

INTRAMITOCHONDRIAL FIBERS WITH DNA CHARACTERISTICS

I. Fixation and Electron Staining Reactions

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ABSTRACT

Chick embryo mitochondria, studied with the electron microscope, show crista-free areas of low electron opacity. These areas are observable after fixation with osmium tetroxide, calcium permanganate, potassium permanganate, formaldehyde, acrolein, acrolein followed by osmium tetroxide, uranyl acetate followed by calcium permanganate, and acetic acid-alcohol. Staining of sections with lead hydroxide or uranyl acetate, or with both, resulted in an increased density of a fibrous material within these areas. The appearance of the fibrous structures varied with the fixative employed; after fixation with osmium tetroxide the material was clumped and bar-like (up to 400 Å in diameter), whereas after treatment of osmium tetroxide-fixed tissues with uranyl acetate before dehydration the fibrous structures could be visualized as 15 to 30 Å fibrils. Treatment with ethylenediamine-tetraacetate (EDTA) in place of uranyl acetate coarsened the mitochondrial fibrils. After fixation with calcium permanganate or potassium permanganate, or a double fixation by uranyl acetate followed by calcium permanganate, the fibers appeared to have a pattern and ultrastructure similar to that observed after the osmium tetroxide-uranyl acetate technique, except that some of them had a slightly greater diameter (up to 50 Å). Other fixatives did not preserve the fibers so well. The fibers appeared strongly clumped by formaldehyde fixation, and were difficult to identify after fixation with acrolein or acetic acid-alcohol. The staining of nucleic acid-containing structures by uranyl acetate and lead hydroxide was improved by treatment of osmium tetroxide-fixed sections with hydrogen peroxide, and the mitochondrial fibers also had an increased density in the electron beam after this procedure. The staining characteristics suggest the fibrous material of chick embryo mitochondria to be a nucleic acid-containing structure, and its variable appearance after different fixations parallels that previously reported, or described in this paper, for the nucleoplasm of bacteria and blue-green algae. The results, in addition to those described in the accompanying communication, indicate that these mitochondria contain DNA.

INTRODUCTION

Recent progress in preparative techniques has advanced our knowledge of nucleic acid structure and has led to some understanding of the essential requirements for adequate fixation and visualiza-

tion of nucleic acids and nucleic acid-containing structures in electron microscopy (13, 16, 17, 40, 42, 43).

Some of these techniques have been adapted by

us in an attempt to characterize chemically a fibrous structure first observed within chick embryo mitochondria after the use of electron staining reactions (26, 28). The low contrast of the structures after fixation with buffered osmium tetroxide solutions, their increased density after staining with lead salts and uranyl acetate, and their structural similarity to some known DNA-containing regions in other organisms and organelles led us to attempt a more systematic survey of the similarities between the reactions of the mitochondrial fibers and the reported characteristics of purified DNA and bacterial nucleoplasm.

This first communication describes the appearance of the fibrous material in question after a number of "single" and "double" fixations and also the effects of some electron contrasting agents. Attempts have been made to demonstrate that (a) the areas of low density observed in chick embryo mitochondria are a normal component of these mitochondria, (b) the rod-like fibrous structures in these areas have electron-staining characteristics consistent with the view that they contain nucleic acid, and (c) the effects of various fixatives upon the mitochondrial fibrous structures parallel the reported effects of these agents upon the bacterial nucleoplasm and purified DNA. The report that follows deals with enzymatic treatments of fixed tissues.

MATERIALS AND METHODS

FIXATION: Chick embryos were incubated from 0 hours to 8 days at 37°C. The fixation procedure described refers to the 19-hour embryo (definitive streak stage). This stage has been utilized for most of the illustrative material in order to facilitate comparisons between variously treated tissues. After the egg was opened and the overlying egg white removed, the fixative was quickly pipetted upon the live embryo *in situ*. After 5 minutes' fixation the embryo was excised, transferred to a buffered solution (vehicle of the fixative) where adherent yolk and the vitelline membrane were removed within about 30 seconds, and then fixation was continued. Further dissection was performed during the subsequent 30-minute washing period.

The fixatives and conditions of fixation employed are summarized in Table I. The second fixative was applied either directly after the first fixation and washing period or after some intervening chemical treatment (29).

Escherichia coli cells were fixed with fixatives 1

(0.34 M, pH 8.0) or 8, Table I, at room temperature for 1 hour, washed, and subsequently mixed with agar (17).

The specimens were dehydrated in an ethyl alcohol series (30 to 100 per cent) and embedded in Epon 812 (22).

ELECTRON MICROSCOPY: Sections, approximately 60 to 90 m μ , were cut with a Porter-Blum microtome equipped with glass knives, picked up on uncoated or Formvar-coated grids, and examined in a Siemens Elmiskop I, operated at 60 kv, with a condenser aperture of 100 or 200 μ and an objective aperture of 20 or 50 μ . The electron optical magnifications varied between 1,000 and 30,000.

TREATMENT OF SECTIONS: Most sections were stained with alkalized lead acetate (42) for 1 to 2 minutes, lead hydroxide (14) for 15 minutes, uranyl acetate (13) for 3 to 5 hours, or lanthanum nitrate (40) for 15 minutes, singly or in combination.

Some sections fixed with osmium tetroxide or acrolein followed by osmium tetroxide were oxidized with 2 per cent hydrogen peroxide for 30 minutes before staining (24).

RESULTS

A. Osmium Tetroxide

Fixative 1 (Table I), ~0.34 M, pH 8.0, was found to be most satisfactory for osmium tetroxide fixations. The pH of the fixative was a critical variable for these tissues; at and below pH 7.4 there was a partial disruption of the cells, especially of the nuclear contents and the cell membranes.

Fig. 1 is a survey micrograph which illustrates the ectodermal cells of the 19-hour chick embryo in the region of Hensen's node. The "general" fixation quality, except for cell membranes, appears to be good. In this double-treated tissue (osmium tetroxide followed by uranyl acetate), contrasting of the sections with lead hydroxide increases the electron opacity of many structures, including the nucleoli. Unlike the chromatin of most adult nuclei, however, the chromatinic areas of these large embryonic nuclei are not well defined and consequently not well contrasted by these procedures.

It is to be observed that an area (0.1 to 0.3 μ) of very low electron opacity is present within the matrix of most of the long, slender mitochondria (which are about 0.5 μ in diameter and up to 20 μ in length). These "clear" areas remained unaltered in size when the tonicity of the fixative was raised to 0.45 M.

TABLE I

Fixative	Time and temperature	Second fixative	Time and temperature
1. 2 per cent osmium tetroxide in buffered salt solution, 0.34 to 0.45 M (46), pH 7.0 to 8.6	1 hour at 0 to 2°C	—	—
2.* “ “	“ “	0.5 per cent uranyl acetate (17) in buffered salt solution (46), pH 5.3	2 hours at room temperature
3.* “ “	“ “	0.25 M ethylenediamine-tetraacetate (EDTA) (17) in buffered salt solution (46) as in fixative 2	“ “
4. 1 per cent osmium tetroxide in buffered sucrose solution (4), pH 8.0	1½ hours at 0 to 2°C	—	—
5. 4 per cent formaldehyde, 7.5 per cent sucrose, in phosphate buffer (11), pH 8.0	1 hour and 24 hours at 0 to 2°C	—	—
6. “ “	“ “	Fixative 4	1 hour at room temperature or 0 to 2°C
7. 10 per cent acrolein in phosphate buffer (21), pH 8.0	30 minutes at room temperature	—	—
8. “ “	“ “	Fixative 1 (0.34 M, pH 8.0)	2 hours at room temperature
9. “ “	“ “	Uranyl acetate as in fixative 2	2 hours at room temperature
10. 3 per cent potassium permanganate (20) in buffered salt solution (46), pH 8.0, or in water	10 minutes at room temperature	—	—
11. 3 per cent calcium permanganate (1) in buffered salt solution (46), pH 8.0	10 minutes at room temperature	—	—
12. 2 per cent uranyl acetate, in buffered salt solution (46), pH 4.6	1 hour at 0 to 2°C	Fixative 11	10 minutes at room temperature
13. Acetic acid-alcohol, 1:3 (5)	30 minutes at 0 to 2°C	—	—
14. “ “	“ “	Uranyl acetate as in fixative 2	2 hours at room temperature

* In these experiments, each embryo was bisected across the primitive streak; one half was treated with uranyl acetate, and the other half with EDTA. It was previously ascertained that there was no apparent structural variation between mitochondria from different areas of the primitive streak ectoderm.

1. UNSTAINED SECTIONS OF MITOCHONDRIA

Fig. 2 represents a higher magnification of 2 mitochondria from an osmium tetroxide-fixed embryo (printed for maximum contrast). Within

the mitochondrial areas of low density may be observed clumped fibers of low contrast (arrows). The regions of low electron opacity are not bounded by membranes.

2. SECTIONS OF MITOCHONDRIA CONTRASTED WITH HEAVY METAL IONS

Staining with uranyl acetate and/or lead compounds, based on procedures described by Huxley and Zubay (13) (but after a ½-hour or 1-hour fixation period), or with lanthanum nitrate (40) increases the general contrast of most cell structures, but most dramatically enhances the density of the clumped mitochondrial fibers. In comparing Figs. 2 and 3, it becomes apparent that the mitochondrial fibers after staining with uranyl acetate have increased in contrast relative to the mitochondrial membranes and are now of approximately equal density. The fibrous structures are contrasted to a similar extent by treatment of sections with uranyl acetate or lead hydroxide, but sequential staining further increased their contrast.

In any tissue section there is a wide variation in the number of electron-transparent areas per mitochondrion and in the number, size, and shape of the structures stained by the heavy metal compounds. The diameters of the fibers vary between 15 and 400 Å, and the thicker bar-shaped structures are sometimes resolvable into a number of parallel strands. In many sections the thinner fibers appear to extend or attach to the membranes of the mitochondria (26). Mitochondrial fibers have been observed at all developmental stages studied.

3. TREATMENT OF SECTIONS WITH HYDROGEN PEROXIDE

Treatment of osmium tetroxide-fixed tissue sections with hydrogen peroxide increases the staining of nucleic acid-containing structures by heavy metal compounds (24). Sections were

treated with 2 per cent hydrogen peroxide for 30 minutes (24), rinsed, and then contrasted with uranyl acetate and/or lead hydroxide (Fig. 4). Under these conditions, membranes have a low density, and the ribosomes and mitochondrial fibers are the only cytoplasmic structures which are strongly electron-opaque.

The peroxide treatment removed the mitochondrial dense granules, leaving holes in the section, and thus this reagent is also of value in distinguishing between mitochondrial fibers which have been cut in cross-section and the dense granules.

Sectioned *Escherichia coli* fixed in osmium tetroxide as used for chick embryos, are shown unstained in Fig. 5 and after the peroxide treatment and staining in Fig. 6. The nucleoplasm has the typical clumped appearance seen after osmium fixation. After the peroxide and staining procedures the clumped nucleoplasm and the ribosomes are heavily contrasted (Fig. 6), and previously undetected fine strands can be seen radiating from the dense, aggregated portions of the nucleoplasm. It is apparent that both the mitochondrial fibers, described above for chick embryo, and the bacterial nucleoplasm have a high contrast after these procedures, but they are not visibly altered in structure.

4. URANYL ACETATE AND EDTA TREATMENTS OF TISSUES

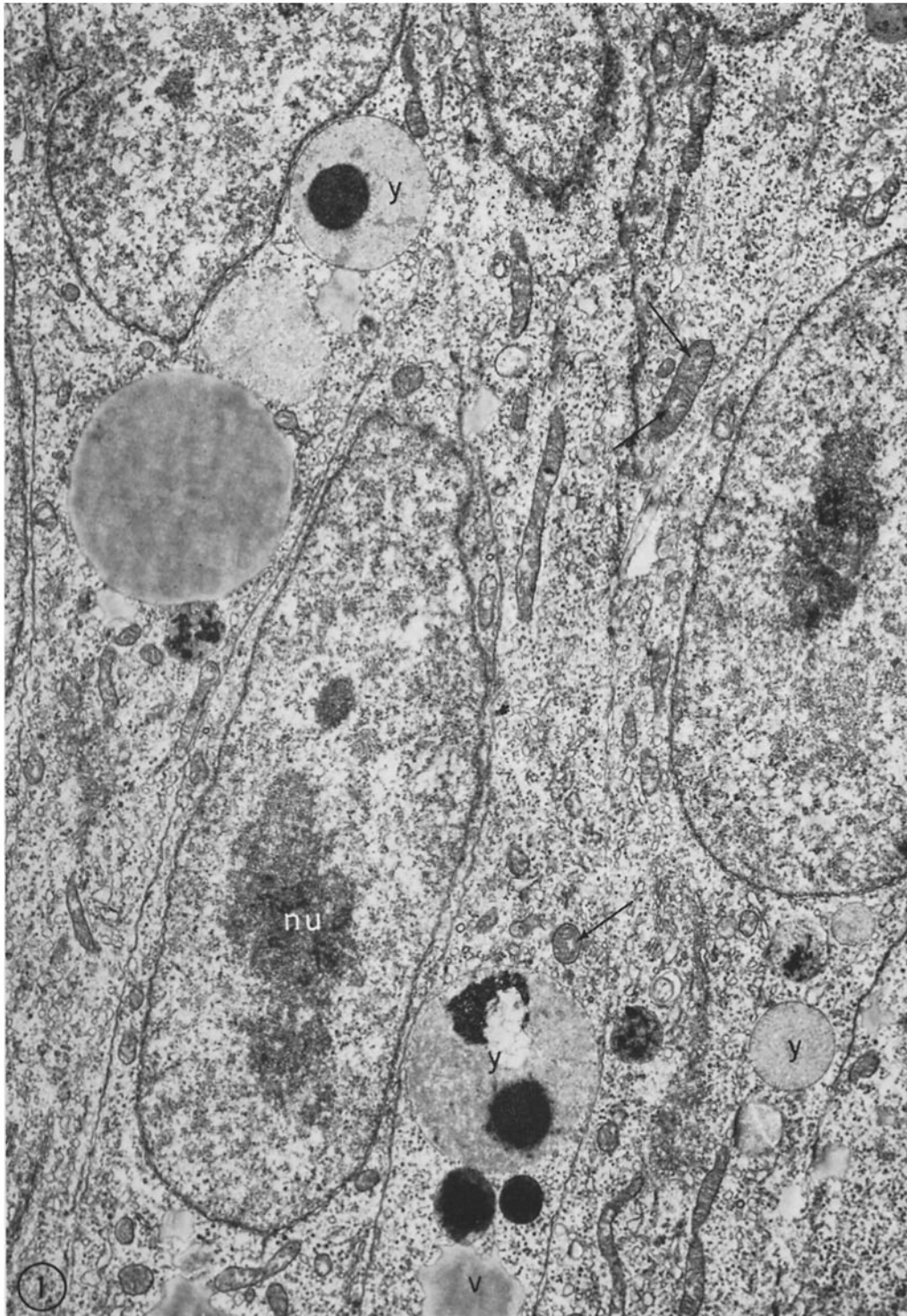
Figs. 7 and 8 represent micrographs from adjacent ectodermal regions of a single embryo after osmium tetroxide-fixation followed by immersion in uranyl acetate or EDTA solutions (17). After these treatments the mitochondrial fibers no longer appear as bar-like structures (up to 400

Explanation of Figures

Arrows, mitochondrial electron-transparent areas and/or mitochondrial fibers
cm, cell membrane
er, endoplasmic reticulum
m, mitochondrion

n, nucleus
nu, nucleolus
v, cytoplasmic vesicle
y, yolk globule, at various stages of digestion

All micrographs of chick embryos depict the 19-hour Hensen's node or primitive streak. FIGURE 1 Survey micrograph of chick node (ectoderm) fixed with buffered 2 per cent osmium tetroxide, with salts added to ~ 0.34 M, at pH 8.0; tissues treated with 0.5 per cent uranyl acetate, sections stained with lead hydroxide. Almost all the mitochondria within the field have areas of low density in their matrices. × 8,200.



A in diameter) typical of osmium tetroxide-fixed mitochondria but may be recognized as clusters of fine fibrils. To facilitate comparison of the effects of these treatments, both micrographs are enlarged to 80,000 diameters. At this magnification the mitochondrial fibrils (15 to 30 A) of the uranyl acetate-treated tissues may not be visible in the reproduction of the print (see Fig. 9 for the fine structure of the fibrils after this treatment), but the EDTA-treated tissues clearly show mitochondrial fibers (30 to 60 A). A higher magnification of a mitochondrion after uranyl acetate rinsing is presented in Fig. 9. The branched and irregular beaded appearance of the fibrils is characteristic of their structure after this treatment or after fixation with calcium permanganate (see section B).

B. Calcium and Potassium Permanganate

Fixation with either 3 per cent calcium permanganate (1) or potassium permanganate (20) in buffered salt solution (46), at pH 8.0, generally resulted in better preservation of membranous components of the cell than did fixation with any of the osmium fixatives employed. The survey micrograph (Fig. 10) shows the junction between ectoderm and mesoderm. The mitochondria appear swollen (diameters up to $1\ \mu$) relative to their appearance after osmium tetroxide-fixation. It is again apparent that most of the mitochondria in the field have an area of low density within their matrix. After either potassium permanganate- or calcium permanganate-fixation the mitochondrial fibers are faintly visible within the low density areas. Uranyl acetate-treatment of tissues, staining of sections with uranyl acetate, lead hydroxide, or double staining produce an over-all increase in contrast of all cell structures. In many sections of mitochondria very fine fibrils approximately 15 to 30 A in diameter and some fibers of slightly greater diameters (up to 50 A) are more readily observed after these contrasting procedures (Fig. 11). The fine fibrils appear beaded and porous

along their length and frequently seem to merge and branch.

C. Acrolein

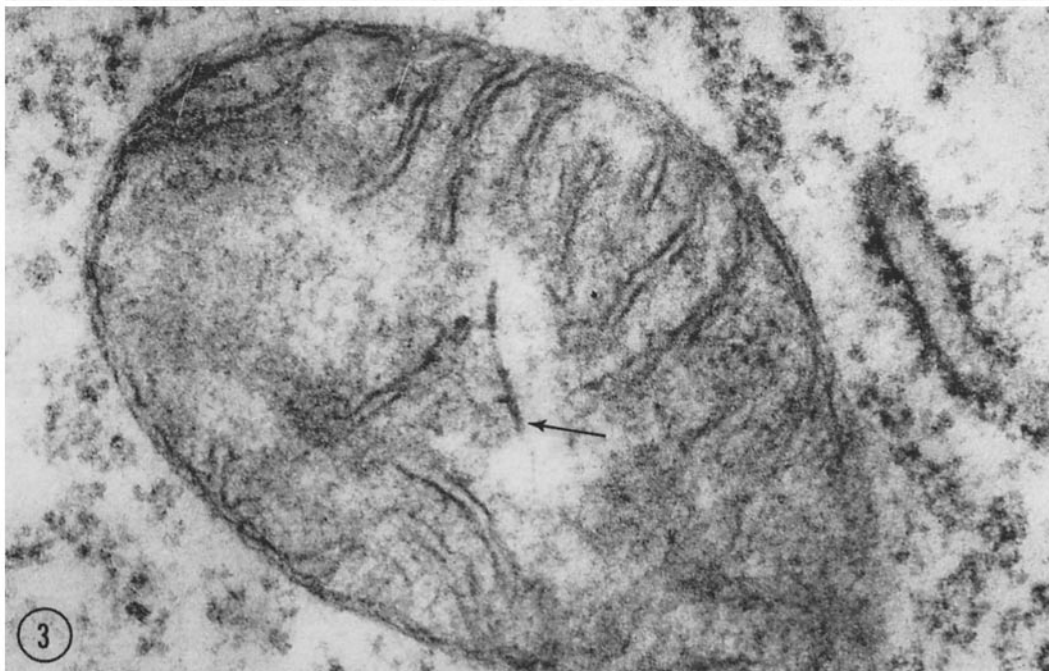
Tissues fixed with 10 per cent acrolein (21) in phosphate buffer, pH 8.0, and postfixed with osmium tetroxide show, in addition to the mitochondrial areas of low density, the excellent preservation of cellular membranes, especially those of the endoplasmic reticulum (Fig. 12). The nucleus, after staining with lead salts, clearly shows a nucleolus and a narrow, indistinct chromatinic material bordering the inner nuclear membrane. Although the mitochondrial fibers are often identifiable as fine fibrils after the staining of acrolein-OsO₄-fixed tissues, they are not preserved in a regular or distinct pattern (Fig. 12). Uranyl acetate treatment of tissues fixed in acrolein also did not preserve the fibers in any definitely recognizable form. Similarly, the bacterial nucleoplasm after double fixation with acrolein and OsO₄ (and staining of sections with lead or uranyl ions) is relatively indistinct (*cf.* Figs. 6 and 13). Nevertheless, fibrillar material (20 to 50 A in diameter) not unlike that observed in mitochondria after these procedures (*cf.* Figs. 12 and 13) is more apparent in the bacterial nucleoplasm than in the chick mitochondria because of the higher concentration of this material in the bacterium.

D. Formaldehyde

Fixation with formaldehyde or with formaldehyde followed by osmium tetroxide causes a marked shrinkage of the mitochondria relative to their appearance after all other fixation methods. Despite the shrinkage and general increase in electron opacity of the mitochondrial matrix, the areas of low density are still apparent (Fig. 14), indicating that these regions are, at least in their response to fixation, relatively rigid structures. A few dense fibers within these areas of low density

FIGURE 2 Unstained sections of mitochondria. Tissue fixed with the osmium tetroxide fixative described in legend to Fig. 1. This micrograph shows some faintly visible material within the electron-transparent areas of the mitochondria. $\times 57,000$.

FIGURE 3 Section of mitochondrion of a 55-hour embryo (tailbud) stained with uranyl acetate. Although there is a general increase in contrast, it is apparent that the now clearly visible clumped mitochondrial fibers are preferentially stained as compared to their appearance in Fig. 2. $\times 105,000$.



in formaldehyde-fixed, lead-stained mitochondria may also be seen in Fig. 14.

E. Other Fixatives

Two other fixatives were studied, acetic acid-alcohol, 1:3, and 2 per cent uranyl acetate followed by calcium permanganate. After staining the acetic alcohol-fixed material with uranyl acetate and/or lead hydroxide, or after uranyl acetate-treatment of tissue blocks, the mitochondria were barely visible (no membrane was seen), the center and inner margins of the nuclei were heavily stained, and the ribosomes showed intense contrast. The mitochondria, sometimes observed as areas of faint density, had traces of what appeared to be the fibrous component.

Some portions of the tissue were preserved after successive treatments with uranyl acetate and calcium permanganate. The uranyl fixative (pH 4.5), known to preserve purified DNA fibers (40), kept many of the 15 to 30 A mitochondrial fibrils intact, despite disrupting most other cell constituents (28).

DISCUSSION

The results presented indicate that electron-transparent areas within chick embryo mitochondria are repeatedly observed after many different fixation procedures. They also show that the various techniques reported to increase the specificity of the staining of nucleic acid-containing structures by heavy metal compounds increase the density of fibrous structures found in the low density regions. Further, reagents known to stabilize the bacterial nucleoplasm and purified DNA fibers have the same effect on the mitochondrial fibers (osmium tetroxide + uranyl acetate, uranyl acetate, and potassium permanganate). Agents, such as EDTA, which partially

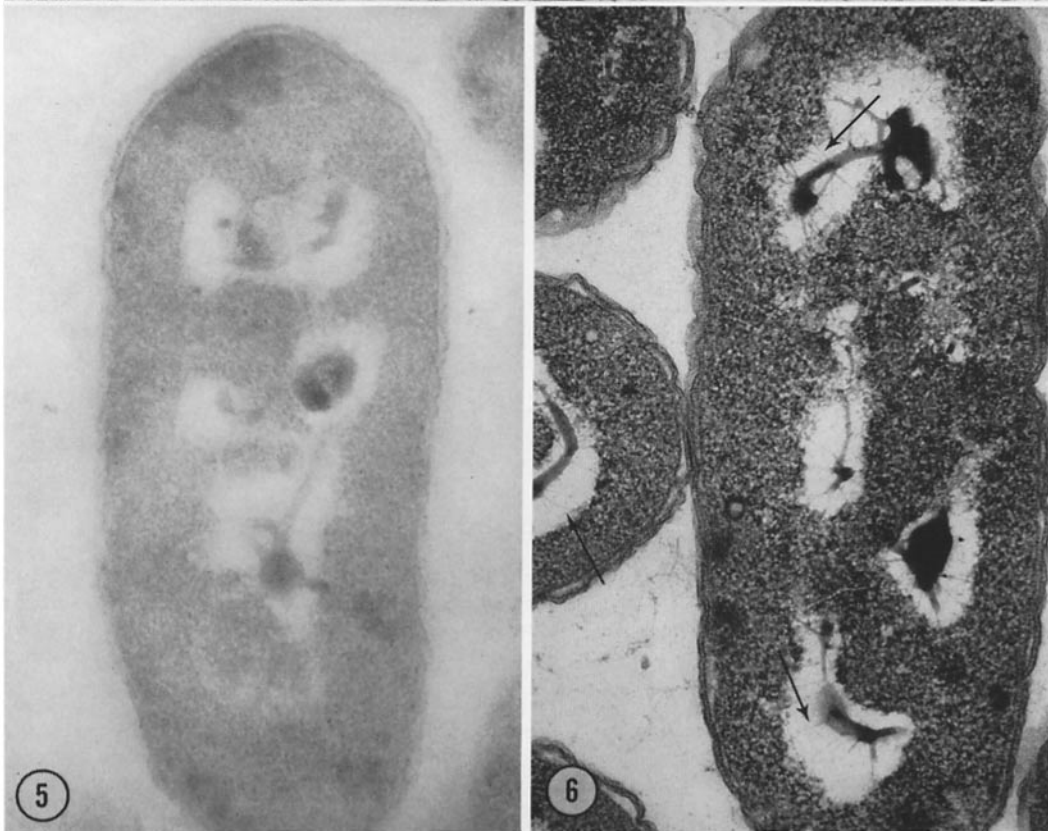
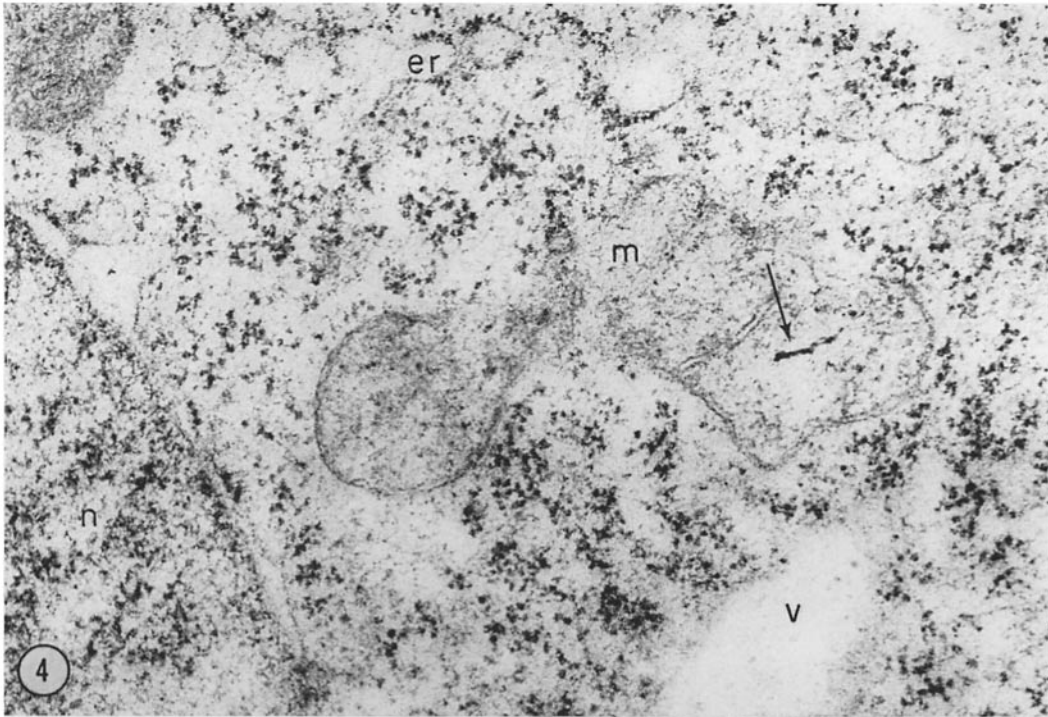
aggregate the bacterial nucleoplasm, or fixation in osmium tetroxide or formaldehyde, which results in strong clumping of the nucleoplasm, or acrolein fixation, all have very similar effects upon the mitochondrial fibers.

MITOCHONDRIAL AREAS OF LOW ELECTRON OPACITY: Although many reports in the literature relate the loss of density of the mitochondrial matrix to fixation artifacts, postmortem changes, neoplastic transformations, and to the effects of certain chemicals administered to animals (15, 46, *cf.* references 30 and 36), the results of the present investigation do not invite such interpretation. The mitochondrial areas of low electron opacity are considered to be a structural component because they were observed after fixation by all the methods employed, and at various pH and salt concentrations. Furthermore, the fact that early chick embryos can be fixed directly without prior killing and dissecting of the embryo, and that the fixatives penetrate very rapidly into all parts of these small organisms seems to rule out a consideration of postmortem changes occurring before fixation. Generally, rarification is observed to occur throughout the matrix of the mitochondria and is frequently accompanied by swelling of the entire organelle, but the micrographs presented here show no over-all loss of density of the mitochondrial matrix, and even the relatively shrunken mitochondria observed after fixation with formaldehyde show clear areas (Fig. 14). The latter finding also suggests that such areas may have some structural rigidity.

SPECIFICITY OF STAINING REACTIONS: The effects produced by heavy metal contrasting agents upon chick embryo tissues fixed in various ways indicate that under certain conditions the known nucleic acid-containing structures of the cell are more highly electron-opaque than are

FIGURE 4 Chick node tissue fixed with the osmium tetroxide fixative described in legend to Fig. 1. Sections treated for 30 minutes with 2 per cent hydrogen peroxide prior to staining with uranyl acetate followed by lead hydroxide. The contrast of the mitochondrial fibers and ribosomes is greatly increased over that of the rest of the cytoplasmic components. The electron-opaque granules of mitochondria are specifically removed by the hydrogen peroxide treatment. $\times 60,000$.

FIGURES 5 AND 6 *Escherichia coli* cells fixed with the osmium tetroxide fixative described in legend to Fig. 1. Comparison between the nucleoplasm in unstained section (Fig. 5) and section treated with hydrogen peroxide and stained as described in legend to Fig. 4 (Fig. 6). It is apparent that the peroxide treatment and staining greatly increases contrast but does not visibly alter the structural appearance. $\times 60,000$.



structures containing little or no nucleic acids (Figs. 2 to 4, and 14). All of the results are consistent with those reported in the literature (13, 17, 19, 24, 41, 42). Thus, after standard osmium tetroxide-fixation and section staining there occurs a general increase in contrast of most or all structures, but the relative increase in density of ribosomes, nucleoli, and mitochondrial fibers after staining appears to be somewhat greater than that of membranous structures (*e.g.*, Figs. 2 and 3), suggesting that there is a higher degree of binding between heavy metal ions and nucleic acid-containing structures than between these ions and the membranes. The generalized staining effect obtained by immersion of osmium tetroxide-fixed tissue blocks in uranyl acetate solutions is also in agreement with the results obtained by Kellenberger *et al.* (17) with bacteria. Staining after potassium or calcium permanganate fixation similarly resulted in a general increase in contrast of most cell structures.

The contrasting by heavy metal ions after fixation with non-metallic reagents (formaldehyde, acrolein, acetic acid-alcohol) appears to be more specific for ribosomes, chromatinic areas, and nucleoli than after metallic fixation (*e.g.*, references 19 and 41). Formaldehyde-fixed and stained mitochondria show the mitochondrial fibers to be densely stained. The oxidation and perhaps removal of the reduced osmium tetroxide compounds by hydrogen peroxide markedly increases the staining specificity of known nucleic acid-containing cell components by heavy metal compounds (24). All of these results are compatible with the view (6) that the relative non-specificity of staining observed after the use of metallic fixatives may be the result of a complex-ion formation between the fixative and the contrasting agent. The removal of reduced osmium tetroxide compounds by hydrogen peroxide could thus account for the fact that after treatment with this

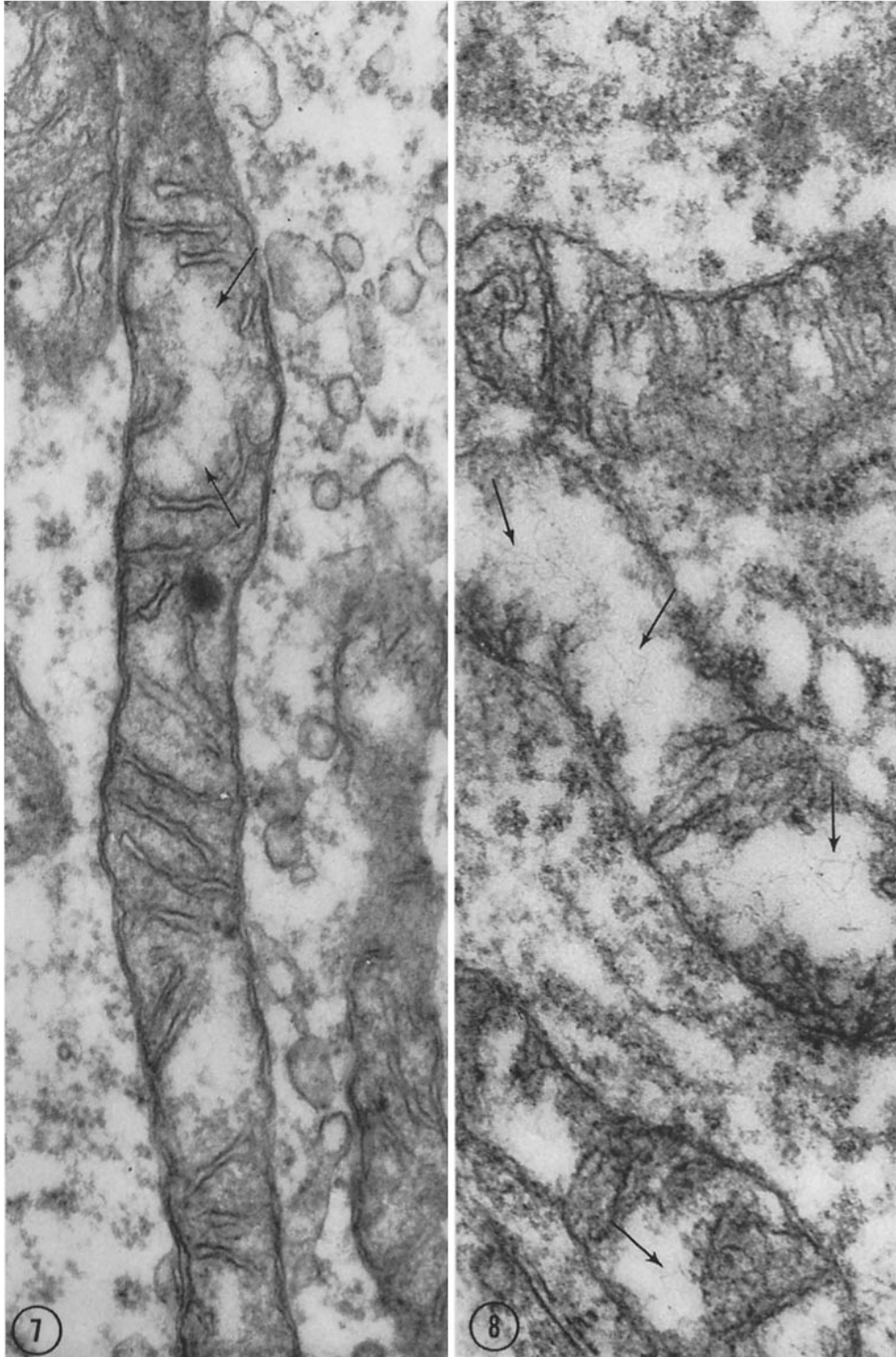
reagent the heavy metal contrasting agents do not produce a general over-all increase in staining but a relatively specific one.

STRUCTURE OF THE MITOCHONDRIAL FIBERS: The appearance of the mitochondrial fibers varies with the fixative employed, and the electron opacity varies with the staining reactions used. After osmium tetroxide-fixation the mitochondrial fibers have a low density (Fig. 2); this is also true of the osmium tetroxide-fixed bacterial nucleoplasm in areas of comparable fiber concentration (Fig. 5), and may perhaps relate to the fact that purified preparations of nucleic acids do not produce a visible reduction product with osmium tetroxide (2). The difference observed in the density of the osmium tetroxide-fixed, unstained, clumped bacterial nucleoplasm and similarly fixed mitochondrial fibers is undoubtedly related to the much greater concentration of the bacterial DNA per unit area and not to its reactions with osmium tetroxide. The clumped bacterial nucleoplasm after fixation with formaldehyde has a density equivalent to that observed after osmium tetroxide-fixation, although the former fixative could hardly increase the weight density of the structures significantly (23). The mitochondrial fibers are also clumped by formaldehyde, but, as with osmium tetroxide, they were not readily visualized until staining with heavy metal ions was carried out.

After all of the staining procedures employed following osmium tetroxide-fixation (Figs. 3, 4), the fibrous component of the mitochondria is recognizable as a bar-like structure (up to 400 Å in diameter), usually with some fine filaments (15 to 50 Å) extending toward the periphery of the mitochondria (26). The bacterial nucleoplasm also shows fibrous strands extending from the larger clumped portions (Fig. 6). It may be noted that in unstained sections of bacteria these fine filaments are not detectable; *i.e.*, they have a low

FIGURE 7 Portion of primitive streak fixed with osmium tetroxide, treated with 0.5 per cent uranyl acetate for 2 hours; sections stained with lead hydroxide. The areas of low density in the mitochondria contain fine fibrils (between arrows), which are barely visible at this magnification (see Fig. 9 for a magnified image of the fibers after this treatment). $\times 80,000$.

FIGURE 8 Portion of primitive streak (from the same embryo as in Fig. 7) treated with 0.25 M EDTA (instead of uranyl acetate) for 2 hours; sections stained with lead hydroxide. Mitochondrial fibers are coarsened. The fibers have a diameter approximately two to several times that of fibers observed after uranyl acetate treatment. $\times 80,000$.



intrinsic density comparable to that of the mitochondrial fibers. Similar fibrous structures with similar properties have been shown to correspond with the DNA-containing portions of the kinetoplast-associated mitochondrion of *Trypanosoma* (39) and *Bodo* (32), and kappa particles of *Paramecium* (7, 10). Some chemically undefined fibrillar structures within the mitochondria of giant amoebae (31) and a marine ciliate (9) have also been reported.

The ultrastructure of the mitochondrial fibers has been found to be stabilized as branching 15 to 30 A fibrils (Figs. 7 and 8) after treatment of tissues by the technique of Kellenberger *et al.* (17), which is the generally accepted method for the preservation of the bacterial nucleoplasm. The fibrils appear markedly similar in substructure to purified DNA, as shown by Stoeckenius (40). Uranyl acetate-treatment of osmium tetroxide-fixed tissues has also been shown to stabilize the DNA-containing regions of blue-green algae (12), chloroplasts (35), and kappa particles of *Paramecium* (33), as well as chemically unidentified fibrils in the mitochondrion of *Micromonas* (34). It is of interest that uranyl acetate treatment of unfixed DNA protein films stabilizes the fibers against alcoholic dehydration, and in the absence of uranyl acetate the fibers are clumped by alcohol (40). It seems plausible that the clumped appearance as well as the low density of the mitochondrial fibers after osmium tetroxide-fixation may be related to the lack or low degree of fixation of nucleic acids by osmium tetroxide, which allows clumping to take place during alcoholic dehydration procedures.

Fixation with either of the permanganate reagents also stabilized the mitochondrial fibers as a branching network of fibrils, and potassium permanganate has been shown to stabilize the bacterial nucleoplasm in a manner comparable to the Kellenberger *et al.* (17) procedure (25, *cf.* reference 16). Further suggestive evidence that the mitochondrial fibers contain DNA is the cytochemical demonstration that RNA, but not DNA, com-

pounds are removed from cells fixed with potassium permanganate (3).

The treatment of tissues with EDTA in place of uranyl acetate partially aggregates the mitochondrial fibers, again mimicking the action of this metal chelator upon the bacterial nucleoplasm. That the aggregation in both bacteria and mitochondria is not so great as after osmium tetroxide-fixation alone indicates that the EDTA treatment effects some degree of stabilization of the fibers.

The similar appearance of the bacterial nucleoplasm and the mitochondrial fibers after acrolein-osmium tetroxide double fixation and staining again appears to reflect the similarities in the composition of these structures.

It is apparent that all the similarities between the fibrous cores of the chick mitochondria, as well as of mitochondria from other organisms (27), and the nucleoplasm of bacteria are not simply morphological. The similarities in structure after many different fixation procedures, rather, are indicative of a set of distinct chemical reactions which these "plasms" share in common. The reactions in bacteria are considered to be specific for highly hydrated DNA (16) not coated by histones (44, 47). Many of these reactions have been demonstrated to occur in other cytoplasmic structures containing DNA, which are considered to be "self-replicating" (*cf.* references 8, 18, 37, 38). The fact that mitochondria, long thought to be "self-replicating" structures (*cf.* references 8, 18, 37, 45), should have a component with similar properties is perhaps not surprising. It seems to be most unlikely that the staining reactions, which are highly suggestive of a nucleic acid content in the mitochondria, and the parallel results obtained in the fixation of mitochondrial fibers and bacterial nucleoplasm are simply fortuitous. When we consider that no other *naturally occurring* structure thus far studied has *all* the properties discussed, with respect to fixation, stabilization and staining, the unavoidable conclusion appears to be that the mitochondrial fibers contain DNA.

FIGURE 9 Higher magnification of a mitochondrion from an osmium tetroxide-fixed, uranyl acetate-treated embryo, as in Fig. 7; section stained with lead hydroxide. The 15 to 30 A fibrils have bulbous and porous enlargements along their length and often appear to branch. (The cell membrane and membranes of the mitochondrion are clearly visible as trilaminar structures, except where the membranes are in intimate association with the irregular vesicular structures observable in these cells.) $\times 225,000$.



The following paper will present more direct evidence to show that this conclusion is warranted.

It is a pleasure to acknowledge the hospitality of Professor O. Lindberg, Director of the Wenner-Gren Institute. The authors are most grateful to Dr. B. A. Afzelius for instructing them in the techniques of electron microscopy and for his helpful advice and comments on the manuscripts. Discussions also with Professor T. Gustafson have been most encouraging and stimulating.

A preliminary report of these findings was pre-

sented at the Symposium on Cytochemical Progress in Electron Microscopy, held in Oxford, England, July, 1962 (28).

Some of the work was carried out while Margit Nass was a Postdoctoral Fellow of the National Science Foundation. She was later aided by a grant for a Postdoctoral Fellowship from the American Cancer Society.

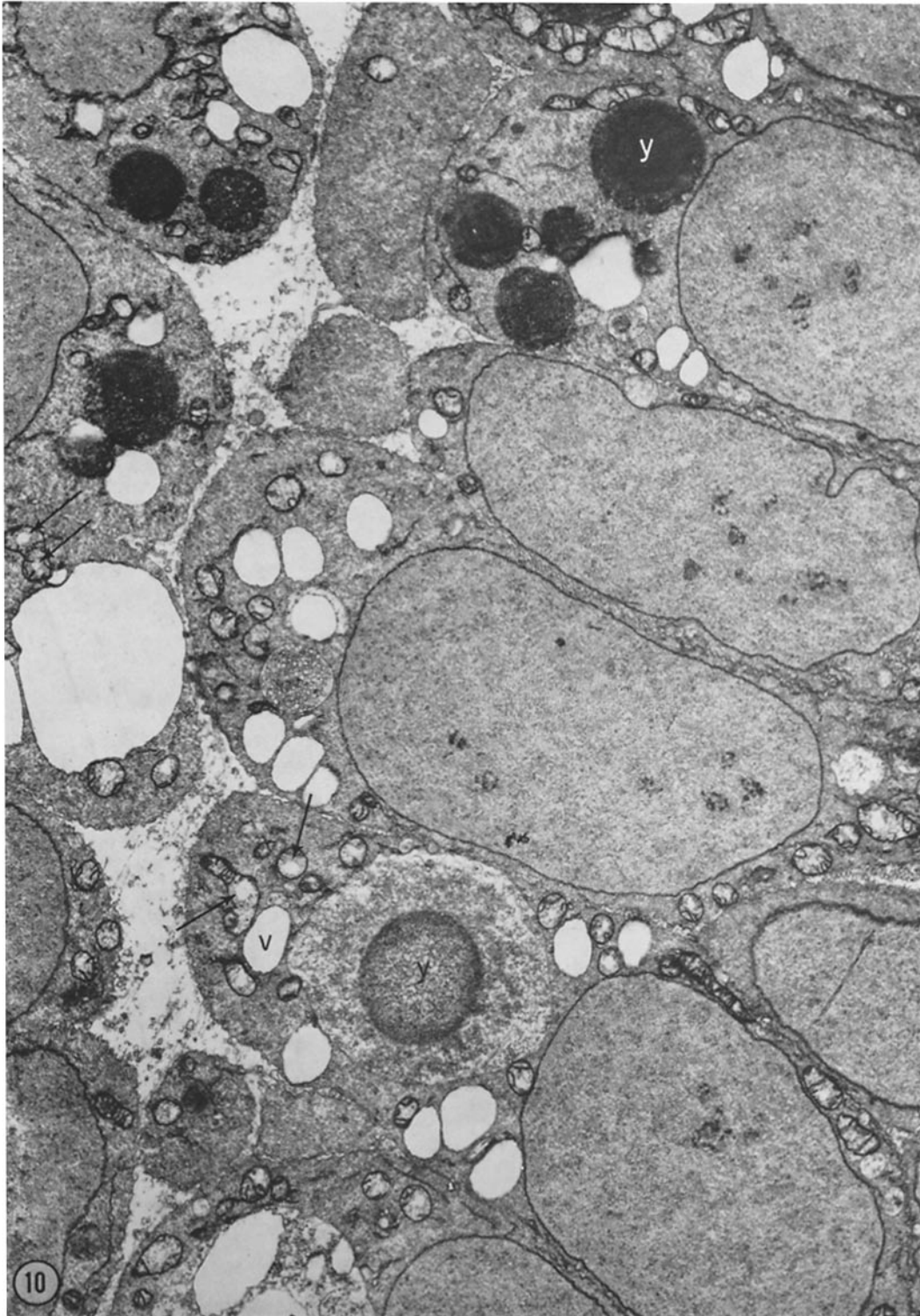
This work was also supported in part by a Public Health Service Fellowship (CA-8573) to Sylvan Nass from the National Cancer Institute, United States Public Health Service.

Received for publication, December 13, 1962.

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FIGURE 10 Survey micrograph of chick node (at ectoderm-mesoderm junction) fixed with 3 per cent calcium permanganate, with salts added, and buffered at pH 8.0; tissues treated with uranyl acetate and sections stained with lead hydroxide. The mitochondria contain areas of very low density in their crista-free regions. $\times 9,200$.



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FIGURE 11 Chick node fixed with 3 per cent calcium permanganate in buffered salt solution, at pH 8.0; sections stained with lead hydroxide. Mitochondrial fibrous structures appear as 15 to 50 A fibrils with porous and bulbous enlargements along their length. $\times 120,000$.



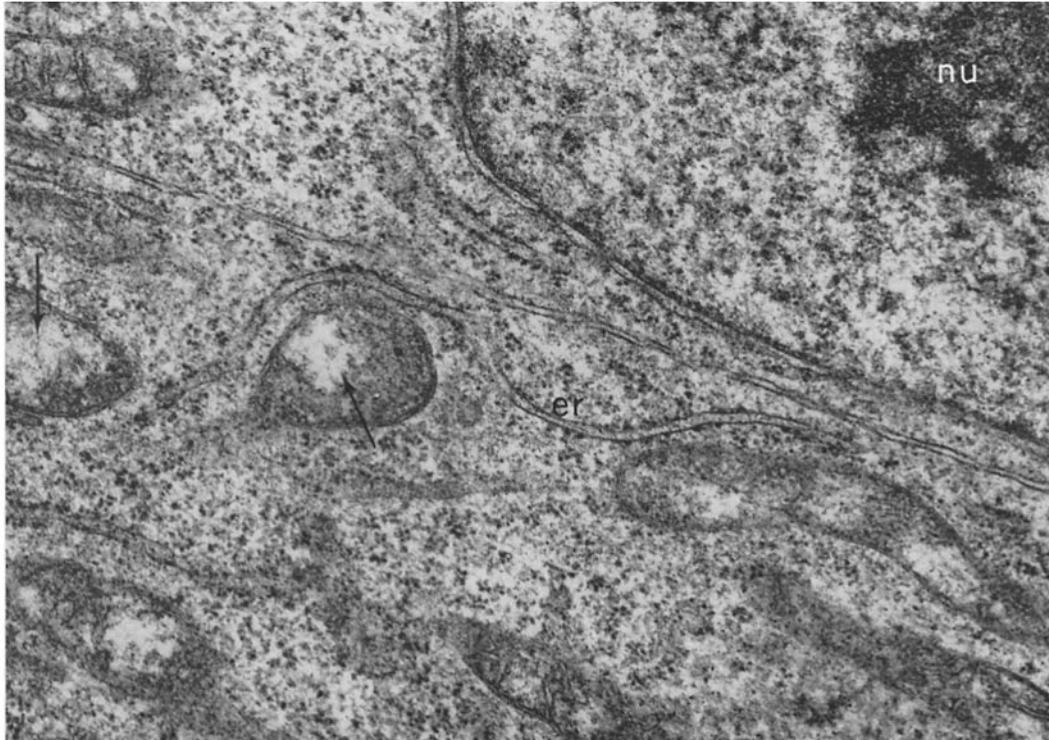


FIGURE 12 Chick node fixed with 10 per cent acrolein buffered at pH 8.0, postfixed with osmium tetroxide, stained with lead hydroxide. Cell membranes and especially the particle-studded membranes of the endoplasmic reticulum are very well defined. The mitochondrial areas of low density are apparent. The mitochondrial fibers are irregularly distributed and not easily recognizable in a definite and reproducible form. $\times 40,000$.

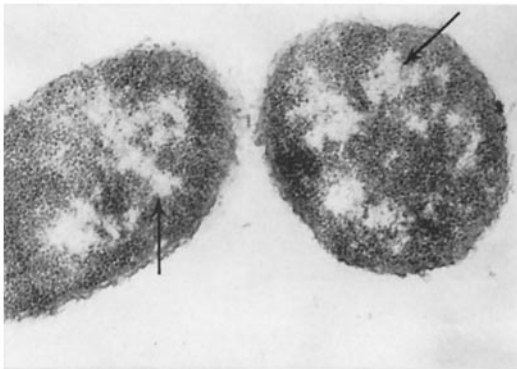


FIGURE 13 *Escherichia coli* fixed and stained as described in legend to Fig. 12. The nucleoplasm forms an irregular fibrous network, which is not strongly contrasted by uranyl or lead staining. $\times 40,000$.



FIGURE 14 Chick node fixed with 4 per cent formaldehyde in sucrose, buffered at pH 8.0, for 24 hours; sections stained with lead hydroxide. The mitochondria appear shrunken. The areas of low density within the mitochondrial matrix are nevertheless apparent. In some of these areas clumped mitochondrial fibers may be seen after staining (arrows). No membranes are visible. At lower left is part of a densely packed cell characteristically seen after formaldehyde and acrolein fixation. $\times 42,000$.