INTRAMITOCHONDRIAL FIBERS WITH DNA CHARACTERISTICS

II. Enzymatic and Other Hydrolytic Treatments

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ABSTRACT

The effects of proteolytic enzymes, ribonuclease, and deoxyribonuclease upon a fibrous component of chick embryo mitochondria, which was previously shown to have many fixation and staining properties characteristic of the bacterial nucleoplasm, are reported. Pepsin digestion of formaldehyde-fixed tissues removed the membranes and matrices of mitochondria, but a pepsin-resistant fibrous material remained which was heavily stained by uranyl and lead ions. Experiments on a DNA "model system" showed that DNA treated with osmium tetroxide can be depolymerized by deoxyribonuclease. Zinc ions strongly inhibited the depolymerization of DNA. Digestion of osmium tetroxide–fixed tissues (fixed only briefly) with deoxyribonuclease for 1 hour greatly reduced the Feulgen staining of the nuclei, and after 4 hours the Feulgen reaction was completely abolished. The reduction and the disappearance of the Feulgen reaction in nuclei was paralleled by partial to complete digestion of the mitochondrial fibers in the regions studied (after 1 and 4 hours, respectively), without any other obvious changes in cellular structures. When deoxyribonuclease was inhibited by the addition of zinc ions, the nuclear Feulgen reaction was not diminished, nor were the mitochondrial fibers removed. Buffer control incubations for deoxyribonuclease and ribonuclease did not alter the structure or staining properties of the mitochondrial fibers, nor did incubation with ribonuclease. The latter reaction digested the cytoplasmic and nucleolar ribosomes after a 4-hour incubation period, in parallel with the abolishment of toluidine blue staining. The results contribute further evidence that these mitochondria contain deoxyribonucleic acid.

INTRODUCTION

In the preceding communication (49) a fibrous component was demonstrated in the matrix of chick embryo mitochondria, which has electron-staining properties consistent with the interpretation that these fibers contain nucleic acid. The markedly similar fixation and stabilization properties of these mitochondrial fibers and the described bacterial nucleoplasm strongly suggested that the fibers contain DNA, and demanded that more direct evidence be obtained to establish the chemical identity of the fibers.

The well established cytochemical techniques for the detection and removal of nucleic acids from tissues were employed or were adapted for studies with the electron microscope. The only interpretation that appears to encompass *all* of the

accumulated evidence presented in this and the previous paper (49) is that these mitochondrial structures are composed mainly, if not purely, of DNA, with properties more closely resembling those of the bacterial nucleoplasm than the chromosomes of nucleated cells.

MATERIALS AND METHODS

The materials used and the routine preparation of tissues for electron microscopy were the same as those described in the preceding report (49). The fixatives employed were numbers 1 (~0.34 M, pH 8.0), 2, 4, 6, 7, and 11 of Table I in that communication.

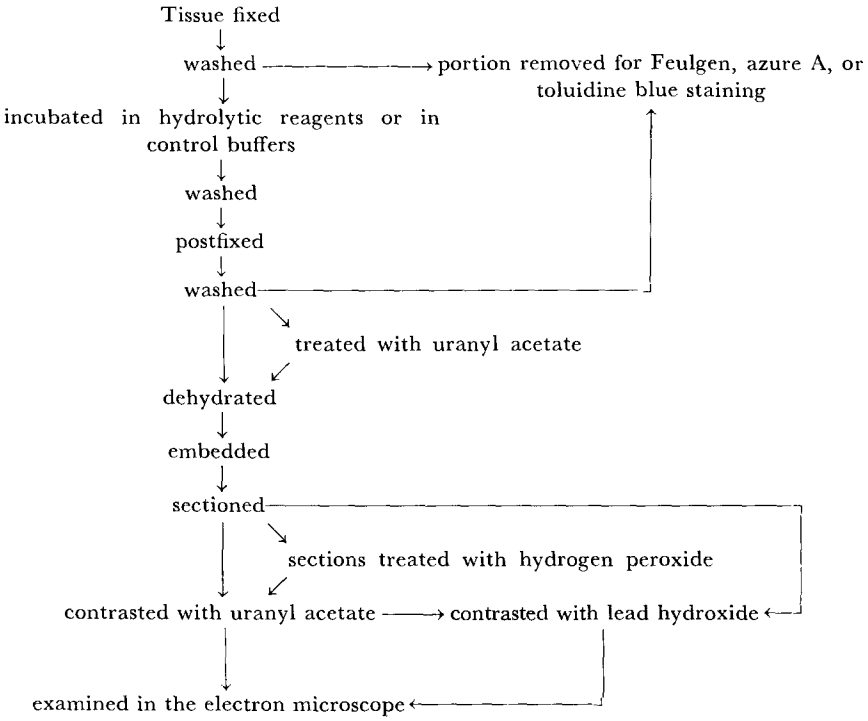
Hydrolytic Treatments

A general flow diagram indicating the major steps of the treatments described below is illustrated in Table I.

The following reagents and conditions were used:—

1. 10 per cent perchloric acid for 18 hours at 5°C.
2. 5 per cent perchloric acid for 15 to 60 minutes at 60°C and 90°C.
3. 2 mg/ml pepsin (3 × crystallized, Sigma Chemical Co., St. Louis, Missouri), in 0.02 N HCl or 0.02 N HCl containing 7.5 per cent sucrose. Incubations for 5 minutes to 24 hours, at 37°C.
4. (*a*) 1 mg/ml crystalline deoxyribonuclease (Sigma), in 0.05 M acetate-Veronal buffer containing 0.003 M $MgSO_4$, at pH 6.4. Incubations for 1 to 8 hours, at 37°C.
   (*b*) the same, with the addition of 0.01 M $ZnSO_4$.
5. 1 mg/ml ribonuclease (5 × crystallized, Sigma), in 0.05 M acetate-Veronal buffer, pH 6.4. Incubations for 1 to 8 hours, at 37°C.

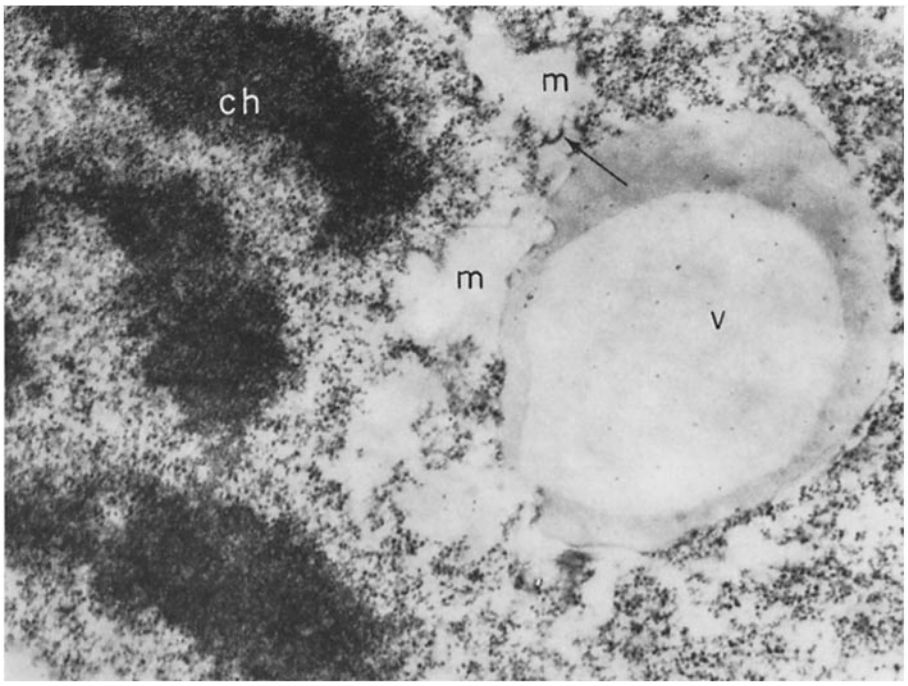
TABLE I



Control tissues were incubated in media lacking the respective enzymes but under otherwise identical conditions. In the perchloric acid experiments, the control tissues were incubated in distilled water.

Experiments with DNA Solutions

10 ml portions of 5 mg/ml deoxyribonucleic acid (sperm, Nutritional Biochemicals Corporation, Cleveland), dissolved in 0.05 M acetate-Veronal buffer (pH 6.4) containing 0.003 M $MgSO_4$, were mixed with 0.2 ml and 1.0 ml of 2 per cent OsO_4 (See Table I fixative 1, reference 49), and dialyzed against several changes of buffer for 18 hours at 5°C. The DNA solutions were warmed to 37°C and then



Explanation of Figures

arrows, mitochondrial electron-transparent areas and/or mitochondrial fibers
ch, chromosomes
er, endoplasmic reticulum
g, space of oxidized mitochondrial electron-opaque granule
go, Golgi vesicles
m, mitochondrion
nu, nucleolus
v, cytoplasmic vesicle

All electron micrographs depict the 19-hour chick embryo Hensen's node or primitive streak outer ectoderm.

FIGURE 1 Embryo was fixed with formaldehyde for 24 hours, treated with pepsin for 30 minutes, post-fixed with osmium tetroxide for 1 hour, and sections were stained with lead hydroxide. The mitochondria and membranes of the endoplasmic reticulum are completely dissolved. The digested mitochondrial areas adjacent to the large partly digested vesicle contain heavily staining fibers. The fibers are not visible elsewhere. Anaphase chromosomes are apparently unaffected by this treatment. × 38,000.

incubated with deoxyribonuclease (0.01 mg/ml final concentration) from 0 to 120 minutes. Additional DNA samples were preincubated for 30 minutes with 0.2 ml and 1.0 ml of 2 per cent OsO_4 or with 0.1 ml of 0.1 M $ZnSO_4$ and the assay performed as above. At various time intervals, 1 ml aliquots were added to 3 ml of cold 10 per cent perchloric acid and examined for the presence or absence of precipitates. The samples were also centrifuged and the supernatants read in a Beckmann spectrophotometer at wavelength 260 mμ. The procedure is based on that described by Kunitz (35).

Experimental Treatments of Tissues

1. PERCHLORIC ACID: Embryos were fixed with 2 per cent osmium tetroxide for 30 minutes at 0°C, washed for 1 hour in several changes of buffer, and extracted for 18 hours with 10 per cent perchloric acid at 5°C or extracted for 15 to 60 minutes with 5 per cent perchloric acid at 60°C and 90°C. Tissues were subsequently rinsed in uranyl acetate for 2 hours, dehydrated, and embedded in the standard manner.

2. PEPSIN: (*a*) Osmium tetroxide–fixed tissues were processed as in condition 1 above, but a 3- or 24-hour pepsin treatment replaced or followed the cold perchloric acid extraction.

(*b*) Tissues fixed with formaldehyde for 1 or 24 hours were washed in several changes of 7.5 per cent sucrose for 30 minutes, then incubated in sucrose-pepsin medium from 5 to 30 minutes. Postfixation was performed, after brief washing in pH 8.0 sucrose

buffer, with 1 per cent osmium tetroxide (See Table I, fixative 4, reference 49), at pH 8.0, for 1 hour.

3. DEOXYRIBONUCLEASE (DNASE): Chick embryos were fixed with 2 per cent osmium tetroxide (49) at 0°C for 4 minutes (fixed on the egg) or for 15 minutes. The tissues were excised and then washed in at least 5 changes of buffer, pH 8.0, 0°C, for at least 1 hour, rinsed in DNase buffer solution (minus the enzyme) at room temperature for 10 minutes, and subsequently incubated in the DNase medium for 1, 4, and 8 hours, at 37°C. In some cases, 0.01 M $ZnSO_4$ was added to the incubation medium. The specimens were then washed for 5 minutes in the buffer, postfixed in 2 per cent OsO_4 for 30 minutes at room temperature, washed for 30 minutes, dehydrated and embedded in the usual manner. In a few experiments, the tissue blocks were exposed to 0.5 per cent uranyl acetate (33, 49) prior to dehydration; sections from these tissue blocks were not treated with hydrogen peroxide before staining.

Tissue sections were treated for 30 minutes with 2 per cent hydrogen peroxide and stained with uranyl acetate followed by lead hydroxide, a method shown to increase markedly the contrast of the structures under investigation (42). Micrographs were taken of the outer ectoderm layer of the primitive streak, where penetration of reagents was most complete.

4. RIBONUCLEASE (RNASE): The procedures, except for the enzyme solution and buffer, were identical with those described for the DNase experiments.

Feulgen Reaction

Chick blastoderms were fixed for 4 minutes with 2 per cent OsO_4 and washed in buffer for 10 minutes, or portions of the same embryos used for electron microscopic examination were studied after the hydrolytic and buffer treatments. The cells were partially separated by teasing on a glass slide and then spread with the aid of a coverslip. The samples were hydrolyzed in 1 N HCl for 6 or 12 minutes and transferred to the Feulgen solution for 1 hour or to a solution of azure A (14) for 6 to 10 hours. They were then rinsed, dehydrated, and mounted.

TABLE II

Effect of OsO_4 on the Depolymerization of DNA by DNase

+++++, maximum precipitation in perchloric acid; −, no precipitation in perchloric acid.

	Minutes of incubation at 37°C				
	0	15	30	60	120
DNA solution* + buffer (1.0 ml), dialyzed, + DNase‡	+++++	±	−	−	−
DNA solution + OsO_4 (0.2 ml, 2 per cent), dialyzed, + DNase	+++++	±	−	−	−
DNA solution + OsO_4 (1.0 ml, 2 per cent), dialyzed, + DNase	+++++	±	−	−	−
DNA solution + OsO_4 (0.2 ml, 2 per cent) + DNase	+++++	+	±	−	−
DNA solution + OsO_4 (1.0 ml, 2 per cent) + DNase	+++++	++++	++++	++++	++++
DNA solution + $ZnSO_4$ (0.1 ml, 0.1 M) + DNase	+++++	++++	++++	+++	++
DNA solution + buffer (1.0 ml)	+++++	+++++	+++++	+++++	+++++

* 10 ml of 5 mg/ml DNA in 0.05 M acetate-Veronal buffer, pH 6.4, containing 0.003 M $MgSO_4$.
‡ 0.1 ml of 1 mg/ml DNase in buffered medium, as for DNA.

RESULTS

Pepsin

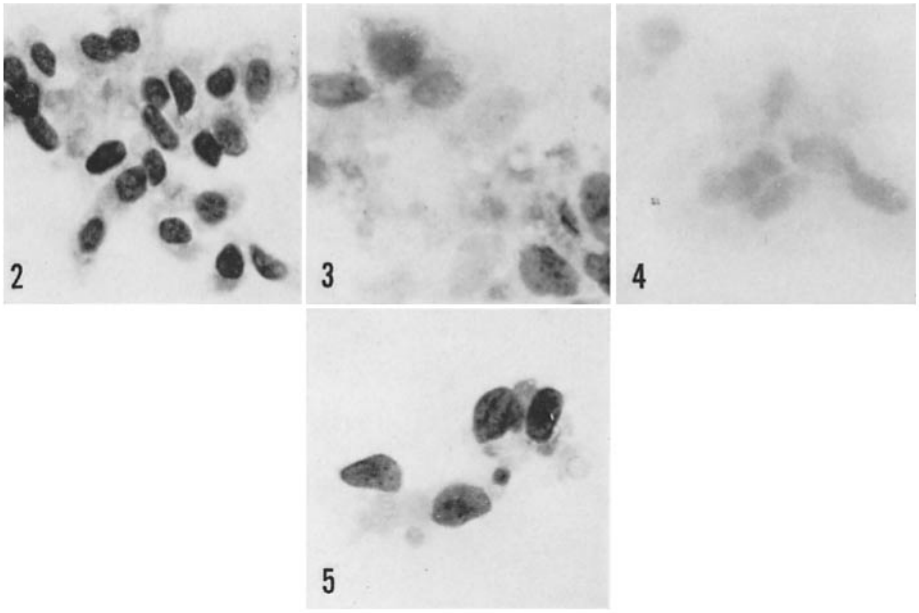
When tissues were fixed for 30 minutes or 4 minutes in osmium tetroxide and washed for 1 hour, incubation in pepsin solutions up to 24 hours did not result in the dispersion of cells nor the digestion of cell structures from the tissues.

Embryos fixed with formaldehyde for 1 hour, however, started to disintegrate after a 5-minute pepsin hydrolysis, and the cells of tissues fixed for 24 hours became very fragile after about 20 to 25 minutes of pepsin treatment. Fig. 1 shows a section of a tissue that was fixed for 24 hours with formaldehyde and treated with pepsin for 30 minutes. Mitochondria of this tissue are frequently found associated with certain large cytoplasmic vesicles

(*e.g.* Fig. 9 in reference 49 and reference 48), and therefore the areas where mitochondria were originally situated were readily recognized. The mitochondrial membranes and matrices are completely digested (Fig. 1), but a densely staining material is observable in these otherwise structureless areas. This densely staining material is not apparent elsewhere in the cytoplasm. The control tissues, incubated in dilute hydrochloric acid–sucrose solutions, were considerably disrupted, but mitochondria were discernible.

Effect of Osmium Tetroxide on the Depolymerization of DNA by DNase

Experiments were performed to test whether OsO_4, at concentrations which are sufficient to fix (blacken) most tissues, would inhibit the action of DNase upon its substrate. It may be observed from Table II that, at the enzyme concentration used in these experiments, the digestion of 50 mg of DNA was almost complete within 15 minutes, as judged by the absence of precipitation by 10



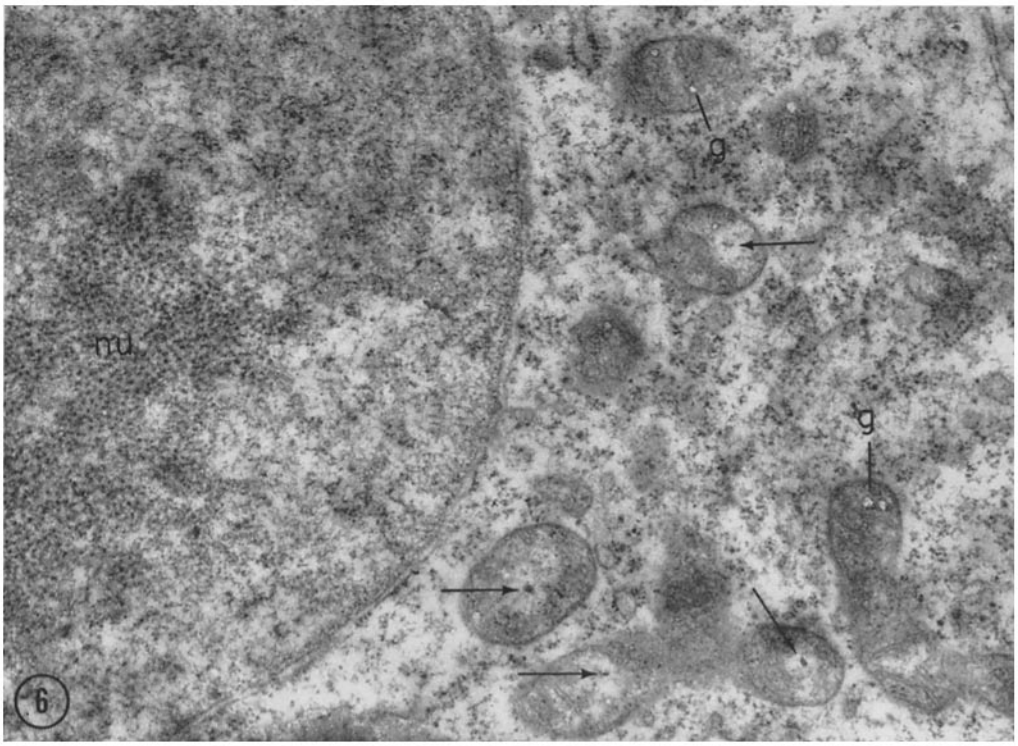
FIGURES 2 THROUGH 5 Feulgen staining of chick embryo cells. Fig. 2, control cells incubated in buffer (minus DNase) for 4 hours; Fig. 3, incubated in DNase for 1 hour; Fig. 4, incubated in DNase for 4 hours; Fig. 5, incubated in DNase + inhibitor ($ZnSO_4$) for 4 hours. × 950.

Perchloric Acid

The first indication that the extractive procedures could be used on osmium tetroxide–fixed tissue blocks was that treatment with cold 10 per cent perchloric acid for 18 hours appeared to extract most of the ribosomes, while the mitochondrial fibers remained unextracted and showed increased contrast after electron staining with uranyl acetate as compared with mitochondrial fibers from control tissues.

After extraction of osmium tetroxide–fixed tissues for 15 to 60 minutes with hot perchloric acid, however, the cellular components were too badly disrupted to allow reliable interpretation.

per cent perchloric acid and verified by spectrotrophotometry of the supernatants after centrifugation. To simulate tissue fixation, 0.2 ml or 1.0 ml of 2 per cent OsO_4 was added to 10 ml of DNA solution and the solution was then dialyzed. There was no inhibition of the subsequent action of the enzyme. Even when 0.2 ml of OsO_4 was added directly to the incubation medium, the enzymatic activity was only slightly inhibited. An approximately equimolar concentration of $ZnSO_4$ caused a pronounced inhibition of DNase activity, confirming a previous report (23). The observed differences between the effects of 1.0 ml of 2 per cent osmium tetroxide (added to DNA and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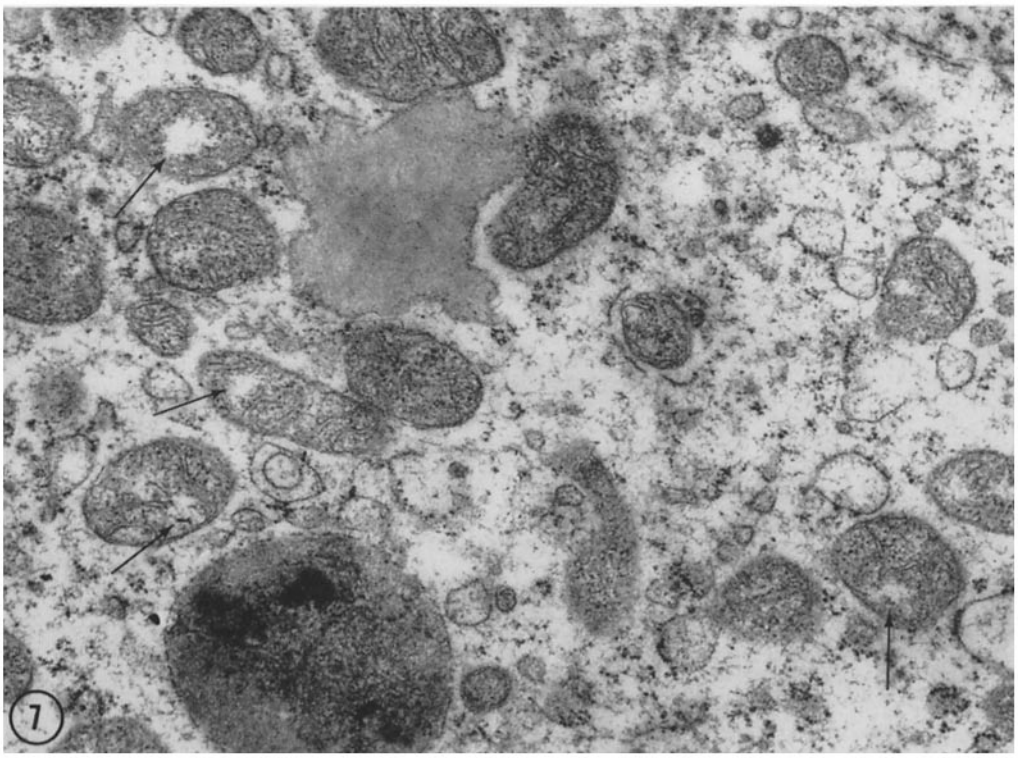
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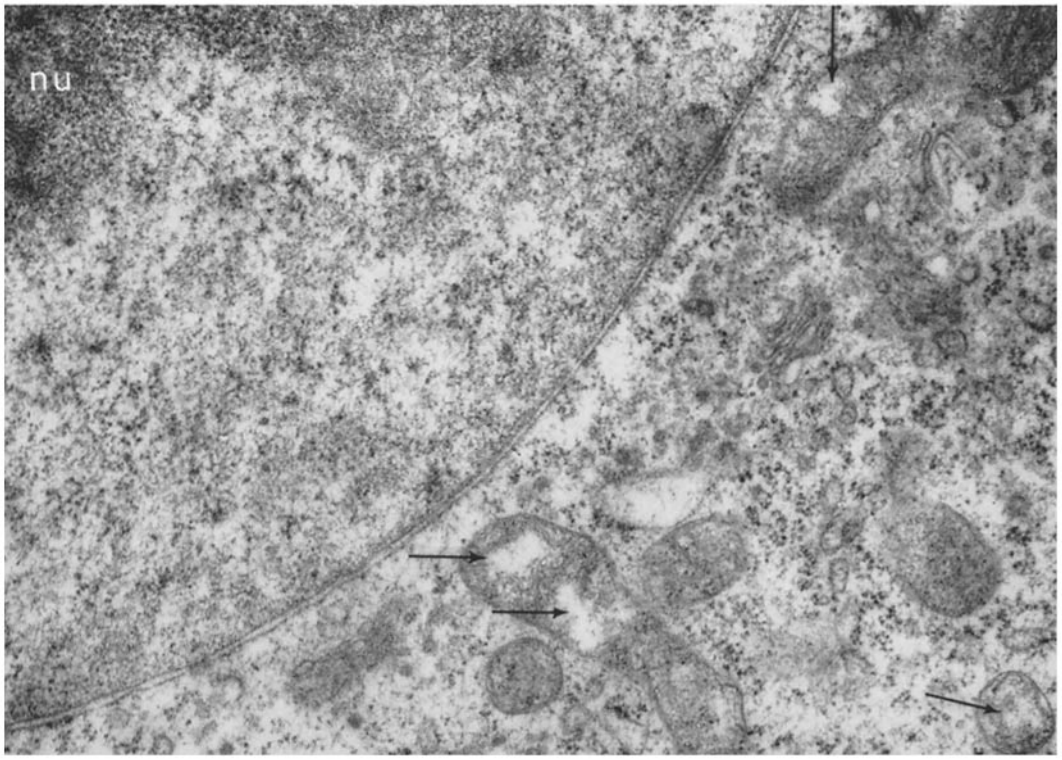


FIGURE 8 Portion of same embryo as in Fig. 6. Outer ectoderm incubated with DNase for 4 hours; subsequent treatments as in Fig. 6. The mitochondrial fibers are not visible in the areas of low density after this treatment. × 35,000.

the solution then dialyzed *versus* direct addition to the incubation tubes) is obviously a result of the action of OsO_4 upon the enzyme and not on the substrate. The results demonstrate that DNA, treated with OsO_4, can be depolymerized by DNase. This fact has been exploited in the cytochemical studies described below.

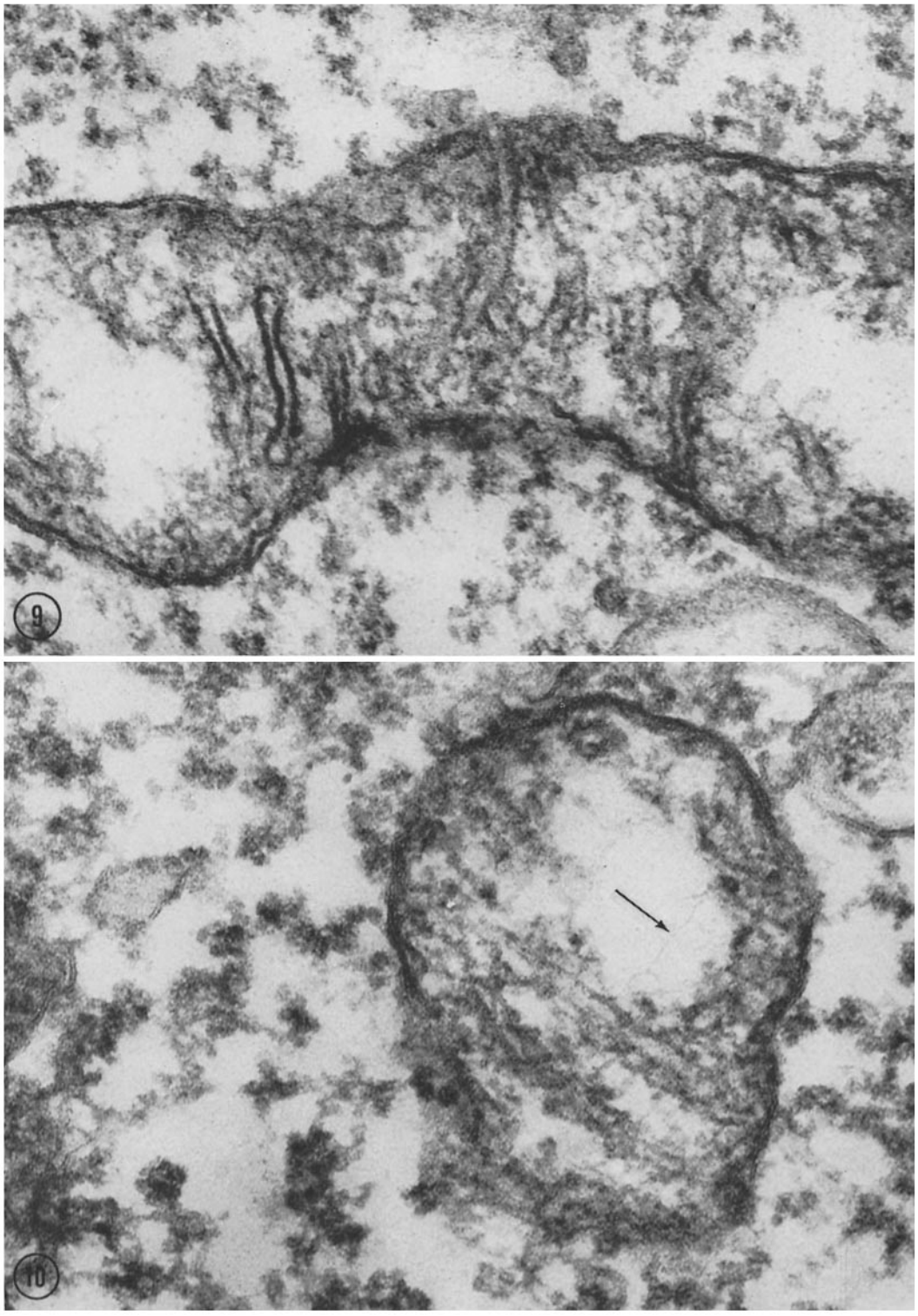
Nucleases

The most successful removal of nucleic acid–containing structures was observed after brief (4 to 15 minutes) fixation of the tissues in buffered osmium tetroxide, followed by extensive washing prior to enzymatic treatment.

Figs. 6 to 13 represent electron micrographs of tissues digested with deoxyribonuclease or ribonuclease and of tissues treated with DNase + inhibitor or of control embryos incubated in the respective buffers. After postfixation with OsO_4, the tissues (in Figs. 6 to 8, 12, and 13) were not rinsed in uranyl acetate so that the mitochondrial fibers, when present, would appear clumped

FIGURE 6 Control for DNase experiment. Portion of primitive streak ectoderm. Embryo was fixed for 4 minutes with osmium tetroxide, washed, incubated in buffer (containing Mg^{++}), postfixed with osmium tetroxide, and sections were treated with hydrogen peroxide, uranyl acetate, and lead hydroxide. Mitochondrial fibers are clearly apparent. Mitochondrial electron-opaque granules have been bleached out. × 35,000.

FIGURE 7 Primitive streak ectoderm (a few cell layers from the dorsal surface of the embryo), incubated with DNase for 1 hour; fixed and stained as in Fig. 6. A few mitochondria in this cell layer show fibers of variable staining intensity after this treatment; in most of the mitochondria no fibers can be seen. × 35,000.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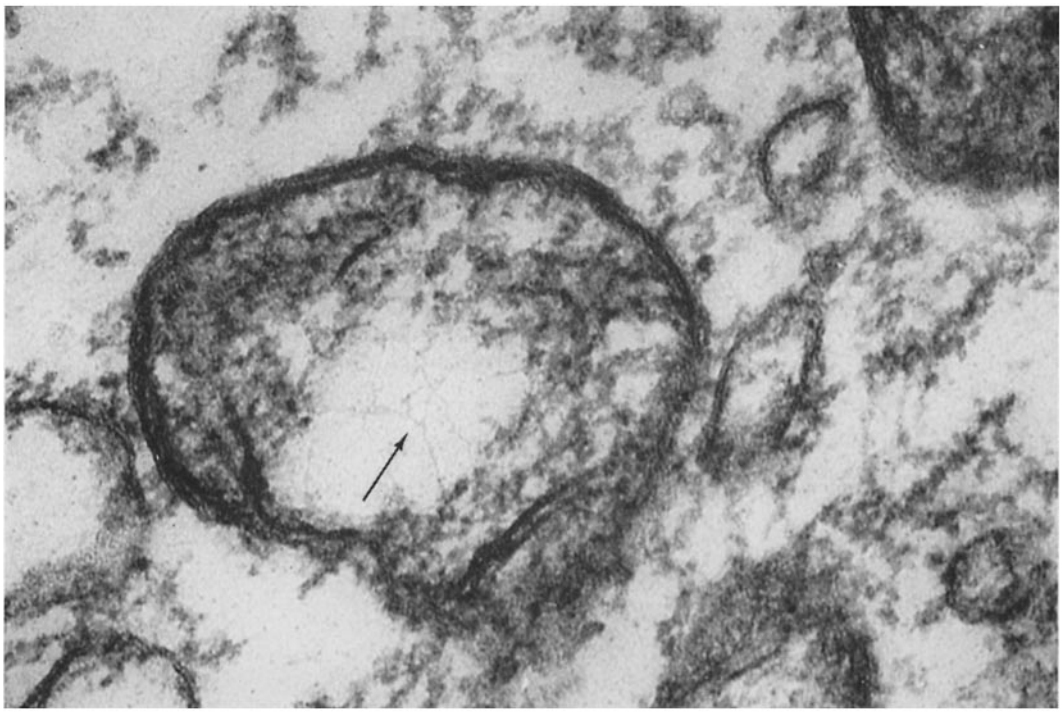


FIGURE 11 RNase treatment for 4 hours. Other conditions as in Fig. 9. Mitochondrial fibrils are visible; no typical ribosomes are apparent. × 150,000.

instead of dispersed and therefore more readily visible. Furthermore, these sections were treated with hydrogen peroxide before staining with uranyl acetate and lead hydroxide in order to increase the contrast of the fibers to the greatest possible extent and to remove the mitochondrial dense granules, which might otherwise interfere with the interpretation of the results.

All the electron microscopic evidence is based on observations of the first 2 or 3 ectodermal cell layers (rather than the deeper lying ectodermal and mesodermal cells), where penetration of enzymes was optimal and the results therefore consistently reproducible. Most of the micrographs are presented at moderate magnifications (× 35,000, at which the mitochondrial fibers, when present, may still readily be seen in their clumped form), in order to observe the effects of various treatments upon a representative number of mitochondria, as well as to demonstrate the reasonably good preservation of structures not specifically digested by the enzyme employed.

1. CONTROL INCUBATIONS FOR DNASE EXPERIMENTS: There were no marked changes in the intensity of the Feulgen reaction in the nuclei after incubation in buffer for 1 or 4 hours (Fig. 2). (Incubation for 8 hours had some disruptive effect on the cells and caused a diminution of Feulgen staining.) In a few unincubated and incubated control tissues, a faintly detectable positive reaction in the cytoplasm was observed after azure A staining, but it was not possible to

FIGURE 9 DNase treatment for 4 hours. Tissue fixed as in Fig. 6, but rinsed in uranyl acetate following the postfixation; sections stained with lead hydroxide. Typical "empty" appearance of mitochondria in the outer ectoderm region. Ribosomes are visible and well stained. × 150,000.

FIGURE 10 Treatment with DNase + inhibitor ($ZnSO_4$) for 4 hours. Other conditions as in Fig. 9. Mitochondrial fibrils are apparent. × 150,000.

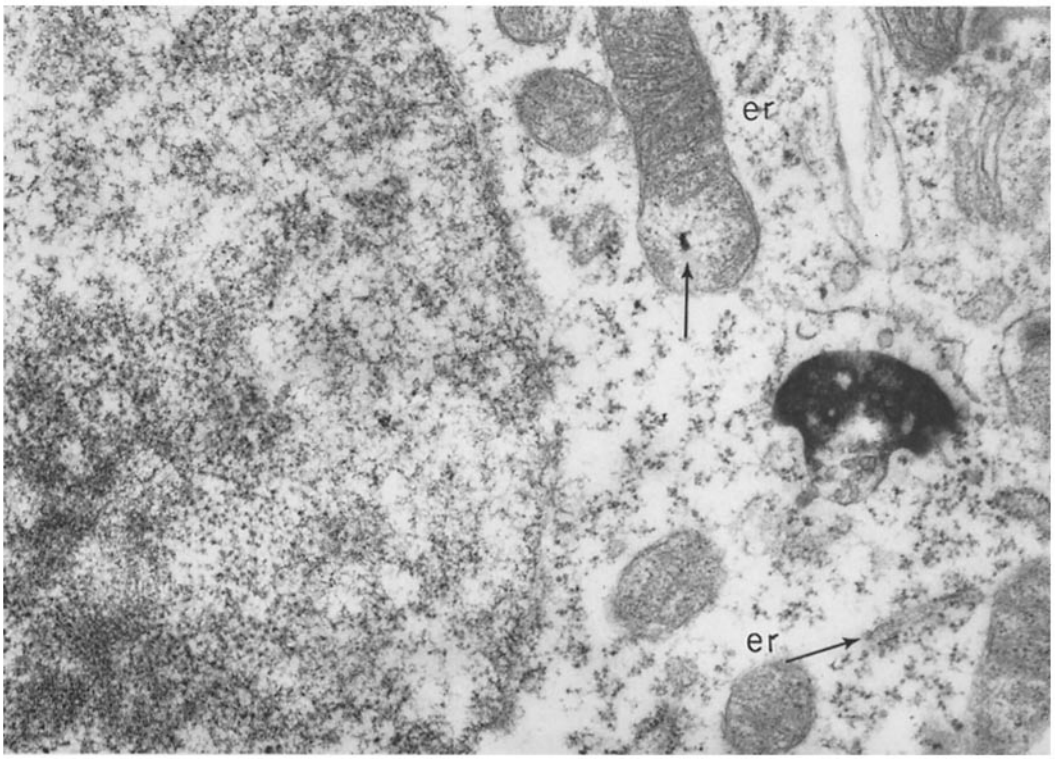


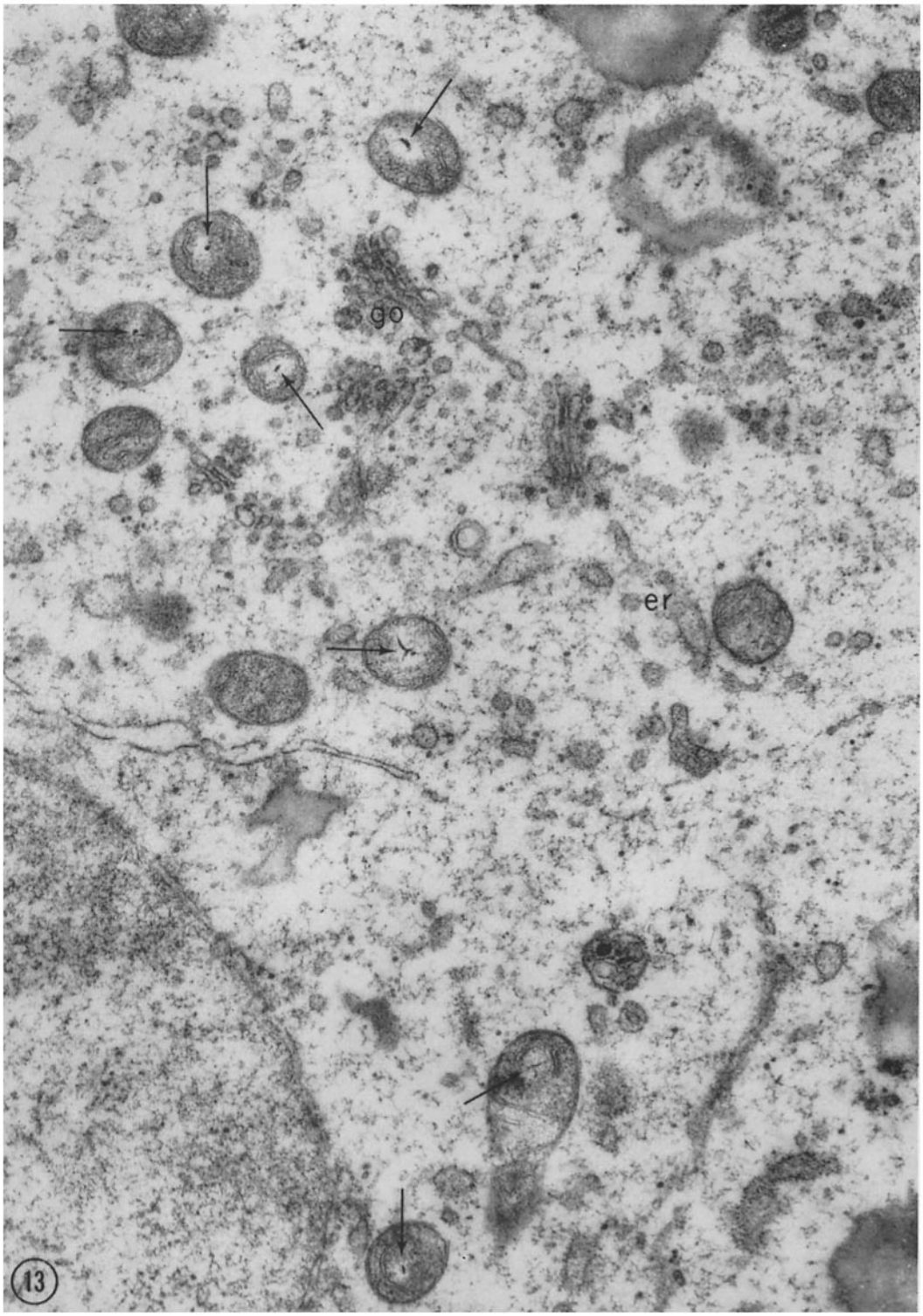
FIGURE 12 Control for RNase experiment. Fixation and staining procedures the same as in Fig. 6. Some swelling of the tissue is observed after incubation in (RNase) buffer. Mitochondria and nuclei are well preserved, and mitochondrial fibers are heavily stained. Ribosomes are visible in the cytoplasm. × 35,000.

identify the structures which were at the limit of resolution of the light microscope.

The electron micrograph of a 4-hour (DNase) control tissue (Fig. 6) shows that the cell membranes are partly disrupted, which is also characteristic of unincubated osmium tetroxide–fixed tissues (49). Other cellular constituents are well preserved, and the mitochondrial fibers, nucleoli, and cytoplasmic ribosomes have the characteristic staining patterns of unincubated tissues.

2. DNASE INCUBATION FOR 1 HOUR: Incubation with DNase for 1 hour decreased the Feulgen reaction (and azure A staining) of many nuclei to various degrees and completely abolished the reaction in some nuclei (Fig. 3). In the cells of the first ectodermal cell layer (of the same embryo used for Feulgen staining) the mitochondria had no detectable fibers, while cells lying slightly farther from the surface of the embryo showed variably stained mitochondrial fibers (Fig. 7). Most of the mitochondrial fibers of the inner ectoderm and mesoderm appeared well stained. Ribosomes and nucleoli, presumably RNA-containing, are heavily contrasted. In these cells, with their diffuse chromatinic regions, as judged by both Feulgen and electron staining methods, it was not possible to detect by electron microscopy the removal of the nuclear chromatin. The difficulties in detecting DNA-containing structures in control nuclei, of course, makes it impossible to detect

FIGURE 13 Embryo tissue treated with RNase. Ribosomes are no longer apparent after this treatment, and the staining reactions are abolished. The mitochondria and their densely staining fibers are clearly apparent. × 35,000.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their digestion after DNase treatment, despite the positive indication of the enzyme's action, as judged by removal of Feulgen staining.

3. DNase incubation for 4 hours: A 4-hour incubation of tissues in DNase solutions completely abolished the Feulgen reaction (Fig. 4), except for a few nuclei, presumably from the inner portions of the tissue, which stained a very faint pink. Figs. 8 and 9 represent electron micrographs of tissues so treated. No densely staining fibers are visible in mitochondria of the outer ectoderm, although it is apparent that many of the mitochondria have been sectioned through their electron-transparent areas. Higher magnification micrographs showed that the absence of the densely staining, clumped mitochondrial fibers in the outer ectoderm was caused by the digestive activity of the DNase and not related to a possible dispersion of the fibers into finer strands. The remainder of the mitochondria and all other cellular structures are not perceptibly affected by this incubation.

In order to be certain that the removal of Feulgen staining by DNase and the removal of the mitochondrial fibers were specifically related to the action of this enzyme upon DNA, incubations were performed with DNase in the presence of 0.01 M $ZnSO_4$, a known DNase inhibitor (23). The Feulgen reaction was observed to be equivalent to that of the controls (Fig. 5), and the structure of the mitochondrial fibers remained intact (Fig. 10), although some of the DNase + Zn^{++}-treated fibers were not so well stabilized by uranyl acetate as were unincubated fibers.

4. Ribonuclease and control incubations: Ribonuclease control incubations (Fig. 12) had a more disruptive effect upon the cell constituents than did the DNase control experiments (the latter contained magnesium ions in the medium). Nevertheless, the mitochondria remained intact and the fibers stained intensely. Although the cytoplasm in some cells appears less compact than in normal sections, the ribosomes are visible along membranes of the endoplasmic reticulum and "free" in the cytoplasm.

After incubation with ribonuclease for 4 hours, the cytoplasmic basophilia, as determined by staining with toluidine blue, was completely abolished. The ribosomes were no longer apparent (Fig. 13), and the nucleolar ribosomes were also digested. The remnants of both ribosomal types were completely disorganized. In the cytoplasm the membranous structures and a fibrous network, presumably protein in nature, are well preserved. The mitochondrial fibers are unaffected by this enzymatic treatment and show their characteristic staining properties, which readily differentiate them from the cytoplasmic fibrous material.

After these tissues have been treated with uranyl acetate, the mitochondrial fibers show their usual stabilization as 15 to 30 A fibrils (Fig. 11), while the other cytoplasmic fibrous structures are unaffected.

DISCUSSION

The results presented show that the mitochondrial fibers are specifically digested by deoxyribonuclease. Control incubations, ribonuclease, and pepsin hydrolyses did not act upon the structures, although ribonuclease digested the cytoplasmic ribosomes and some nucleolar material, and pepsin digested all of the mitochondrial structure except for the fibers. When DNase was inhibited by zinc ions, the mitochondrial fibers and the nuclear Feulgen reaction were apparent. The only conclusion that appears to be consistent with these results and those reported in the previous communication (49) is that the mitochondrial fibers contain DNA.

The arguments presented in the preceding paper (49), which suggested that osmium tetroxide does not appreciably bind and does not "fix" nucleic acids and hence would not interfere with DNase action, led eventually to the study of the nucleases upon osmium tetroxide–fixed tissues. The studies of DNase activity upon DNA solutions which had been pretreated with osmium tetroxide confirmed the view that OsO_4 would not inhibit the enzymatic action because of its nonfixation of DNA. The digestion of DNA-containing structures after fixation with OsO_4 has also been shown by Jurand on *Paramecium* (32). It is of interest that formaldehyde fixation also results in a clumped (non-stabilized) appearance of mitochondrial fibrils and the bacterial nucleoplasm (*cf.* reference 49) and that DNase has been reported to depolymerize the DNA of chloroplasts after this fixation (61).

Pepsin did not act upon the cells to any recognizable extent after fixation with osmium tetroxide, probably because the fixative binds to protein or lipoprotein substrates of pepsin. Fixation by formaldehyde, on the other hand, renders protein

structures accessible to the action of pepsin, as was previously shown on tissue sections by Leduc and Bernhard (37). The digestion of the ribosomes by ribonuclease is also in agreement with more recent studies by these investigators (38), despite the differences in the techniques used.

The diffuse Feulgen reaction shown by the nuclei of chick embryo cells and the absence of any clearly defined electron-opaque regions other than nucleoli in the nuclei of these cells (after staining) is a further indication of the diffuseness of the chromatinic material. The same procedures greatly increase the electron opacity of chromatinic regions in nuclei of many adult vertebrate tissues (29, 42, 50). It was therefore not unexpected that DNA digestion by DNase, as shown by the loss of Feulgen staining, did not obviously alter the nuclear fine structure. Ris and Plaut (61) and Swift (74) have reported that the action of DNase upon formaldehyde-fixed tissues abolishes the nuclear Feulgen reaction but does not appreciably alter the ultrastructure of the nucleus.

The dimensions of the mitochondrial fibers studied, the very small quantity of fibers per mitochondrial clear area, and the probability that the fibers are in a highly hydrated and therefore diffuse state appear to account for the inability to detect a positive Feulgen reaction. With the use of azure A, which is approximately 3½ to 4 times as sensitive as the Schiff reagent (73), faint cytoplasmic staining at the theoretical limit of resolution of the light microscope was occasionally observed. There have been reports of the presence of large quantities (up to 500 times the amount in the nucleus) of cytoplasmic DNA in the chick embryo as well as in other eggs and embryos (4, 16, 17, 20, 25), but a positive Feulgen reaction generally has not been observed in the cytoplasm (*cf.* reference 76), and, indeed, it is often difficult or imposible to demonstrate a positive Feulgen reaction in some nuclei (6, 70). Swift (72) has stated that one should not conclude on the basis of a negative Feulgen reaction that DNA is absent from a nucleus. It appears most reasonable to conclude that a negative Feulgen reaction is not indicative of the absence of DNA from any structure but that other methods must be devised to detect these macromolecules when they are present in low concentration.

Alternative methods (other than those employed here) for detecting small quantities of DNA include: (*a*) bulk isolation of cellular components; (*b*) physiological alteration of the cell in order to increase the quantities of DNA to levels ordinarily detectable by Feulgen staining or by H^3-thymidine incorporation; and (*c*) incorporation of radioactive precursors using the reasonably specific H^3-thymidine for electron microscopic radioautography of "DNA" (with DNase digestion experiments as verification of the chemical constitution of the labeled material).

Some biochemical analyses upon isolated mitochondrial fractions indicate that certain mitochondria contain up to 12 per cent of the cell's DNA (*e.g.* 27, 39, 44). The difficulties in determining whether this DNA is a component of the mitochondria or is a nuclear contaminant have not permitted a definitive conclusion from these techniques, although the DNA found in mitochondrial fractions is generally assumed to be a contaminant (*cf.* references 27, 52). It may, however, be profitable to determine whether any DNA components in mitochondrial fractions have unique properties or composition before their presence is disregarded as artifact. Evidence for the heterogeneity of DNA's from the same tissue and organism with regard to composition, molecular weight, and incorporation rates of radioactive precursors has been shown by a variety of methods (3, 9, 21, 71). Durand (16) has presented evidence that the cytoplasmic and nuclear DNA fractions of insect eggs and embryos have different properties and base ratios. In most of these studies, however, attempts have not yet been made to determine whether the reported heterogeneity may be partly accounted for by non-nuclear DNA (See Note Added in Proof).

An alternative method of demonstrating the presence of normally low concentrations of DNA is to alter the cell physiologically so that it builds up DNA to detectable concentration. This has apparently been achieved by Chèvremont *et al.* (10). Incubation of living chick fibroblasts in acid DNase resulted in the appearance of Feulgen-positive cytoplasmic structures, which also incorporated tritiated thymidine, and which were interpreted to be mitochondria.

Tritiated thymidine incorporation into mitochondria of HeLa cells has also been shown by Meek and Moses (46), and these cells contain mitochondrial fibers with structural and staining properties similar to those found in the chick embryo (50). The interpretation that Meek and Moses prefer to place upon the incorporation is

that the thymidine may be bound to the mitochondria as a DNA precursor. Selective digestion experiments with nucleases might help in reaching a definitive conclusion.

Table III has been compiled in order to show that the DNase digestion of the fibrous structures with characteristics similar to those described in this and the previous communication (49, and *cf.* reference 60) is a sensitive and discriminative cytochemical method for detecting small quantities of DNA *in situ*. It is apparent from the Table that there is a direct positive correlation between the presence of clumped or dispersed (~25 A) fibrils, which are digestible by DNase, and the presence of DNA as shown by other cytochemical tests. The evidence presented by Ris and Plaut (61) and by us indicates that the electron microscopic cytochemical method for detecting bacterial-type DNA (lacking histone?) is much more sensitive than the Feulgen procedure and much more precise in localizing the DNA material than any other available method.

The compilation presented in Table III does not include all the pertinent evidence available. Despite occasional alternative interpretations of some of the data, we consider that the summary presented speaks very strongly in favor of the view that all of the listed cytoplasmic particles, which have been presumed, suggested, or "demonstrated" to be "self-duplicating," contain DNA. [Although the typical fibers have not as yet been demonstrated in kinetosomes (centrioles and basal bodies), there is abundant evidence that these structures also fit the above generalization (56, 65, *cf.* also references 40 and 45).]

As reasonable as these correlations may appear to be, other interpretations for the role of mitochondrial DNA must also be considered. Chèvremont *et al.* (10) have postulated that DNA is manufactured by the mitochondria and transported to the nucleus. Such transport could fit the scheme proposed by Moore (47), that nuclei incorporate cytoplasmic DNA into chromosomes during early development. The evidence that DNA-polymerase is localized mainly in the cytoplasm (5) may also be interpreted in favor of the hypothesis of Chèvremont *et al.* (10). Mitochondrial-nuclear contacts, which have been demonstrated *in vivo* and by electron microscope studies (*e.g.* 1, 7, 22), have been thought to indicate transport of DNA (10).

TABLE III

Summary of Some Properties of Cytoplasmic DNA-Containing Structures

+, positive results; D, removed by DNase; 0, evidence unknown to the authors.

	Presence of fibers or bar-like structures with characteristics of the bacterial nucleoplasm§	Feulgen reaction or related cytochemical method	H^3-thymidine incorporation	Biochemical analyses of DNA	Cytological and genetical studies suggesting "self-duplication"
Mitochondria	+ (48–50)‡ D (this paper)	+ (10)*	+ (10, 46)*	+ (27, 39, 44)*	+ (8, *cf.* 18; 19, 41, 59, *cf.* 77)
Chloroplasts	+ (61) D (61)	+ (11, 61) D (61)	+ (70)	+ (30, 12)	+ (13, *cf.* 18, *cf.* 36, 58)
Kinetonucleus (Kinetoplast)	+ (54, 68)	+ (63, 69) D (69)	+ (69) D (69)	0	+ (53)
Kappa particles of *Paramecium*	+ (15, 24, 60)	+ (55)	0	+ (66)	+ (*cf.* 67)

* See text for discussion of these results.

‡ Numbers in parentheses refer to references in Bibliography.

§ Similar fibers have also been demonstrated in the nucleoplasm of blue-green algae (28, 62).

Further, some electron microscopists have hypothesized the formation of mitochondria from the nuclear membrane (7, 26). Although nuclear-mitochondrial contacts may certainly be interpreted in other ways, as noted in the preceding paragraph, the generation of mitochondria from the nucleus would appear to be the simplest mechanism to account for nuclear control of mitochondrial activity. At present, however, there is little supporting evidence available for this view.

A number of important problems must be solved before any definitive understanding of the function of the mitochondrial DNA is possible. Among these we might list (*a*) whether such fibers are a regular feature of most or all mitochondria, (*b*) whether there are quantitative variations in the fibers that are related to cell division, differentiation, or neoplastic transformations,[1] (*c*) whether there are recognizable differences between cytoplasmic and nuclear DNA's with regard to composition or rate of precursor uptake, (*d*) what the significance is of the frequently observed nuclear-mitochondrial associations (1, 7, 22, 51) which appear to be more frequent during early developmental stages than later (51), (*e*) what relationship mitochondrial DNA may have to mitochondrial protein synthesis (64, 75), and (*f*) what the relation is between mitochondrial DNA and the reported presence in mitochondria of alkaline DNase (2) as well as some of the enzymes involved in nucleic acid synthesis (34, 57).

Experiments in progress indicate that mitochondria from many species contain fibers with a location and staining properties similar to those reported here, *e.g.* mitochondria of various functional organs (heart, liver, brain) of developing chicks, skeletal muscle of the human foetus, eggs and embryos of sea urchins and tunicates, gills of adult clam, ear epidermis of adult rat, and Ehrlich ascites tumor cells of mouse (50).

We have already stressed the similarities between mitochondrial fibers and the bacterial nucleoplasm with regard to their structural appearance and their fixation and stabilization properties. There is a great deal of modern biochemical and ultrastructural evidence that may be interpreted to suggest a phylogenetic relationship between blue-green algae and chloroplasts (60) and bacteria and mitochondria. For example, Marr (43) concluded in his survey of enzyme localization in bacteria: "For those who have sought homologies in the localization of respiratory enzymes in bacterial cells and in cells of higher organisms, the most appropriate choice of a structure homologous with the mitochondrion is the entire bacterial cell. . . ." If these homologies may be extended further, we might ask whether the mitochondrial DNA is involved in cellular enzyme regulatory mechanisms similar to those described to occur in bacteria (31). In any case, the presence of DNA in mitochondria appears to require modification and extension of some generally accepted hypotheses of cell function which consider the nucleus to be the exclusive site of cellular DNA and genetic information.

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Note added in proof: Chemical studies, in progress, indicate that isolated rat liver mitochondrial fractions contain a small quantity of DNA (40 to 60 μg/100 mg protein, in 4 separate experiments). Repeated washing did not decrease the quantity of DNA measured. Experiments have also shown that there was negligible contamination by nuclei and that purified high polymer DNA, when added to a mitochondrial preparation, was not bound to the fraction. The quantity of mitochondrial fibers with DNA characteristics in rat liver tissues, observed in the electron microscope, appears to be much less than that found in embryonic chick tissues (50).

[1] A characteristic structural alteration of the mitochondrial fibers of Ehrlich ascites tumor cells has been observed repeatedly. These observations will be the subject of a future communication.

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