conditions and with corrected magnification. The results of this are shown in Fig. 14A. It can be seen in Fig. 14 that the mode is the same in all three cases. The means were 4.2, 4.6 and 3.9 μm for mDNA of hamster, rat and turtle, respectively. The variation in the size of the mDNA is much smaller in the case of the hamster which was measured under stable magnification conditions.

Discussion:

Deoxyribonuclease treatment of mitochondria was necessary to remove nDNA contamination. To reduce the possibility of increased nicking of mDNA, due to residual enzyme activity and activity of any endogenous enzymes after lysis of the mitochondria, proteinase K was added to the lysing solution. Since, especially in rat and hamster preparations, few linear molecules and a low incidence of open circles was observed, this treatment appears effective and does not seem to harm the DNA in any way.

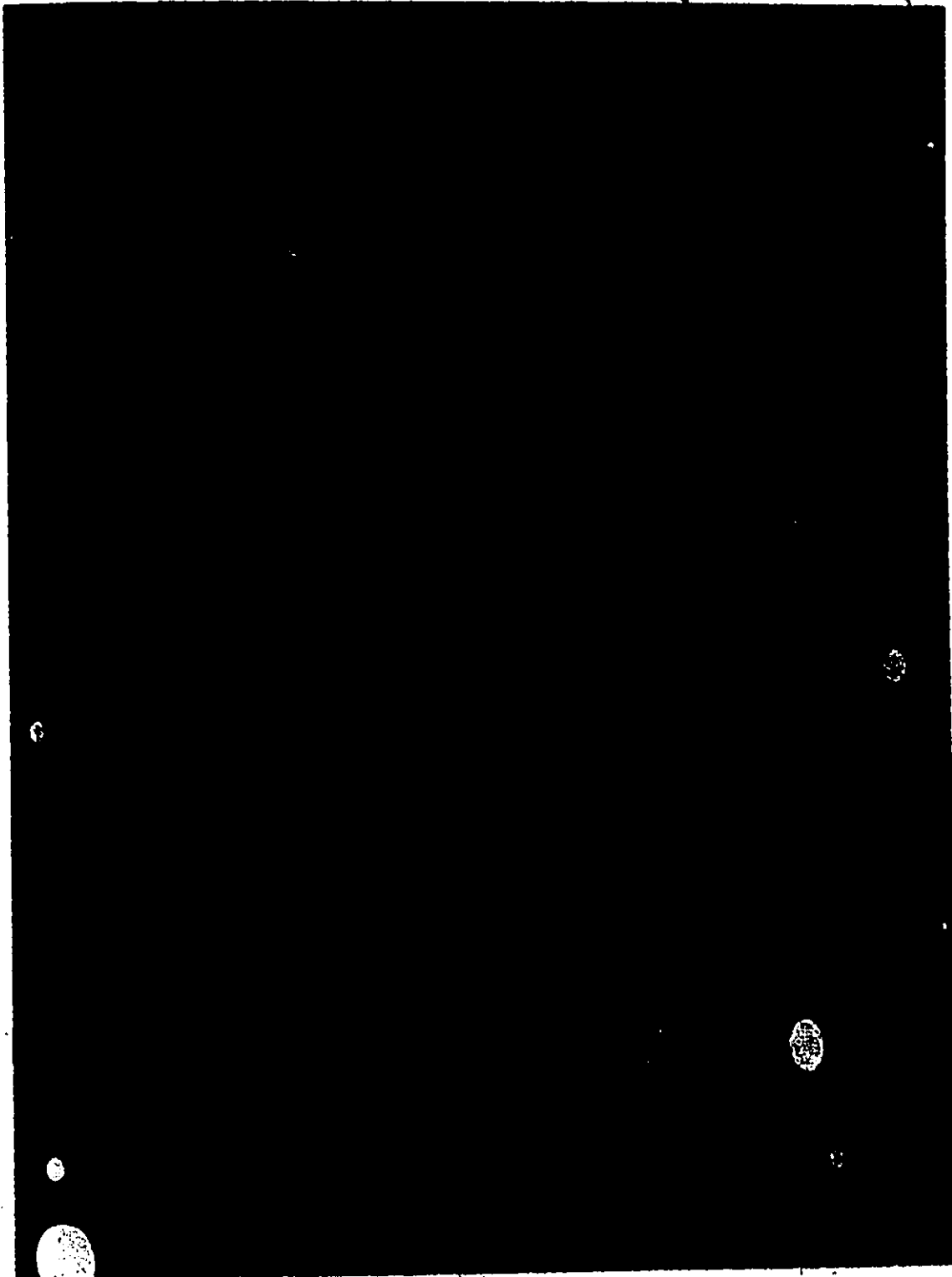


Fig. 13: Hamster liver mRNA spread by aqueous method. Magnification 41,000.

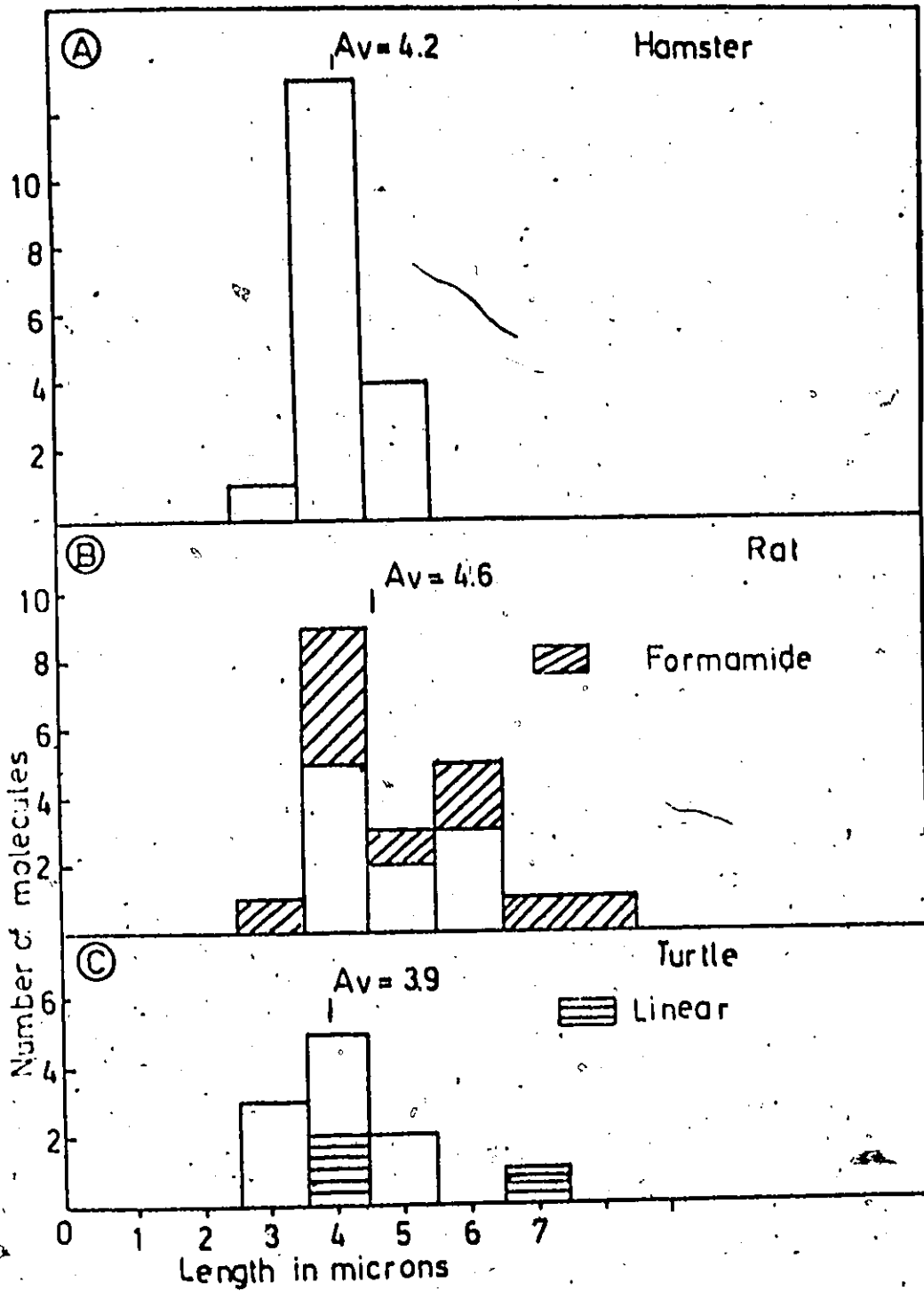


Fig. 14: Length of mRNA from hamster (A), rat (B), and turtle (C)

Referring to Fig. 14, it can be seen that turtle mDNA is of a size similar to that of rat and hamster. It can also be seen, however, in comparison with Table 1, that these molecules are about 20% too short. There is no explanation for this since the magnification of the microscope should be correct within $\pm 5\%$. Ideally, these measurements should be made with an internal standard DNA such as λ phage DNA. Similarly, to compare the lengths of two populations of DNA (e.g. turtle and rat), these DNAs should be mixed and spread together. This was the intended procedure; however, as mentioned before, turtle mDNA was lost when concentration for this purpose was attempted.

It is concluded that, although exact measurements could not be made, turtle mDNA is of the 5 μ m length type found in animals. This result was not unexpected, but demonstrates for the first time this size of mDNA in reptiles.

Cnidaria "mDNA"

When one wishes to examine mDNA from an organism in which no reference to the isolation of mitochondria can be found, the first problem is to demonstrate that mitochondria have been isolated. Usual methods, such as assay of cytochrome c oxidase activity and oxygen consumption as measured with an oxygen electrode, were unsuccessful in this case. Activity could be found in the homogenate and sometimes in the crude 4,300 x g pellet, but further purification of the pellet resulted in loss of activity both in the pellet and in the supernatant. For this reason, the supposed mitochondrial pellet as well as a high speed pellet were monitored by electron microscopy as described in the Method section.

Hydra mDNA

Results:

Fig. 15 and 16 are representative fields from the 6,600 x g pellet of the hydra homogenate. Fig. 15, although rather heavily negatively stained, indicates that this pellet contained many mitochondria. Fig. 16 is an enlargement of another field that was less stained. These two figures indicate that, although containing some fragments and a few unknown structures (Fig. 16), the pellet did contain mostly mitochondria. The high speed (23,400 x g) pellet showed no mitochondria; only small electron dense particles were observed. Neither osmotic shock nor extraction of this pellet revealed any DNA, under electron microscopy.

Grids prepared from osmotic shock of the 6,600 x g pellet from hydra showed ghosts with much DNA in the vicinity, most of which appeared to be still attached to the membranes. Fig. 17 shows a typical example. These grids, although difficult to interpret, demonstrate the presence of DNA in hydra mitochondria. Here, one can see, by comparing the osmotically shocked hydra mitochondria in Fig. 17 to that of the BHK mitochondria Fig. 10, that there appears to be more DNA in Fig. 17. The DNA in Fig. 17 is very tangled near the ghost and in some areas the ghost prevented shadowing. All molecules that could be identified were measured even if they were apparently obscured by the ghost. On these grids, two highly twisted molecules of length 4.2 and 4.8 μm were observed. The one in Fig. 17, indicated by the arrow, is about 5 μm long. It disappears near the ghost and could therefore be longer. When a molecule is highly twisted, it indicates circularity may be present. Since most of the DNA was too tangled to distinguish many individual



Fig. 15: Negatively-stained preparation of 6,600 x g fraction of hydra homogenate. Magnification 15,000



Fig. 16: Negatively-stained preparation of 6,600 x g fraction of hydra homogenate. Magnification 29,000

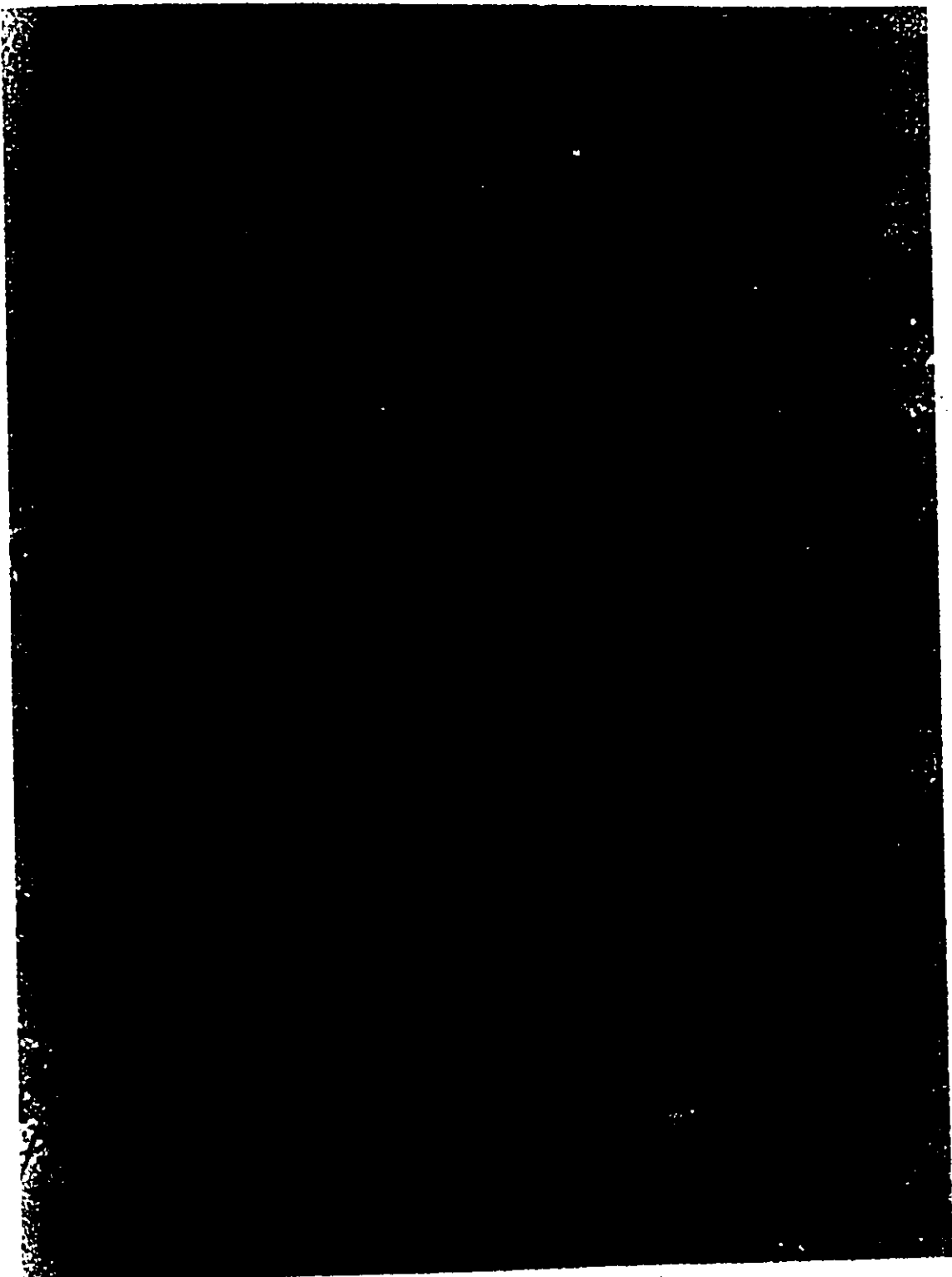


Fig. 17: Osmotically shocked hydra mitochondrion. Magnification 27,000

molecules, the ones that could be measured were usually small fragments and there were many molecules between 1.0 and 1.5 μm long. These measurements have been included in Fig. 18.

When the DNA from the 6,600 x g pellet was extracted and spread many small linear pieces were observed. The unusual feature of these grids (i.e. not observed with vertebrate mDNA) was that there seemed to be segments of about 1.2 μm linked together with unknown structures. Fig. 19 shows a good example of the type of molecule seen. It appears to be interrupted by unknown structures at about 1.2 μm intervals, or at distances which are multiples of 1.2 μm . One interpretation of this phenomenon might be that contamination on the grids obscures the molecules at these points. If this were true, this molecule has a total length of 9.8 μm . Three other molecules that appeared like this would have had total lengths close to 7 μm . In other grids, isolated DNA pieces of the size order 1.2, 2.4 or 3.6 μm were seen. In Fig. 18 only lengths of those molecules, or segments, that could be unambiguously identified as a single molecule are included. This includes segments from molecules like the one in Fig. 19. In order to establish that these molecules were DNA, two preparations were made: one containing 5 mM MgCl_2 , the other containing 5 mM MgCl and deoxyribonuclease. After 60 sec incubation these preparations were examined for DNA by electron microscopy. The grids from the preparation containing the nuclease were void of DNA; on those from the preparation containing MgCl_2 many long molecules were observed. Due to the fact that the grids contained many holes and, in many cases, the course of the molecule could not be determined, many molecules could not be measured. Three that could had lengths of 5.1, 4.7

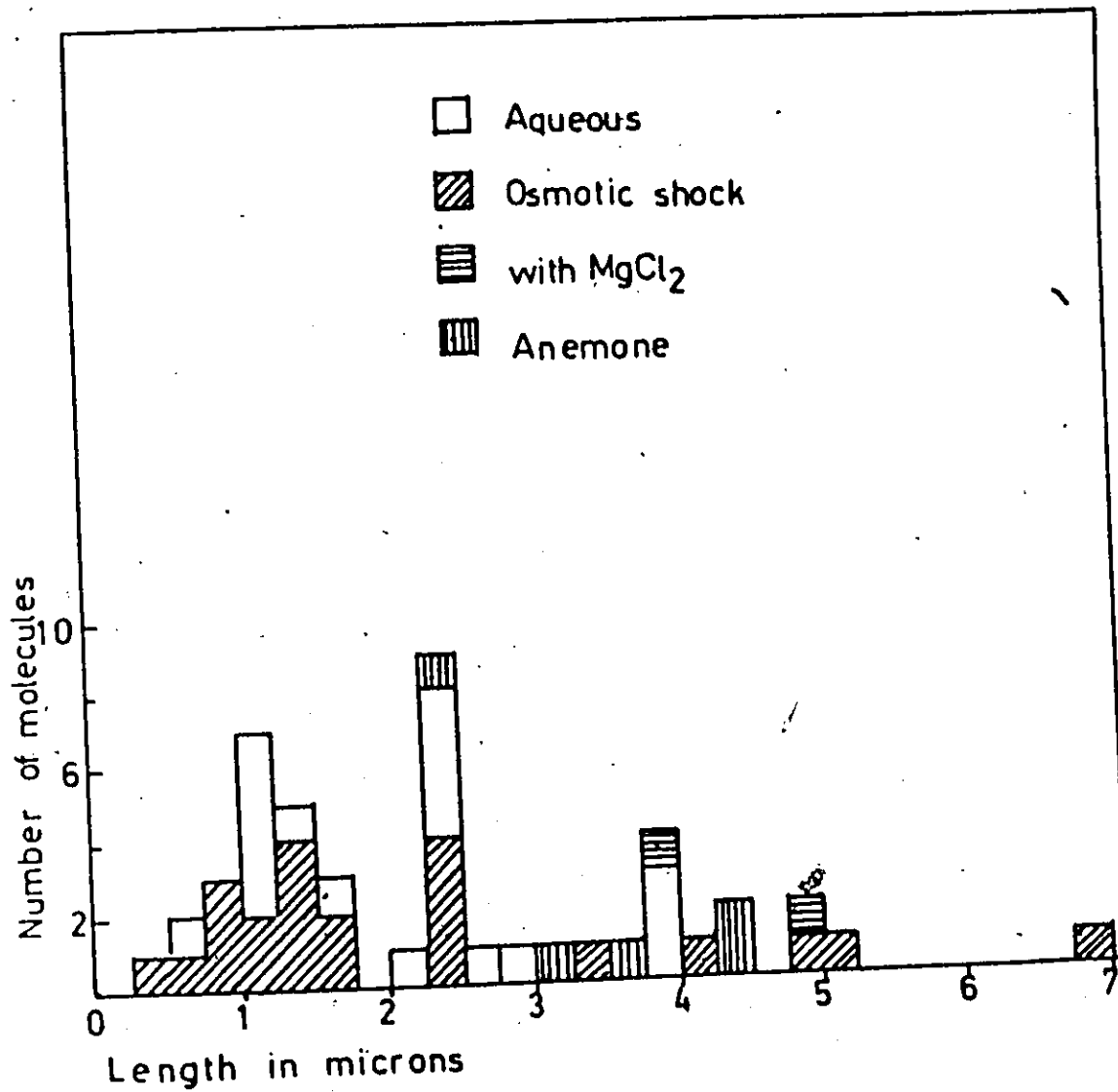


Fig. 18: Length of mRNA from hydra and anemone

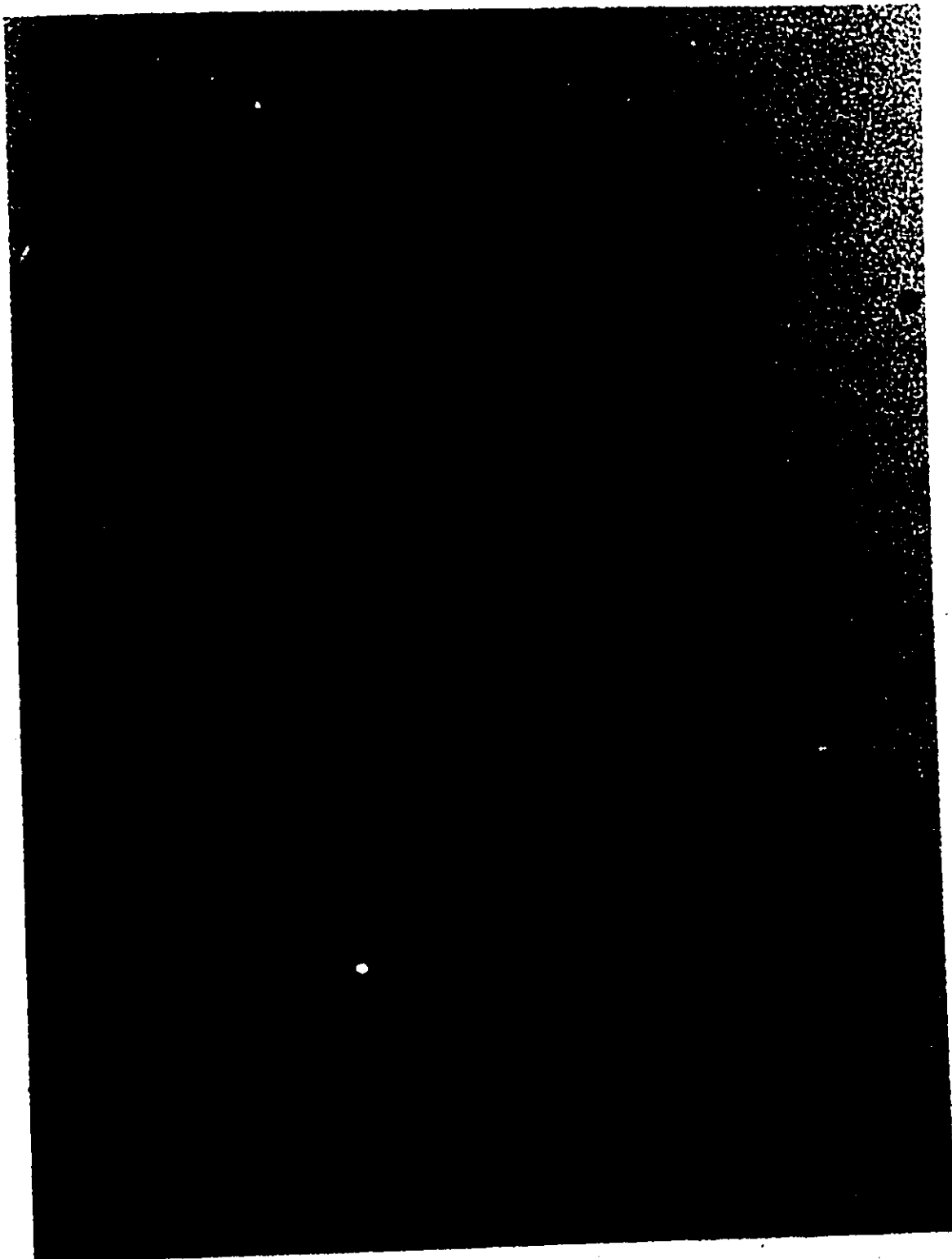


Fig. 19: Hydra ³²P-DNA spread by the aqueous method. Magnification 41,000

and 4.0 μm . The DNA in the centre of Fig. 20 appeared to be three molecules - two longer segments of 4.7 and 5.1 μm and shorter one. If this, in fact, was one molecule the total length would be about 11 μm . These grids also showed contamination but not attached to the DNA at regular intervals. In these grids, discontinuity was taken as the end of a DNA molecule.

Anemone "mDNA":

As explained at the beginning of this section, biochemical methods could not be used to identify anemone mitochondria. It was also found that electron microscopy of pellets from these organisms was unsuitable for the demonstration of mitochondria. With the high sucrose concentration used in the preparation of anemone subcellular fractions, there was charring and bubbling which left large electron dense areas. The film was also found to be very fragile under these conditions and would split if left too long under the beam as was required for photography. A subjective view of the 6,600 x g pellet was that in a few places structures that could have been mitochondria were seen, but the film broke before a picture could be taken. The 23,400 x g pellet did not appear to contain any mitochondrial structures.

When osmotic shock was tried on the 6,600 x g pellet, few ruptured structures were seen and none with visible released DNA. A solution of 8 M ammonium acetate was tried to increase the concentration gradient with the hypophase; the result was the same as that found with the usual concentration of 4 M. The DNA spread directly from the extracted pellet showed few molecules that could be definitely identified as DNA by diameter and conformation. The measurements made on these

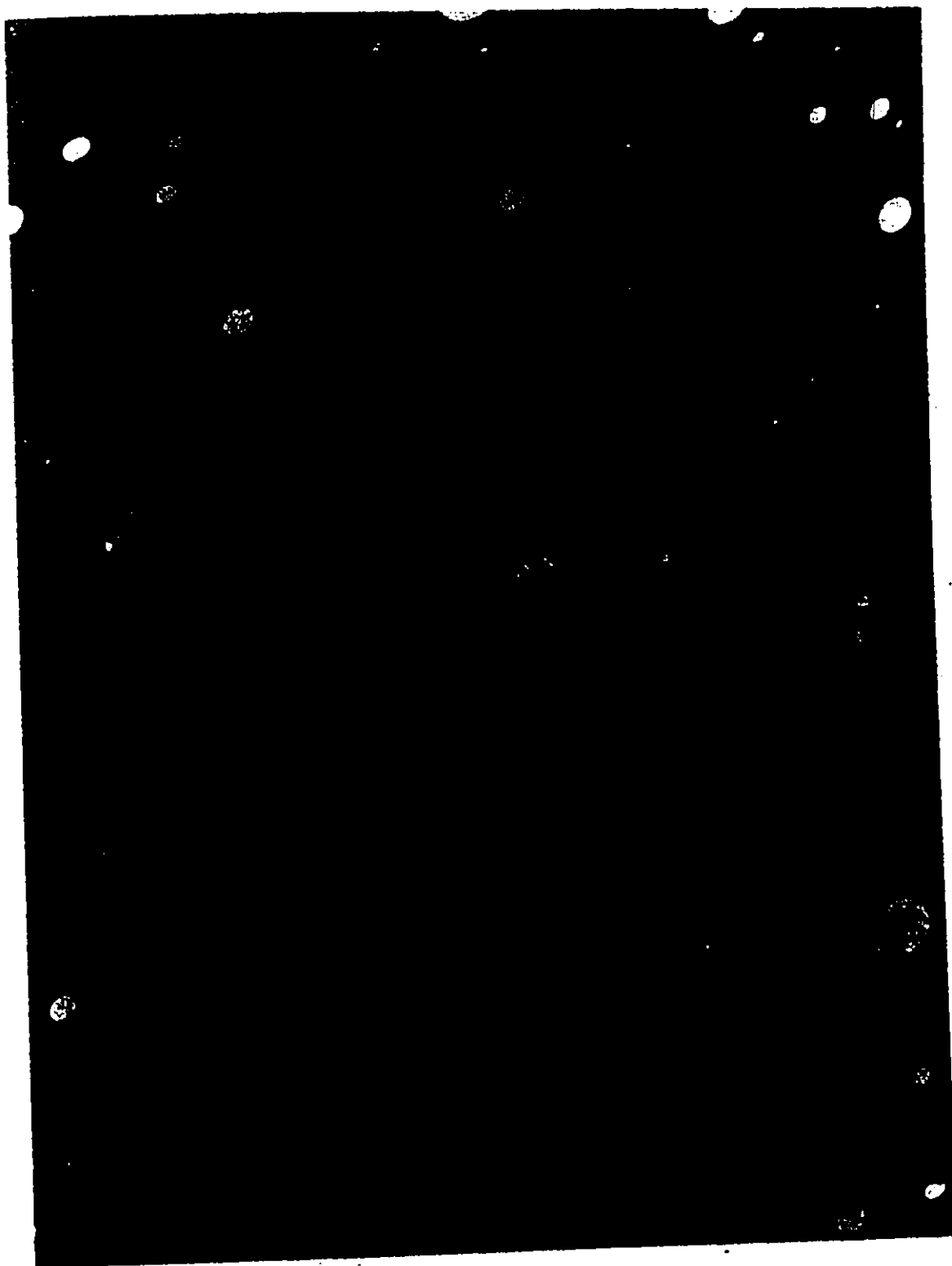


Fig. 20: Hydra "mDNA" spread with MgCl₂. Magnification 29,000

molecules were included in Fig. 18 for comparison.

Discussion:

Preliminary experiments with osmotic shock on both hydra and L-cell mitochondrial preparations indicated that the abundance of nuclear contamination made identification of the DNA from ruptured mitochondria impossible. One alternative, the use of EB-CsCl gradients to separate the two species of DNA, was not used since it was not known whether mDNA from the Cnidaria would be circular. These two considerations made necessary the use of deoxyribonuclease treatment of the pellets. With this treatment any DNA seen is unlikely to have been nuclear in origin. As in the vertebrate mitochondrial preparations, proteinase K was used to insure that residual deoxyribonuclease was removed. Since this treatment did not damage the circular molecules from vertebrate mitochondria, it is unlikely that it would affect circular DNA from the Cnidaria. This does not, however, eliminate the risk of deoxyribonuclease nicking the DNA during the incubation since Cnidaria mitochondria may be more permeable to the enzyme than vertebrate mitochondria. Also, endogenous nucleases may be present.

Fig. 15 and 16 indicate that, whereas other structures may have been present in the 6,600 x g pellet from the hydra, the pellet did contain mostly mitochondria. The mitochondria measure about 1 μ m in diameter and, in some cases, the inner and outer membranes are clearly visible.

The DNA extracted from this pellet yields ambiguous results. The short segments seen in molecules, such as the one in Fig. 19, could be caused by contaminants obscuring the DNA. Two things argue against this.

First of all, one would expect contaminants to obscure the molecules at random intervals, rather than regular intervals. Secondly, short segments were observed in isolation as well. The fact that longer molecules were seen on other grids containing $MgCl_2$ supports the idea that this segmentation is an artefact, although the possibility that short pieces may have existed as well in $MgCl_2$ preparations cannot be excluded.

In the osmotic shock preparation, the two twisted molecules indicate that circularity might exist in vivo; and the fact that these molecules were obscured by the ghost and the fact that the microscope was used under the same conditions as used with hamster mDNA and, therefore, be 20% too short, indicate that the twisted molecules may be longer than 5 μm . Since most of the DNA in the vicinity of the ghosts was too tangled to identify separate molecules, most of the ones that could be measured were short and easily identified. The identification of long open circles would have been impossible under these conditions. These measurements, therefore, tend to select for short pieces and easily identified twisted molecules. There appears to be more DNA in hydra osmotically shocked mitochondria than in BHK mitochondria (Fig. 10) but this is a qualitative observation that could not be quantitated.

The origin of the short pieces is not known. They could be a unit genome, the longer ones being multimers. They could be caused by nuclease nicking during treatment with deoxyribonuclease if the membranes were permeable to the enzyme. There might also be an endogenous nuclease. If the short pieces were caused in this way one would expect random nicking. More work would be required to be certain, but from the work done it is concluded that hydra mitochondria contain DNA and

the molecules could be slightly longer than 5 μm and are probably circular in vivo. The implication of the 1.2 μm segments (or multiples) remains unexplained.

If correct, this result has interesting implications. If the mDNA of the hydra is longer than 5 μm , it might represent an intermediate length of molecule between protists and higher metazoans. This is consistent with the theory that the 5 μm genome arose from a longer molecule and makes the establishment of the 5 μm length very early in evolution unlikely.

Summary

Proteinase K allows circular DNA to be isolated after deoxyribonuclease treatment of mitochondrial fractions.

Turtle mDNA has a buoyant density of $1.704 \text{ gm cm}^{-3} \pm 2 \text{ gm cm}^{-3}$, is circular and similar in size to mDNA of rat and hamster. The buoyant density of turtle nDNA is about the same as that of turtle mDNA.

Mitochondria from hydra were isolated and it was demonstrated that these organelles contain DNA.

The mDNA of the hydra might be circular in vivo and seems to have a length of 5 μm or slightly more.

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