

ROLE OF THE MITOCHONDRIAL GENOME DURING EARLY DEVELOPMENT IN MICE

Effects of Ethidium Bromide and Chloramphenicol

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ABSTRACT

The role of the mitochondrial genome in early development and differentiation was studied in mouse embryos cultured *in vitro* from the two to four cell stage to the blastocyst (about 100 cells). During this period the mitochondria undergo morphological differentiation: progressive enlargement followed by an increase in matrix density, in number of cristae, and in number of mitochondrial ribosomes. Mitochondrial ribosomal and transfer RNA synthesis occurs from the 8 to 16 cell stage on and contributes to the establishment of a mitochondrial protein-synthesizing system. Inhibition of mitochondrial RNA- and protein-synthesis by 0.1 $\mu\text{g}/\text{ml}$ of ethidium bromide or 31.2 $\mu\text{g}/\text{ml}$ of chloramphenicol permits essentially normal embryo development and cellular differentiation. Mitochondrial morphogenesis is also nearly normal except for the appearance of dilated and vesicular cristae in blastocyst mitochondria. Such blastocysts are capable of normal postimplantation development when transplanted into the uteri of foster mothers. Higher concentrations of these inhibitors have general toxic effects and arrest embryo development. It is concluded that mitochondrial differentiation in the early mouse embryo occurs through the progressive transformation of the preexisting mitochondria and is largely controlled by the nucleocytoplasmic system. Mitochondrial protein synthesis is required for the normal structural organization of the cristae in blastocyst mitochondria. Embryo development and cellular differentiation up to the blastocyst stage are not dependent on mitochondrial genetic activity.

INTRODUCTION

The mitochondrial DNA in higher animals consists of a uniform population of circular molecules of about 5- μm perimeter (7). This DNA codes for the RNA components of a mitochondrial protein-synthesizing system: 12S and 16S ribosomal RNA's and 4S transfer RNA's (2, 5, 6, 23, 24, 58). The mitochondria contain unique ribosomes of about 60S which form polysome-like complexes active in protein synthesis (9, 10, 60, 63, 64, 78). The role

of mitochondrial protein synthesis is incompletely known, but the available evidence indicates that the mitochondria of as widely different species as yeast and mammals synthesize only a small number of polypeptides which are essential for the structural and functional integrity of mitochondrial cristae (5, 9, 34, 44, 70).

Animal eggs and early embryos are of special interest for the study of mitochondrial genetic ac-

tivity because of the presence of large amounts of mitochondrial DNA in these eggs as shown in frog (22), sea urchin (68), and echiuroid worm (25). Biochemical evidence indicates that the mitochondrial DNA is transcribed during early development in sea urchins (16, 17, 20, 39, 71), but the role of this transcriptive activity is not clear. On the other hand, in frog eggs the mitochondrial genome may be inactive until gastrulation (19).

One approach to the analysis of the genetic function of mitochondrial DNA has been the use of specific inhibitors of mitochondrial RNA-, DNA-, and protein-synthesis, such as ethidium bromide and chloramphenicol. We have employed these drugs in the present study using mouse embryos cultured *in vitro* from the 2 to 4 cell stage to the blastocyst (about 100 cells). During the 3-day culture period the mouse embryo undergoes both morphological and biochemical differentiation but does not increase in cytoplasmic volume or protein content (12). The mitochondria themselves differentiate as shown by their fine structural appearance (15, 40, 51, 76), changes in energy metabolism (4, 13, 43), and increase in oxygen consumption (54).

The main question we have sought to answer is to what extent the development and differentiation of the preimplantation mouse embryo is dependent on the expression of the mitochondrial genome. We have monitored the effects of ethidium bromide and chloramphenicol by electron microscopy and biochemical analyses of RNA- and protein-synthesis; background studies of control embryos were also conducted. A particular effort was made to ascertain that the drug concentrations used specifically inhibit mitochondrial synthetic activity without affecting the nuclear and cytoplasmic systems. The results indicate that the mitochondrial genome is transcribed during cleavage and contributes, through the synthesis of ribosomal RNA and transfer RNA, to the establishment of a mitochondrial protein-synthesizing system. Mitochondrial protein synthesis is required for the normal structural organization of the cristae in blastocyst mitochondria. However, the inhibition of mitochondrial RNA- and protein-synthesis does not interfere with normal development and cellular differentiation of the mouse embryo up to the blastocyst stage. Mitochondrial differentiation during this period is also largely controlled by the nucleocytoplasmic system. Preliminary reports of some of the data presented here have been given elsewhere (18, 67).

MATERIALS AND METHODS

Culture of the Embryos

The conditions of embryo culture and handling were the same as described earlier (66). Eggs at the two to four cell stage were recovered on the second day (day 1 of culture) after mating of superovulated Swiss mice (Simonsen Laboratories, Gilroy, Calif.) and were cultured in 100- μ l droplets of medium under mineral oil at 37°C in 5% CO₂ in air. The Krebs-Ringer bicarbonate medium contained 1 mg/ml crystalline bovine serum albumin, 100 U/ml penicillin G, 50 μ g/ml streptomycin sulfate, and 2.5×10^{-4} M sodium pyruvate and 2.5×10^{-2} M lactic acid as energy source (11). The glassware and media were sterilized and aseptic conditions were maintained during the manipulations. The development of the embryos was evaluated by examination under a dissecting microscope. In some cases the number of cells in the embryo was determined by phase microscopy after staining the embryo with acetocarmine and gently squashing it under a coverslip.

Treatment with Inhibitors

Stock solutions of ethidium bromide (Boots Pure Drug Co., Ltd., Nottingham, England) of 50 μ g/ml were prepared in culture medium without albumin and kept for several weeks in the refrigerator in a glass container wrapped in aluminum foil. Stock solutions of chloramphenicol (Calbiochem, San Diego, Calif.), 500 μ g/ml, and cycloheximide (Sigma Chemical Co., St. Louis, Mo.), 1 mg/ml, were made up in culture medium the day of the experiment. Before the experiment, the stock solutions were diluted to the desired concentrations in culture medium and the embryos in the culture droplet were washed in three changes of these solutions (with a dilution rate of about 10:1 each).

Labeling Conditions

For incubation with radioisotopes, groups of embryos were set up in a measured volume of medium by means of a micropipetting device using disposable glass pipets (Drummond Scientific Co., Broomall, Pa.). Inhibitor and isotope solutions were then added to give the desired concentration in a 100- μ l culture drop. The following isotopes were used: [³H]uridine (Amersham/Searle Corp., Arlington Heights, Ill.), 22.7 mCi/ μ mol, and a mixture of three uniformly labeled [¹⁴C]amino acids (New England Nuclear, Boston, Mass.), L-aspartic acid, 167 μ Ci/ μ mol, L-lysine, 223 μ Ci/ μ mol, and L-valine, 190 μ Ci/ μ mol. The isotope solutions were evaporated and redissolved in culture medium before use. The final concentrations of isotopes in the culture drop were the following: [³H]uridine, 200 μ Ci/ml, and [¹⁴C]amino acid

mixture, 1 $\mu\text{Ci}/\text{ml}$ of each of the three amino acids giving a total of 3 $\mu\text{Ci}/\text{ml}$.

At the end of incubation the embryos were washed in six changes of ice-cold medium and either were transferred directly on filter paper strips (Whatman no. 1) or used for the isolation of mitochondria and RNA extraction (see below). In the studies of protein synthesis the uptake of radioactive amino acids was measured by counting the dried papers directly in toluene-PPO (2,5-diphenyloxazole, Packard Instrument Co., Inc., Downer's Grove, Ill.) (5 g of PPO per 1 liter of toluene) scintillation fluid; an aliquot of the final wash fluid was counted similarly and these counts were deducted from the uptake radioactivity. For the measurement of incorporated label, the same papers were passed through two changes each of 100% ethanol, 95% ethanol, hot (100°C) and cold 5% TCA, dehydrated in ethanol and ether, and counted.

Isolation of Mitochondria

The procedure was carried out in the cold. The embryos were transferred in about 50 μl of medium into 0.4 ml of 0.01 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 7.6, and allowed to swell for 10 min. They were homogenized by hand in a 1-ml Potter-Elvehjem homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.) by 10 strokes. The homogenate was mixed with 50 μl of 2 M sucrose in Tris-EDTA buffer and 1 ml of the following: 0.21 M mannitol, 0.07 M sucrose, 0.01 M Tris, and 0.001 M EDTA, pH 7.6 (MS buffer). After centrifugation at 700 g for 5–7 min, the supernate was pipetted off and recentrifuged at 12,000 g for 5–7 min. The supernate was discarded and the mitochondrial pellet was washed either once (for RNA preparations shown in Fig. 17) or three times (Fig. 16) in MS buffer by repeating the latter centrifugation.

Isolation and Analysis of RNA

For the extraction of total cellular RNA, the washed embryos were transferred into 1 ml of 2% sodium dodecyl sulfate (SDS) (Matheson, Coleman, and Bell, Cincinnati, Ohio) in 0.01 M sodium acetate, pH 5, and shaken at room temperature for about 10 min. For the extraction of mitochondrial RNA, the mitochondrial pellet was lysed similarly in 1 ml of SDS-acetate buffer. After the addition of 20–50 μg of mouse liver carrier RNA, the lysate was extracted at room temperature with an equal volume of phenol (Fisher Scientific Co., Pittsburgh, Pa.) saturated with acetate buffer and containing 0.1% hydroxyquinoline (Fisher). The phases were separated by centrifugation, the water phase was collected, and the phenol phase was reextracted with an equal volume of acetate buffer. The pooled water phases were brought to 0.1 M in NaCl and the RNA was precipitated with 4 \times volume of 95% ethanol at -20°C . The pre-

cipitate was washed three times in cold ethanol and once in ether, air dried, and dissolved in 0.036 M Tris, 0.03 M NaH_2PO_4 , 0.001 M EDTA, and 0.2% SDS, pH 7.7 (electrophoresis buffer [49]).

Electrophoresis was carried out on polyacrylamide-agarose gels (61) containing 2.5% acrylamide (electrophoresis grade, Eastman Kodak Co., Rochester, N.Y.), 0.12% bisacrylamide (recrystallized, Kodak) and 0.5% agarose (Seakem brand, Bausch & Lomb Inc., Scientific Instrument Div., Rochester, N.Y.). The gels were made up in electrophoresis buffer without SDS in 5-mm inner diameter siliconized glass tubes. The gels were extruded a short distance before use and the tip was sliced off with a razor blade to provide a flat meniscus. After a prerun for 30 min at 5 mA/gel, a sample of 30 μl RNA solution containing 8% sucrose was layered on the gel surface and electrophoresis continued at room temperature at 5 mA/gel for 2½ h. The gels were scanned immediately after the run at 260 nm in a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with linear transport. The gels were cut in 1.5-mm slices which were digested overnight in 0.3 ml of 0.5 N NaOH each in scintillation vials. The lysate in each vial was then shaken with 0.8 ml of NCS solubilizer (Amersham/Searle) for 1 h, mixed with 9 ml of toluene-PPO scintillation fluid and counted for radioactivity.

For sedimentation velocity analysis 100 μl of RNA solution in electrophoresis buffer was layered on 12 ml of 5–20% (wt/vol) linear sucrose (RNase-free, Schwarz Bio Research, Inc., Orangeburg, N.Y.) gradient in 0.1 M NaCl, 0.01 M Tris, 0.001 M EDTA, and 0.2% SDS, pH 7, and centrifuged in a Beckman SW41 rotor (Beckman Instruments, Inc., Spincro Div., Palo Alto, Calif.). The conditions of centrifugation are specified in the figure legends. The contents of the tube were pumped through a 2-mm Anderson flow-cell (Pyrocell Manufacturing Co., Inc., Westwood, N.J.) and the absorbance at 260 nm was recorded on a Gilford 2000 spectrophotometer. For radioactivity measurements equal size fractions of 0.2–0.3 ml were collected either directly into scintillation vials (mitochondrial RNA) or onto filter paper strips (total embryo RNA). The fractions in the vials were suspended in a mixture of 0.5 ml Beckman Biosolv-3 and 9.5 ml toluene-PPO. The filter papers were washed in three changes of ice-cold TCA, dehydrated in ethanol and ether, and counted in toluene-PPO.

In the sedimentation experiments the sedimentation coefficients of the RNA bands were calculated on the basis of their distance of migration from the meniscus as compared with the marker RNA's (52). In the electrophoresis runs, s_E values were calculated on the basis of a linear relationship between the logarithm of s_E and electrophoretic mobility, using the 18S and 28S marker RNA's from mouse liver ribosomes as reference values (6).

Electron Microscopy

Embryos were washed in a balanced salt solution, fixed for 30–60 min at room temperature in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, rinsed in 0.1 M phosphate buffer, postfixed for 1 h in 2% osmium tetroxide in the same buffer, and washed in distilled water. These operations were performed while the eggs were in an aqueous drop under mineral oil (in the same way as they were set up for culturing) and involved three exchanges for each change of solution described. Before dehydration each egg was placed in a 2% agar sandwich to facilitate further handling. Rapid dehydration in alcohol was followed by embedding in Epon 812. Silver and grey sections were cut on an LKB Ultratome I (LKB Instruments, Inc., Rockville, Md.) and stained for 20 min with saturated aqueous uranyl acetate and for 1 min in lead citrate; they were examined with a Philips EM 200 electron microscope operated at 60 kV.

In some experiments a greater differentiation of ribosomes from background structures was achieved by omitting the postfixation of embryos in osmium tetroxide and then staining sections from this material in saturated aqueous uranyl acetate for 60 min at 60°C before the usual lead citrate staining. All counts of mitochondrial ribosomes were performed on material processed in this manner. Prints were made at a total magnification of 38,000 and the ribosomes in randomly selected mitochondria were counted with the aid of a 7× magnifying lens. Sections of at least 20 different mitochondria per experimental group were counted.

Transfer of Blastocysts into Foster Mothers

The procedure was that of McLaren and Michie (53). Swiss female mice that had one previous litter were used as recipients. They were mated with vasectomized males (tested for sterility) three days before the embryo transfer. The uteri were pulled out through a small lumbar incision, the uterine wall was pierced with a 25-gauge needle and six to eight blastocysts (day 4) were transferred to each horn with a fine glass pipet; care was taken not to blow air into the uteri. The females were operated on under Nembutal (Abbott Scientific Products Div., Abbott Laboratories, North Chicago, Ill.) anesthesia and received the antidote Megimide (Abbott) for faster recovery.

RESULTS

Embryo Development In Vitro

In a first series of experiments the general effects of ethidium bromide and chloramphenicol on em-

bryo development were explored. A range of concentrations for each drug was established, the effects of which varied from virtually no interference with embryo development to a complete arrest of development.

Table I summarizes the effect of different concentrations of ethidium bromide on embryo development from two to four cells (day 1) to blastocyst (day 4). At a concentration of 0.1 $\mu\text{g/ml}$ of ethidium bromide the percentage of embryos reaching the blastocyst stage is similar to that in the control group, but 0.2 $\mu\text{g/ml}$ reduced the proportion of blastocysts by about 50%. Concentrations of 0.4 and 0.8 $\mu\text{g/ml}$ of ethidium bromide essentially completely inhibited blastocyst formation; cell counts showed that embryo development was arrested at the 8–16 cell (0.4 $\mu\text{g/ml}$) and 4–8 cell (0.8 $\mu\text{g/ml}$) stages, respectively. Although those embryos which cavitated appeared morphologically normal, a slight delay in development and a reduction in the average size of the blastocyst at day 4 were noted. Less than 10% of the ethidium bromide-treated blastocysts hatched (lost their zonae pellucidae) by the afternoon of day 4 by which time about one-half of control blastocysts had hatched.

The effect of chloramphenicol on embryo development from day 1 to day 4 is shown in Table II. At a concentration of 31.2 $\mu\text{g/ml}$ of chloramphenicol blastocyst formation is nearly normal, but it is progressively inhibited at 62.5 and 125 $\mu\text{g/ml}$. In the presence of 250 $\mu\text{g/ml}$ of chloramphenicol development did not proceed beyond

TABLE I
Effect of Ethidium Bromide on Embryo Development In Vitro*

Concentration	Number of embryos	Blastocysts	Inhibition‡
$\mu\text{g/ml}$		%	%
0.8	101	0	100
0.4	93	1	99
0.2	127	50	46
0.1	76	93	0
Control	124	93	0

* Embryos were cultured from the two to four cell stage (day 1) to the blastocyst stage (day 4) in the presence of different concentrations of ethidium bromide.

‡ Percent inhibition of blastocyst formation as compared with the control.

the 8–16 cell stage. As with ethidium bromide, chloramphenicol-treated blastocysts also showed some delay in development and very few of them hatched by the afternoon of day 4. By day 5, however, the blastocysts grown in the presence of inhibitors (ethidium bromide or chloramphenicol) very nearly caught up with the control blastocysts in their general appearance, and electron microscopy of thin sections revealed a normal pattern of cytological differentiation (Fig. 1).

Cellular RNA- and Protein-Synthesis

The purpose of these experiments was to determine to what extent the developmental effects of ethidium bromide and chloramphenicol could be attributed to a general toxic effect on cellular RNA- and protein-synthesis rather than to a specific inhibition of mitochondrial synthetic activity.

Blastocysts were cultured in different concentrations of ethidium bromide for 24 h (from day 3 to day 4) and then pulse-labeled with [³H]uridine for 2 h in the continued presence of ethidium bromide. Total cellular RNA was extracted and analyzed by sedimentation in a sucrose density gradient (Fig. 2). Incubation in the presence of 0.2 $\mu\text{g/ml}$ of ethidium bromide reduced the rate of RNA synthesis by about one-half but did not significantly change the pattern of the RNA's synthesized. Concentrations of 0.4 and 0.8 $\mu\text{g/ml}$ of ethidium bromide suppressed the rate of RNA synthesis by 85% and 96%, respectively. In a similar experiment (not shown), 0.1 $\mu\text{g/ml}$ of

ethidium bromide reduced the quantity of RNA synthesized only slightly (by an average of 14% in three experiments) without affecting the pattern of RNA synthesis. In agreement with the biochemical data, electron micrographs of day 3 embryos grown in the presence of 0.8 $\mu\text{g/ml}$ of ethidium bromide and arrested at the four to eight cell stage showed an abnormal nuclear structure and an absence of nucleolar differentiation (Fig. 4) as compared with control embryos of similar age (Fig. 3). Nucleolar differentiation in embryos grown in the presence of 0.1 or 0.2 $\mu\text{g/ml}$ of ethidium bromide was essentially normal; however, the nucleoli of embryos treated with 0.2 $\mu\text{g/ml}$ of ethidium bromide were generally more compact and contained a reduced amount of granular element.

Table III shows the effect of chloramphenicol on total cellular protein synthesis in day 4 blastocysts. Blastocysts were pulse labeled with a non-competitive amino acid mixture (79) for 1 h in the presence of different concentrations of chloramphenicol (added to the culture 15 min before the isotope). The presence of 31.2 $\mu\text{g/ml}$ of chloramphenicol had no effect on the uptake and incorporation of amino acids, but higher concentrations progressively suppressed amino acid incorporation into acid-precipitable counts.

Mitochondrial Fine Structure and Mitochondrial Ribosomes

Preliminary studies showed that while the cytological and fine structural differentiation of embryos grown in the presence of low concentrations of inhibitors, which permit blastocyst formation in a high proportion of the embryos (Tables I and II), was essentially normal, there were characteristic differences from the control in the fine structure of mitochondria at the expanded blastocyst stage (Fig. 1). To further evaluate this effect, we followed the fine structural differentiation of mitochondria in control and inhibitor-treated embryos cultured *in vitro* from the two to four cell stage (day 1) to the blastocyst (day 4 and 5).

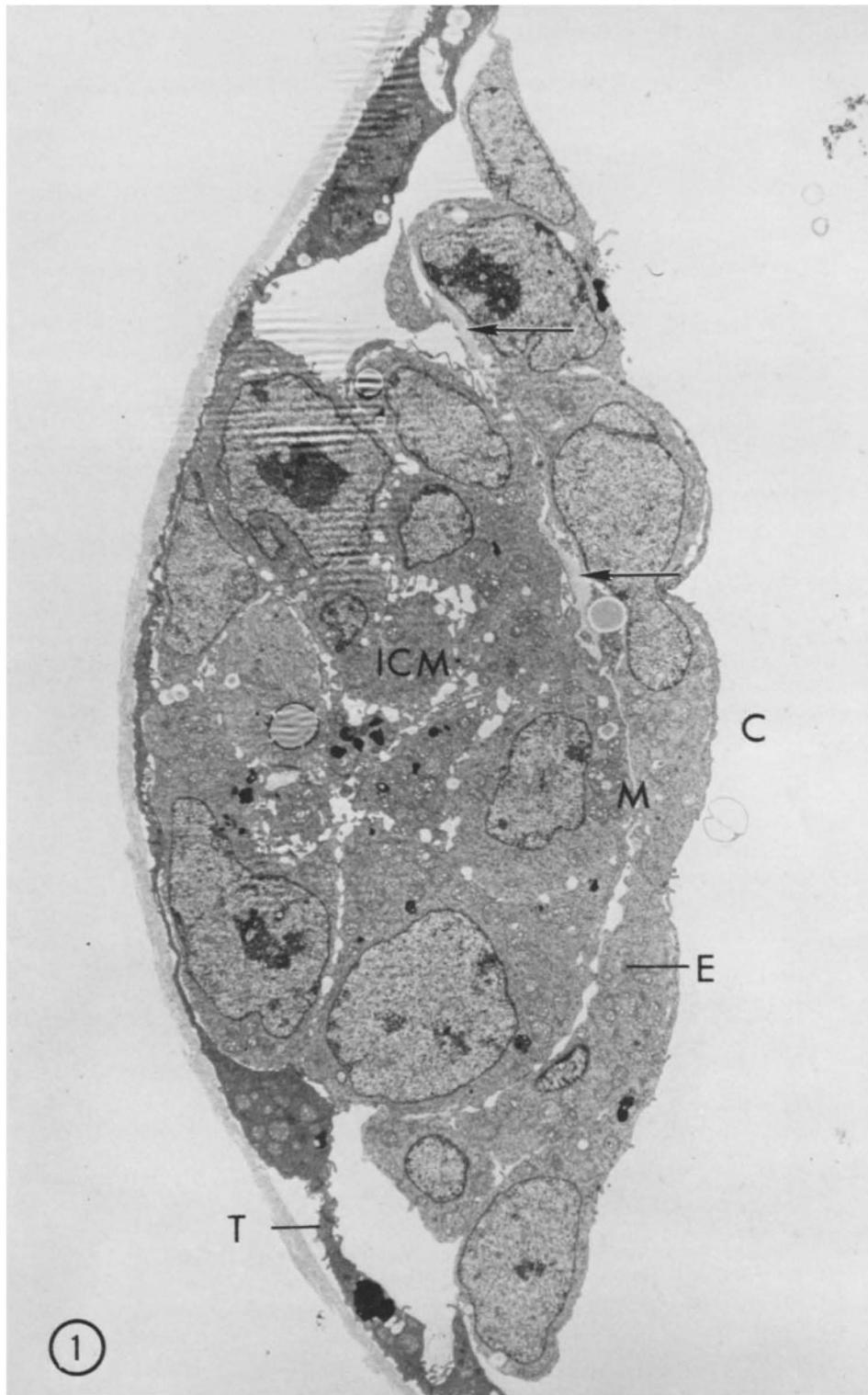
CONTROL EMBRYOS: During early development *in vitro* the mitochondria of control embryos undergo conspicuous changes in their fine structural appearance (see Figs. 5–8). In two to four cell embryos the mitochondria are small, vacuolated, with a very dense matrix and with few cristae which are often arranged concentrically

TABLE II
*Effect of Chloramphenicol on Embryo Development In Vitro**

Concentration $\mu\text{g/ml}$	Number of embryos	Blastocysts %	Inhibition‡ %
250	66	0	100
125	105	47	51
62.5	129	79	17
31.2	129	88	7
Control	168	95	0

* Embryos were cultured from the two to four cell stage (day 1) to the blastocyst stage (day 4) in the presence of different concentrations of chloramphenicol.

‡ Percent inhibition of blastocyst formation as compared with the control.



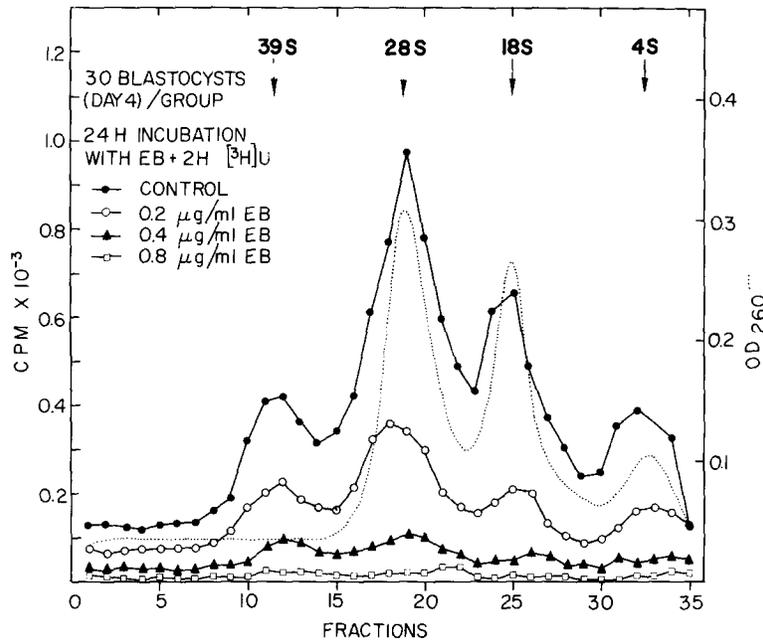


FIGURE 2 Effect of ethidium bromide on the sedimentation pattern of total cellular RNA extracted from day 4 blastocysts. The embryos were incubated in the presence of different concentrations of ethidium bromide for 24 h (day 3 to day 4) preceding a 2-h pulse with $[5\text{-}^3\text{H}]$ uridine ($200\ \mu\text{Ci/ml}$; $22.7\ \text{mCi}/\mu\text{mol}$). RNA was extracted and analyzed on a 5–20% sucrose gradient as described in Materials and Methods. Centrifugation was in the Beckman SW41 rotor at 40 krpm, 20°C , for 140 min. The OD_{260} peaks of the carrier RNA were coincident in the four samples, but only one tracing is shown (dotted line). Background counts have been subtracted from the counts per minute values shown in these and subsequent diagrams.

(Fig. 5). Beginning with the late four cell embryo the mitochondria progressively increase in diameter and decrease in matrix density (Fig. 6). These changes are particularly pronounced in the 16–32 cell embryo (morula to early blastocyst): the mitochondria are large and electron lucent but the number of cristae is still sparse (Fig. 7). In the mid-to-late blastocyst the mitochondria undergo an increase in matrix density and in the number of cristae. They also become quite pleomorphic in size and general appearance: some are rounded

and vacuolated; others are elongated with transverse cristae (Fig. 8).

The mitochondria of expanded blastocysts (day 4) contain numerous dense particles of about $150\text{-}\text{\AA}$ diameter, presumed to be mitochondrial ribosomes (Fig. 8). The particles are located in the intracristal space and are usually closely apposed to the cristal membrane. Their staining properties are similar to those of cytoplasmic ribosomes; their contrast can be increased under certain conditions of fixation and staining (Figs. 12–15).

FIGURE 1 Thin section through the inner cell mass of an expanded blastocyst (day 5 of culture) grown in the presence of $31.2\ \mu\text{g/ml}$ chloramphenicol. The mitochondria (*M*) are enlarged and spherical, with vesicular cristae. Cytological differentiation is normal. The trophoblast (*T*) consists of a single layer of cells surrounding the inner cell mass (*ICM*) and the blastocyst cavity (*C*). The cells of the endoderm (*E*) are more elongated and appear to be migrating out peripherally along the trophoblast. In some areas there is an accumulation of extracellular material, possibly the precursor of Reichert's membrane (arrows), between the endoderm and inner cell mass. $\times 4,000$.

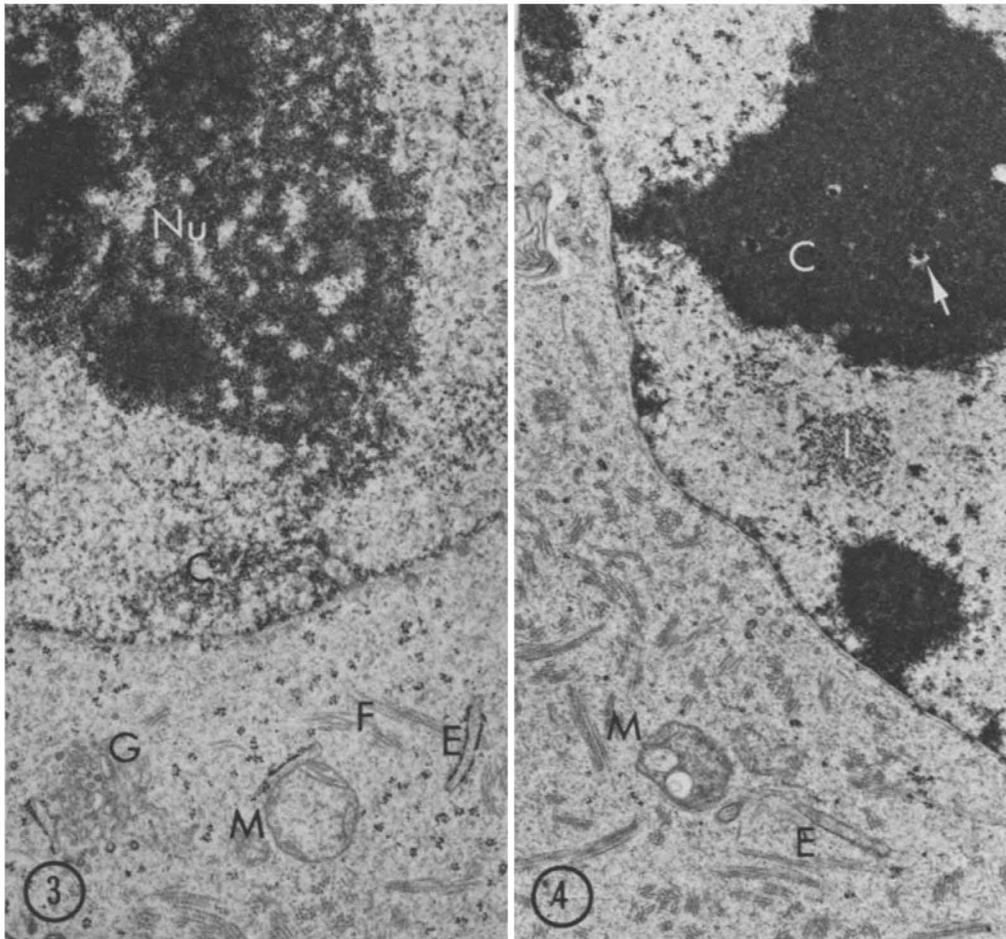


FIGURE 3 Portions of the nucleus and cytoplasm of a control morula of approximately 16 cells (day 3 of culture). The nucleolus (*Nu*) is well developed and forms a typical fibrillogranular network. Chromatin (*C*) is very sparse. In the cytoplasm a mitochondrion (*M*), a Golgi dictyosome (*G*), endoplasmic reticulum (*E*) with attached ribosomes, free polysomes, and fibrous yolk (*F*) are seen. $\times 17,000$.

FIGURE 4 Nuclear and cytoplasmic regions of an embryo cultured in the presence of $0.8 \mu\text{g/ml}$ ethidium bromide and arrested at the four to eight cell stage of development; it was fixed on the 3rd day of culture. The most characteristic nuclear features are irregular masses of dense chromatin (*C*) and clusters of interchromatin granules (*I*). Perichromatin granules (arrow) are also seen. In comparison with Fig. 3 note that the mitochondrion (*M*) in this embryo has a higher matrix density and that the number of free polysomes and ribosomes bound to endoplasmic reticulum (*E*) is smaller. $\times 17,000$.

There is a conspicuous change in the number of mitochondrial ribosomes during early development. At the two to four cell stage, their presence or absence cannot be ascertained because of the high matrix density of the mitochondria. Mitochondrial ribosomes are very sparse from the eight cell stage through the morula, but their number increases about 15-fold from the morula stage to the expanded (day 4) blastocyst (Figs. 12 and 13; Table IV).

EMBRYOS GROWN IN THE PRESENCE OF INHIBITORS: Low concentrations of inhibitors (0.1 or $0.2 \mu\text{g/ml}$ of ethidium bromide and 31.2 or $62.5 \mu\text{g/ml}$ of chloramphenicol) have no effect on the early fine structural changes in the mitochondria (enlargement and decrease in matrix density) that take place from the eight cell stage to the early blastocyst (Fig. 9). At the higher concentrations of ethidium bromide and chloramphenicol which stop embryo development at an early stage

TABLE III
Effect of Chloramphenicol on the Uptake and Incorporation of [¹⁴C]Amino Acids by Day 4 Blastocysts*

Concentration	Uptake	Incorporation	Inhibition of incorporation†
μg/ml	cpm per 20 blastocysts		%
250	10,272	1,171	55.2
125	12,837	2,153	17.6
62.5	13,338	2,251	13.9
31.2	12,852	2,778	0
Control	12,841	2,613	0

* Average of five experiments. Approximately 30 embryos per 100 μl culture drop were incubated in the presence of chloramphenicol for 15 min. Then 5 μl of a stock solution (total radioactivity 60 μCi/ml) of three uniformly labeled [¹⁴C]amino acids (L-lysine, L-valine, and L-aspartic acid) were added to each drop and incubation continued for 1 h. Uptake is total cpm; incorporation is acid-precipitable cpm. For details, see Materials and Methods.

† Percent reduction in the amount of incorporated radioactivity as compared with the control.

(after one to three cell divisions), the mitochondrial changes proceed to a stage which corresponds to that of control embryos of comparable cell numbers (see Fig. 4).

In contrast, the mitochondria of blastocysts (day 4 and 5) that have been grown in the presence of ethidium bromide or chloramphenicol show characteristic differences from control mitochondria. They enlarge and increase in matrix density, in a manner similar to the control. Subjectively, there also appears to be an increase in the number of cristae. However, the mitochondria are more rounded and the cristae are dilated and vesicular rather than flattened saccular structures as in the control (Figs. 10 and 11). The gross mitochondrial changes were essentially similar for the two inhibitors; there was no difference either between the two concentrations tested for each drug (0.1 and 0.2 μg/ml for ethidium bromide and 31.2 and 62.5 μg/ml for chloramphenicol). However, it should be noted that there was no complete uniformity in the mitochondrial changes of drug-treated embryos. Although most mitochondria showed the above changes in any one of the treated blastocysts, some essentially normal-appearing mitochondria were also present in each case, even at the higher concentrations of inhibitors.

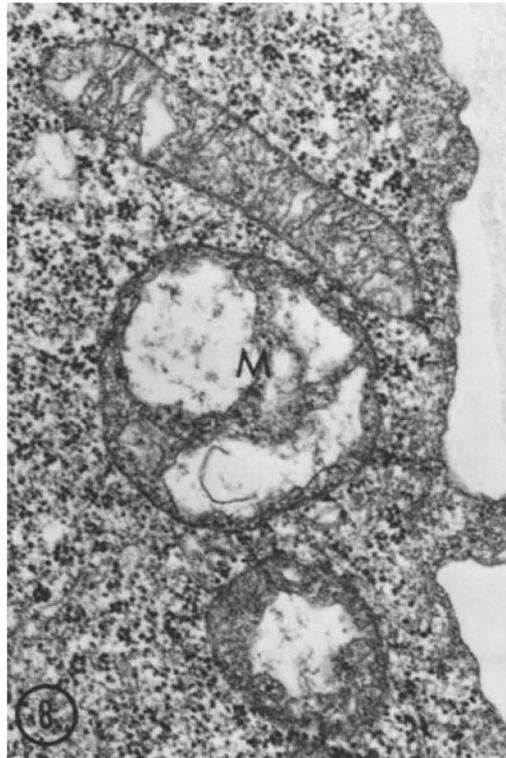
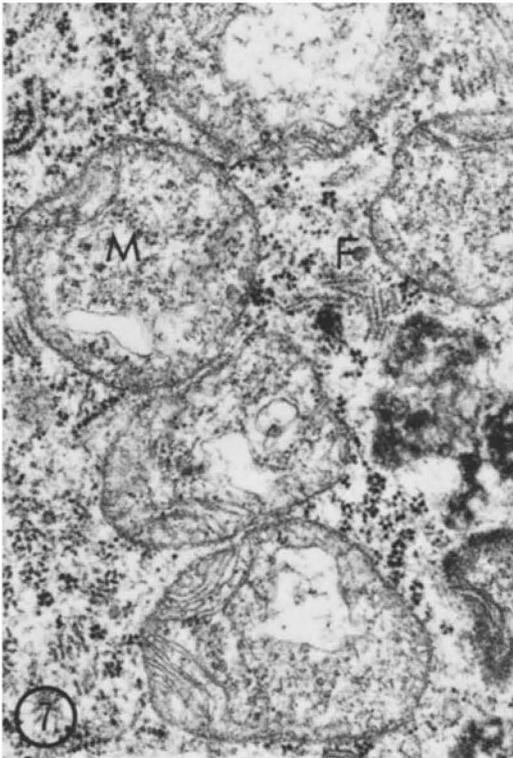
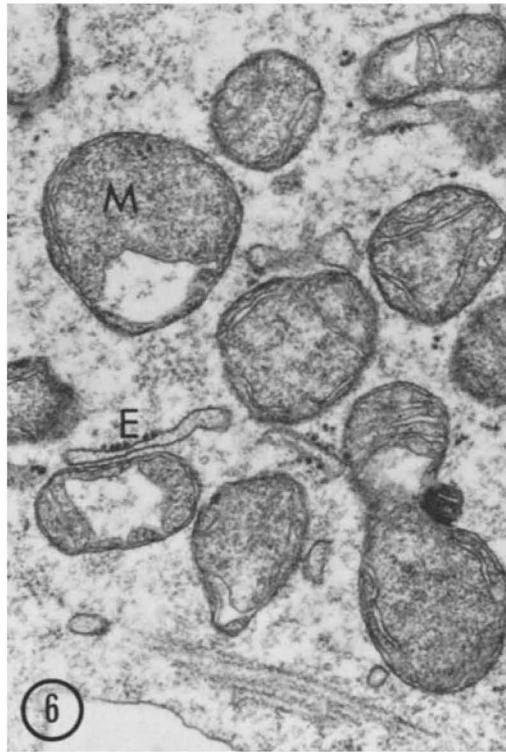
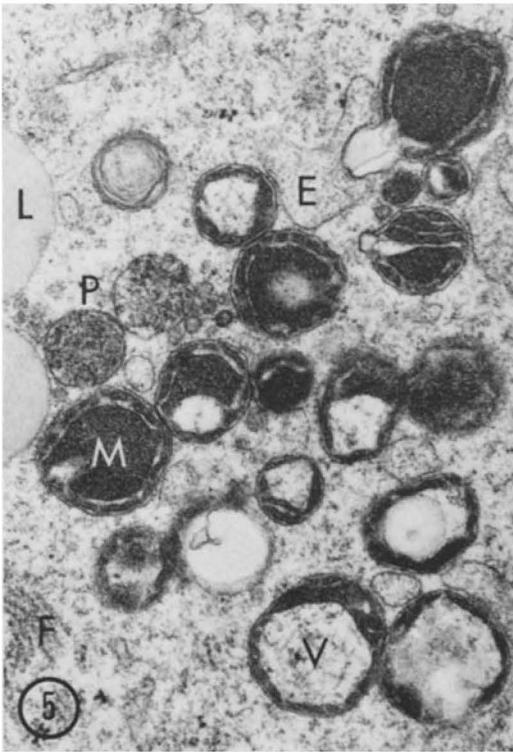
A consistent difference between the effect of ethidium bromide and that of chloramphenicol on blastocyst mitochondria was found regarding the number of mitochondrial ribosomes. In ethidium bromide-treated blastocysts the number of mitochondrial ribosomes is very low and is similar to that found in control morulae; on the other hand, in chloramphenicol-treated blastocysts mitochondrial ribosomes are nearly as numerous as in control blastocysts (Figs. 12–15 and Table IV). These observations indicate that the formation of new mitochondrial ribosomes is inhibited by ethidium bromide but is relatively unaffected by chloramphenicol.

Mitochondrial RNA- and Protein-Synthesis

These studies were aimed at obtaining further information on the functioning of the mitochondrial genome and its suppression by the inhibitors used. In particular, the pattern of mitochondrial RNA synthesis was examined in both control and inhibitor-treated embryos in order to determine whether the changes in the number of mitochondrial ribosomes were correlated with corresponding changes in RNA synthetic activity.

CONTROL EMBRYOS: When mouse embryos were cultured in the presence of [³H]uridine for 24 h, from the late morula (day 3) to the expanded blastocyst (day 4), the sedimentation profile of the RNA extracted from the mitochondrial fraction (Fig. 16a) revealed three major RNA species that became labeled, of 4S, 12S, and 16S, evidently representing transfer RNA and mitochondrial ribosomal RNA (2, 5, 6, 23, 24, 59). The mitochondrial origin of these peaks is indicated by the fact that their synthesis was essentially completely inhibited when the embryos were incubated under similar conditions except that the culture medium also contained 0.1 μg/ml of ethidium bromide (Fig. 16b).

In an attempt to determine the time of initiation of mitochondrial ribosomal and transfer RNA synthesis, mouse embryos were incubated with [³H]uridine, and the RNA extracted from the mitochondrial fraction was similarly analyzed, at the following stages of development: (a) 24-h labeling from the two to four cell to mid-eight cell (day 1 to day 2), (b) 24-h labeling from the mid-eight cell to late morula (day 2 to day 3), and (c) 10-h labeling from the mid-eight to late eight cell (day 2). In the first group there was no evidence of label in any of the mitochondrial



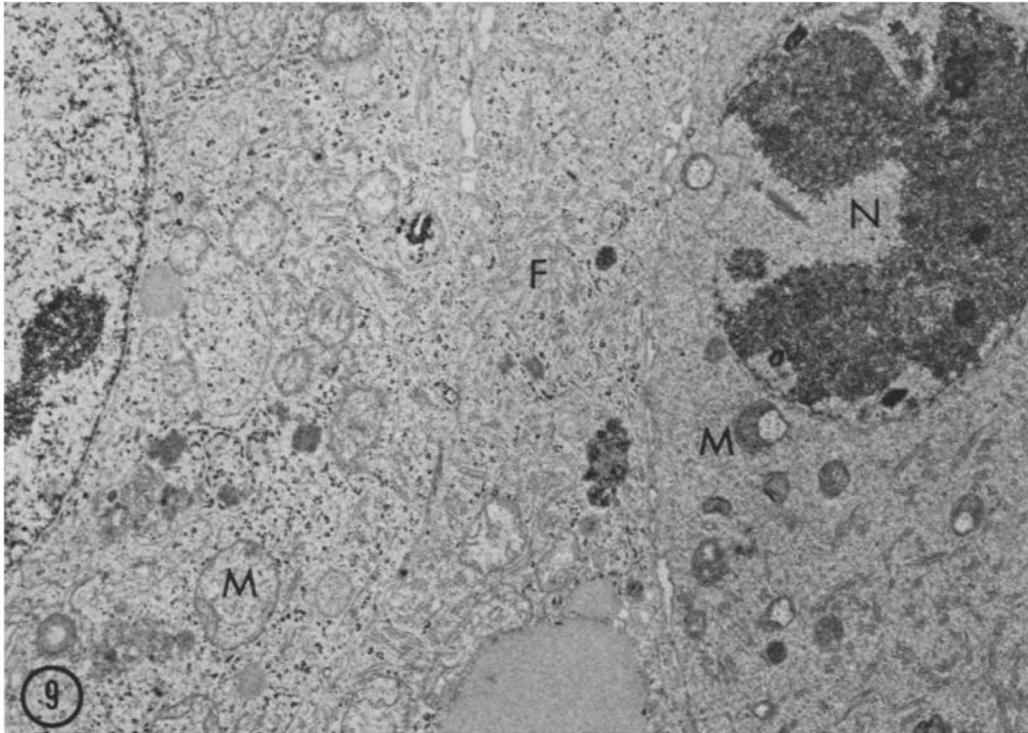


FIGURE 9 An early blastocyst grown in the presence of $0.2 \mu\text{g/ml}$ of ethidium bromide. On the right an undifferentiated cell, presumably a persistent polar body, containing small dense mitochondria (*M*) is shown. On the left, portions of the cytoplasm of normally differentiated cells are seen; they contain large, lucent mitochondria (*M*) typical for this stage. Other features to be noted are fibrillar yolk (*F*), polysomes which are essentially absent in the polar body, and the pycnotic polar body nucleus (*N*). $\times 10,000$.

FIGURES 5-8 Figs. 5-8 illustrate the fine structural changes in the mitochondria (*M*) of control mouse embryos cultured in vitro from the two to four cell stage to the blastocyst. $\times 38,000$.

FIGURE 5 The mitochondria of two-cell embryos (at the beginning of embryo culture) have few, often concentrically arranged, cristae, no visible ribosomes, and an exceptionally high matrix density. Intra-mitochondrial vesicles (*V*) present at this and later stages represent dilated cristae. Other features shown are granules presumed to be peroxisomes (*P*), lipid droplets (*L*), fibrous yolk (*F*), and endoplasmic reticulum (*E*) which is closely associated with the mitochondria.

FIGURE 6 The mitochondria of eight-cell embryos (fixed on the 2nd day of culture) are larger on the average than those of two-cell embryos and show a conspicuous decrease in matrix density; other features, including the sparseness of cristae and mitochondrial ribosomes and the association of mitochondria with endoplasmic reticulum (*E*), remain similar.

FIGURE 7 Mitochondrial matrix density continues to decrease while average size increases up to the early blastocyst stage shown here (day 3 of culture). Other mitochondrial features show little or no change. Fibrous yolk (*F*) is seen predominantly in cross-sections.

FIGURE 8 Mitochondria from a trophoblast cell of an expanded blastocyst fixed on the 4th day of culture. Large, round and slender, elongate mitochondrial profiles partially illustrate the morphological and size variations characteristic at this stage. Note that matrix density and number of both mitochondrial and cytoplasmic ribosomes have increased relative to the preceding stage.

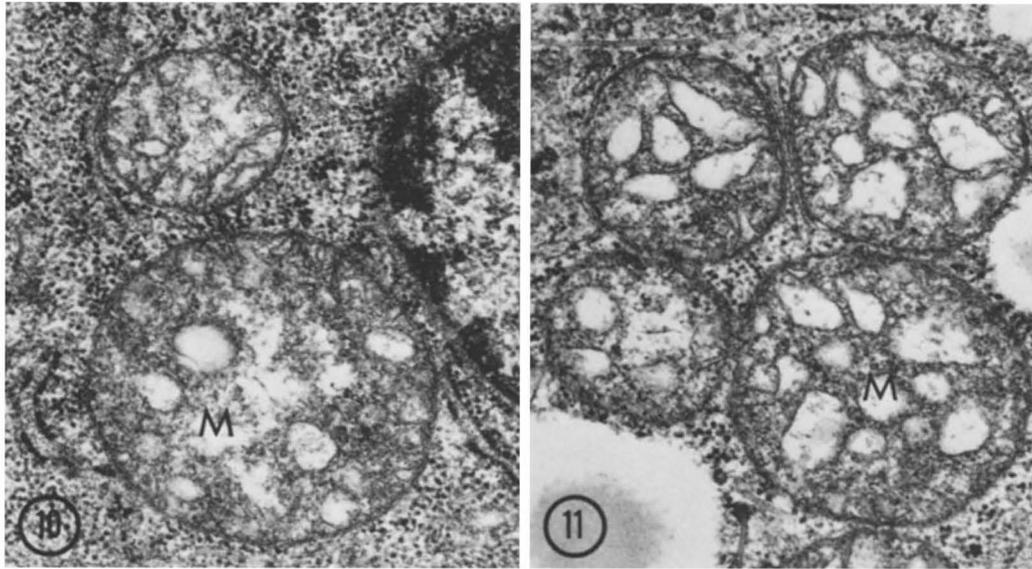


FIGURE 10 Mitochondria (*M*) in a trophoblast cell of a blastocyst grown in the presence of 0.1 $\mu\text{g}/\text{ml}$ ethidium bromide (day 4 of culture). The mitochondria are swollen and have predominantly vesicular cristae. There is a normal density of ribosomes in the cytoplasm but ribosomes are absent in the mitochondria (see also Fig. 14). $\times 38,000$.

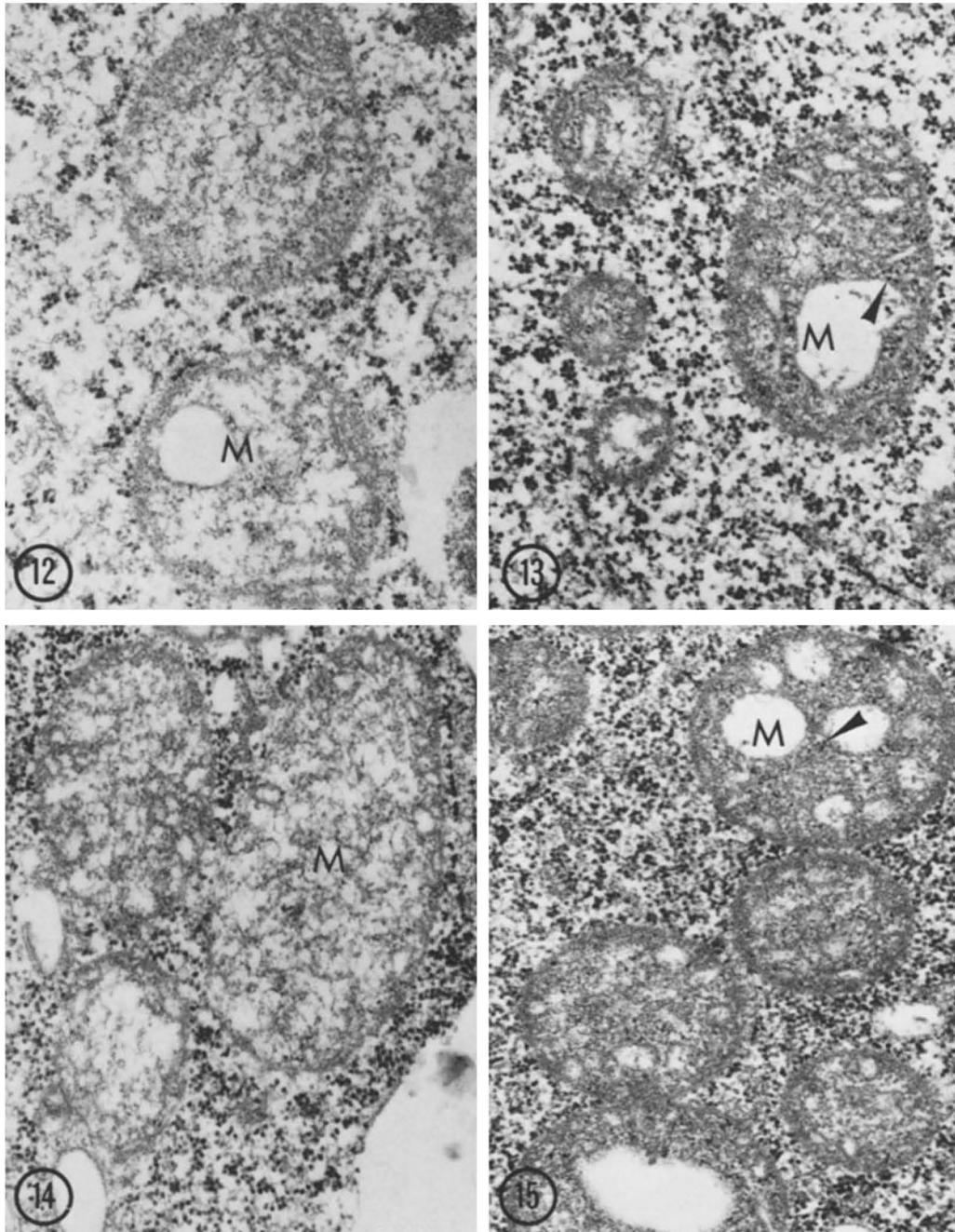
FIGURE 11 Mitochondria (*M*) in an inner-cell-mass cell of a blastocyst grown in the presence of 31.2 $\mu\text{g}/\text{ml}$ chloramphenicol (day 4 of culture). The mitochondrial cristae are dilated. Ribosome-like particles are discernible in the mitochondria (see also Fig. 15). $\times 38,000$.

RNA's (diagram not shown); in the second group, all three peaks (4S, 12S, and 16S RNA's) became labeled (Fig. 16 *c*); in the third group, there was label in 12S and 16S RNA but not in 4S RNA (Fig. 16 *d*). These results suggest that the synthesis of 12S and 16S ribosomal RNA begins earlier, apparently at the late eight-cell stage, than that of mitochondrial transfer RNA which is first detected at the morula stage.

EMBRYOS GROWN IN THE PRESENCE OF INHIBITORS: In a study of the effect of ethidium bromide and chloramphenicol on the synthesis of mitochondrial ribosomal RNA, control and inhibitor-treated embryos were labeled with [^3H]uridine for 24 h (day 3 to day 4) and the RNA extracted from the mitochondrial fraction was analyzed by acrylamide gel electrophoresis (Fig. 17). In the control embryos, besides residual 18S and 28S cytoplasmic RNA's, there are two peaks of radioactivity with relative electrophoretic mobilities of 16 s_E and 21 s_E , assumed to represent the 12S and 16S mitochondrial ribosomal RNA's (Fig. 17 *a*). A similar discrepancy between sedimentation coefficients and electrophoretic mobilities has been observed for the ribosomal RNA's

of other animal mitochondria and is presumably caused by changes in molecular conformation depending on the conditions of electrophoresis (6, 26, 69). The 16 s_E and 21 s_E peaks are also present in the mitochondria of blastocysts that have been cultured in the presence of 31.2 $\mu\text{g}/\text{ml}$ of chloramphenicol (Fig. 17 *b*); however, these peaks are absent in blastocysts that have been grown in the presence of 0.1 $\mu\text{g}/\text{ml}$ of ethidium bromide (Fig. 17 *c*).

The effects of chloramphenicol on mitochondrial fine structure (see above) suggest that (*a*) mitochondrial protein synthesis does play a role in the differentiation of blastocyst mitochondria and that (*b*) it is effectively inhibited by low concentrations of chloramphenicol. For an exploration of the extent of mitochondrial protein synthesis day 4 blastocysts (60–100 per group) were pulse-labeled for 1 h with a [^{14}C]amino acid mixture in the presence of the following inhibitors: (*a*) 100 $\mu\text{g}/\text{ml}$ cycloheximide, (*b*) 100 $\mu\text{g}/\text{ml}$ cycloheximide plus 31.2 $\mu\text{g}/\text{ml}$ chloramphenicol, (*c*) control. The conditions of incubation were the same as described in Table III. In four experiments the average percentage inhibition of incorporated



FIGURES 12-15 Figs. 12-15 illustrate the distribution of ribosomes in control embryos and in embryos treated with mitochondrial inhibitors. Structures interpreted as mitochondrial ribosomes are indicated by arrowheads. Postosmication was omitted and the sections were stained with uranyl acetate at 60°C for 1 h before lead citrate staining (see Materials and Methods). $\times 38,000$.

FIGURE 12 Control embryo at the morula stage (day 3 of culture). Ribosomes are moderately numerous in the cytoplasm. Few ribosomes are seen in the mitochondria (*M*).

FIGURE 13 Control blastocyst (day 4 of culture). Ribosomes are abundant in the cytoplasm and in the mitochondria (*M*).

FIGURE 14 Blastocyst grown in the presence of 0.1 $\mu\text{g/ml}$ ethidium bromide (day 4 of culture). There are numerous ribosomes in the cytoplasm but the mitochondria (*M*) are essentially devoid of ribosome-like particles.

FIGURE 15 Blastocyst grown in the presence of 31.2 $\mu\text{g/ml}$ chloramphenicol (day 4 of culture). There are many ribosomes in the cytoplasm and the mitochondria (*M*) also contain ribosome-like particles.

radioactivity was 96.4 ± 1.5 SD in the presence of cycloheximide and 96.8 ± 1.3 SD in the presence of both cycloheximide and chloramphenicol. Increasing the concentration of cycloheximide to

300 $\mu\text{g/ml}$ and that of chloramphenicol to 62.5 $\mu\text{g/ml}$ did not result in additional inhibition.

In another experiment, the mitochondrial fraction was isolated (see Materials and Methods) from blastocysts that had been similarly labeled with [^{14}C]amino acids for 1 h in the presence of either 100 $\mu\text{g/ml}$ of cycloheximide or 31.2 $\mu\text{g/ml}$ of chloramphenicol; a third group served as control. In chloramphenicol-treated blastocysts, radioactivity incorporated in the mitochondrial fraction was reduced only slightly while in the cycloheximide-treated blastocysts it was reduced by 86%, as compared with the control (average of two experiments). In the control blastocysts the mitochondrial fraction contained 4% of the total incorporated radioactivity, while in the cycloheximide-treated blastocysts this fraction contained 11%.

These results suggest that in day 4 blastocysts mitochondrial protein synthesis is of a low level

TABLE IV
Mitochondrial Ribosomes in Control and Inhibitor-Treated Embryos

Stage of development	Inhibitor	Ribosomes per μm^2 (Average \pm SEM)
Day 3 morula	Control	5.7 ± 0.7
Day 4 blastocyst	Control	91.4 ± 5.4
Day 4 blastocyst	EB \ddagger	4.8 ± 0.9
Day 4 blastocyst	CAP*	56.0 ± 3.3

\ddagger , * Embryos were grown from the two to four cell stage on (day 1) in the presence of 0.1 $\mu\text{g/ml}$ ethidium bromide (EB) and 31.2 $\mu\text{g/ml}$ chloramphenicol (CAP), respectively.

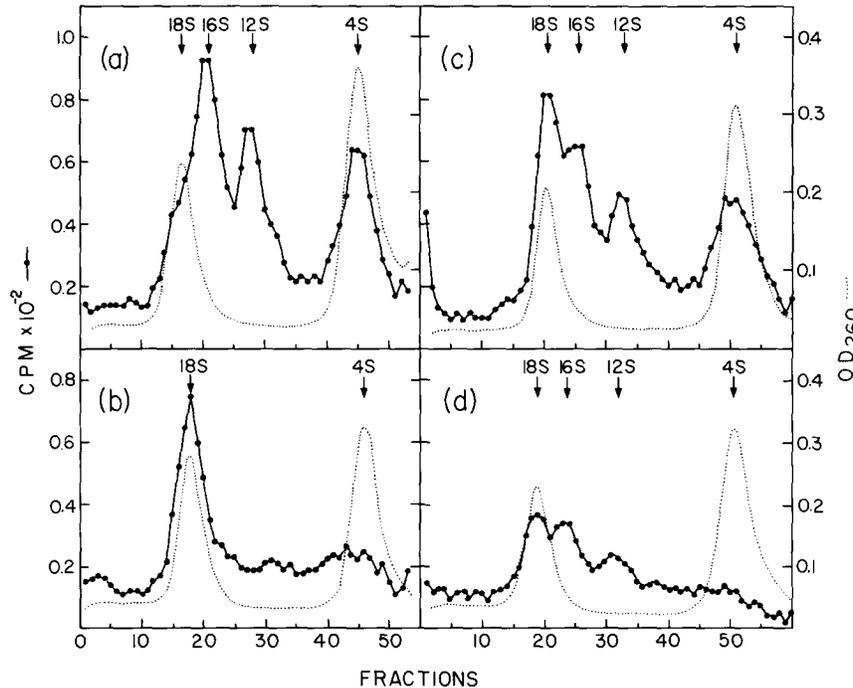


FIGURE 16 Sedimentation pattern of RNA extracted from the mitochondrial fraction of mouse embryos labeled with [^3H]uridine at different stages of development. From 150 to 200 embryos per group were cultured in the presence of [^3H]uridine (200 $\mu\text{Ci/ml}$, 22.7 $\text{mCi}/\mu\text{mol}$) for the following periods: (a) 24-h labeling from the late morula (day 3) to the expanded blastocyst (day 4), (b) experimental conditions as in (a) except that ethidium bromide was added to the culture medium at a concentration of 0.1 $\mu\text{g/ml}$ 30 min preceding the incubation with [^3H]uridine, (c) 24-h labeling from the mid-eight cell (day 2) to the late morula (day 3), (d) 10-h labeling from the mid-eight cell to the late-8 cell (day 2). Isolation of mitochondria, extraction of RNA, and analysis on a 5–20% sucrose gradient are described in Materials and Methods. Centrifugation was in the Beckman SW41 rotor at 40 krpm, 20°C, for about 6 h. The OD $_{260}$ peaks (dotted line) derive from 18S and 4S carrier RNA's isolated from mouse liver.

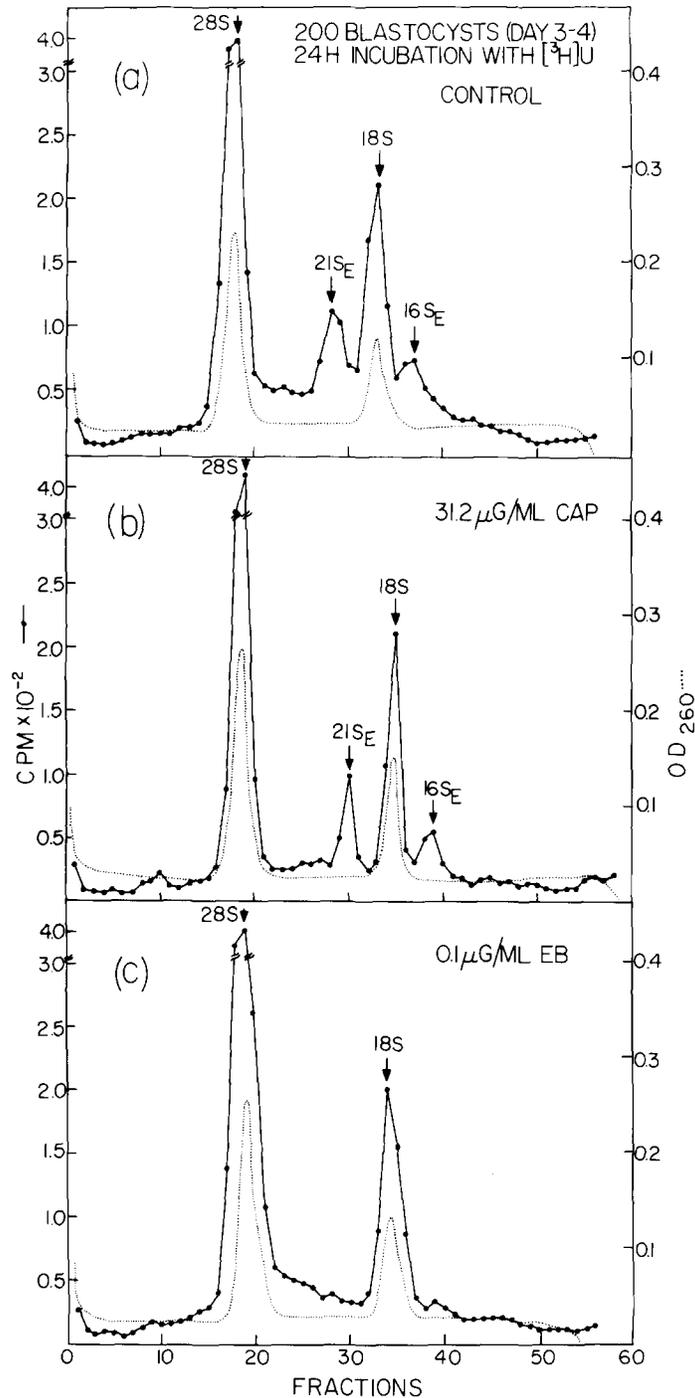


FIGURE 17 Acrylamide gel electrophoresis patterns of RNA's extracted from partially purified mitochondria of control blastocysts (a) and of blastocysts grown from the two to four cell stage in the presence of 31.2 $\mu\text{g}/\text{ml}$ chloramphenicol (b) and 0.1 $\mu\text{g}/\text{ml}$ ethidium bromide (c). About 200 embryos in each group were labeled with [³H]uridine (200 $\mu\text{Ci}/\text{ml}$, 22.7 $\text{mCi}/\mu\text{mol}$) for 24 h (day 3 to day 4); inhibitors continued to be present in groups b and c. Isolation of mitochondria, extraction of RNA and analysis by gel electrophoresis are described in Materials and Methods. The 4S RNA ran off the gels under the conditions of electrophoresis used. The OD₂₆₀ peaks (dotted line) derive from 28S and 18S carrier RNA's isolated from mouse liver.

TABLE V
Effect of Ethidium Bromide and Chloramphenicol on
Postimplantation Development*

Inhibitor	Number of blastocysts	Number of implantations†		
		Live	Resorbing	Total
Ethidium bromide	30	8	4	12
Chloramphenicol	27	9	1	10
Control	36	11	2	13

* Embryos were cultured from the two to four cell stage (day 1) to the blastocyst stage (day 4) in the presence of 0.1 $\mu\text{g/ml}$ of ethidium bromide and 31.2 $\mu\text{g/ml}$ of chloramphenicol, respectively. The blastocysts were then transplanted into the uteri of foster mothers.

† Number of clearly recognizable uterine swellings on day 17 of pregnancy.

and contributes less than 1% of the total cellular protein synthesis. This is roughly comparable to the level of mitochondrial protein synthesis in HeLa cells estimated at about 0.5% of total cellular protein synthesis (9, 63). The approximately 3% residual incorporation that is resistant to the combined effect of cycloheximide and chloramphenicol may be due to cycloheximide-resistant incorporation by the cytoplasmic system as has been observed in HeLa cells (64).

Embryo Development In Utero

Table V shows the capacity of inhibitor-treated blastocysts to undergo normal development when transplanted into the uteri of foster mothers. Day 4 blastocysts that had been cultured in vitro from the two to four cell stage in the presence of ethidium bromide (0.1 $\mu\text{g/ml}$) or chloramphenicol (31.2 $\mu\text{g/ml}$), as well as control blastocysts, were transferred as described in Materials and Methods. The recipient females were sacrificed 2 wk later and the uteri inspected for embryo development. There is no significant difference in the number of implantation sites or the number of live fetuses in the three groups.

DISCUSSION

Mitochondrial Differentiation in Control Embryos

The fine structural changes observed in the present study in the mitochondria of control

embryos developing in vitro from the two to four cell stage to the blastocyst are in general agreement with the changes reported for preimplantation mouse embryos developing in vivo (15, 51, 76) or in vitro (40). Early and late changes in mitochondrial morphology can be distinguished. The first, from the late four cell stage through the early blastocyst, is characterized by a progressive enlargement of the mitochondria; the latter, from the early to the late blastocyst, involves an increase in matrix density and the number of cristae, as well as considerable heteromorphy as to mitochondrial size and shape. The agreement with the earlier findings suggests in itself that these changes are real and not due to artifacts of fixation and handling. This is further corroborated in the present study by the coexistence within the same embryo (Fig. 9) of an undifferentiated cell (presumably a persistent polar body) with dense vacuolated mitochondria, on the one hand, and differentiated cells typical for the early blastocyst stage having greatly enlarged electron-lucent mitochondria, on the other.

Biochemical evidence indicates that the changes in mitochondrial fine structure at the four to eight cell stage coincide with metabolic changes. Oxygen consumption, which is low in the one to four cell mouse embryo, begins to rise at the eight cell stage and increases by about 3.5-fold in the blastocyst (54). In addition, the number of energy substrate compounds that can support development into blastocyst increases at the eight cell stage (14, 82). The increased utilization of several TCA-cycle intermediates (malate, citrate, and 2-oxoglutarate) at the four to eight cell stage has been attributed, in part, to permeability changes and an increase in mitochondrial transport (43, 80). Changes in the activities of several key enzymes, such as hexokinase, have also been observed and are probably involved in the increase of metabolic activity as cleavage proceeds (13).

The present study brings to light another aspect of mitochondrial differentiation in the early mouse embryo; namely, an approximately 15-fold increase in the number of mitochondrial ribosomes between the morula stage and the blastocyst (day 4). The sparseness of mitochondrial ribosomes in the early cleavage stages suggests that the mitochondria are capable of very little intrinsic protein synthesis during this period. The biochemical data indicate that mitochondrial ribosomal RNA, but not transfer RNA, is first

synthesized in the late eight cell embryo; the possibility that the precursor is not taken up by the mitochondria or that the RNA products are rapidly degraded up to this stage has not been excluded. Mitochondrial ribosomal RNA, as well as transfer RNA, are synthesized and accumulate from the morula stage through the blastocyst with a concomitant increase in the number of mitochondrial ribosomes. Therefore, the capacity for mitochondrial protein synthesis is greatly enhanced in the blastocyst. Throughout this period mitochondrial DNA synthesis is either absent or of a low level in the mouse embryo (66). These data suggest that one important function of the mitochondrial genome in the early mouse embryo is to contribute, by the synthesis of ribosomal RNA and transfer RNA, to the establishment of a mitochondrial protein-synthesizing system. The increase in the mitochondrial ribosome population lags about two cell cycles behind the accumulation of cytoplasmic ribosomes which begins at the four to eight cell stage (15, 28, 40, 55, 66, 83).

A similar general pattern of mitochondrial nucleic acid synthesis was observed during early embryogenesis of *Xenopus laevis*. In this species mitochondrial RNA- and DNA-synthesis is absent up to the gastrula stage when synthesis of mitochondrial ribosomal RNA (but not 4S RNA) begins; mitochondrial DNA content and total mitochondrial mass begin to increase in the swimming tadpole (19). In the sea urchin, mitochondrial RNA synthesis occurs both during normal cleavage and in activated nonnucleate fragments (16, 17, 20, 39, 71); however, mitochondrial DNA synthesis appears to be absent during early development (38, 65).

Mitochondrial Differentiation and Embryo Development in the Presence of Ethidium Bromide and Chloramphenicol

The main question the present study has sought to answer is to what extent the morphological and biochemical differentiation of the mouse embryo is determined by the functioning of the mitochondrial genome. It was important, therefore, to ascertain that the concentrations of ethidium bromide and chloramphenicol used specifically inhibit mitochondrial RNA- and protein-synthesis without affecting the nuclear and cytoplasmic systems. Concentrations of

ethidium bromide of 0.4 $\mu\text{g/ml}$ and higher, which caused an arrest of embryo development (Table I), also suppressed total cellular RNA synthesis (Fig. 2) and resulted in an abnormal nuclear fine structure characterized by an excessive clumping of chromatin (Fig. 4). Chromatin clumping has been described in cultured rat embryo cells (72) and sea urchin embryos (36) grown in the presence of ethidium bromide. On the other hand, a concentration of 0.1 $\mu\text{g/ml}$ of ethidium bromide, used in most of these studies, had virtually no effect on the amount and pattern of nuclear RNA synthesis, nuclear fine structure, and the percentage of blastocyst formation; this concentration was nevertheless effective in inhibiting the synthesis of mitochondrial ribosomal RNA (Fig. 17 c) and also prevented an increase in the number of mitochondrial ribosomes (Fig. 14 and Table IV).

A similar preferential inhibition of mitochondrial RNA synthesis by ethidium bromide has been observed in HeLa cells (2, 84) and sea urchin embryos (17, 21). In addition, ethidium bromide was found to preferentially inhibit mitochondrial DNA synthesis in cultured mammalian cells (57, 73) and slime mold (41) and to reversibly inhibit the accumulation of cytochrome oxidase in cultured human fibroblasts (59) and HeLa and L cells (42). The mechanism for the effect of ethidium bromide on the mitochondrial genetic system is not known, but it is probably due, at least in part, to a preferential binding of this drug to mitochondrial DNA in vivo (3, 73).

The concentration of chloramphenicol, 31.2 $\mu\text{g/ml}$, used in this study had no effect on the total uptake and incorporation of amino acids (Table III) or on embryo development from the two to four cell stage to the blastocyst (Table II). The effect of cycloheximide and chloramphenicol on amino acid incorporation by whole embryos and by the mitochondrial fraction suggests that mitochondrial protein synthesis in day 4 mouse blastocysts amounts to less than 1% of total cellular protein synthesis. It is likely that a concentration of 31.2 $\mu\text{g/ml}$ of chloramphenicol inhibited most or all of the mitochondrial protein synthetic activity for the following reasons. First, the fine structural changes in the mitochondria of blastocysts grown in the presence of chloramphenicol were similar to those produced by ethidium bromide treatment (see below). Second, similar concentrations of chloramphenicol were

shown to be effective in inhibiting mitochondrial protein synthesis in isolated rat liver mitochondria (33) and HeLa cells (34) and in inducing a loss of insoluble mitochondrial cytochromes in regenerating rat liver (27, 31) and in HeLa and L-cell cultures (29, 30, 42, 77). The reduction in the rate of protein synthesis and blastocyst formation in the presence of higher concentrations of chloramphenicol (62.5 $\mu\text{g/ml}$ and above) in this study may be attributed to a direct inhibitory effect on mitochondrial respiration by chloramphenicol as observed in rat liver mitochondria (32), HeLa cells (30), and, using very high doses of chloramphenicol, in cleaving frog embryos (45).

The fine structural effects of 0.1 $\mu\text{g/ml}$ of ethidium bromide and 31.2 $\mu\text{g/ml}$ of chloramphenicol are similar and are observed exclusively in the mitochondria of mid-to-late blastocysts (day 4 and day 5) which show an otherwise normal pattern of cytological differentiation (Fig. 1). The mitochondria are somewhat swollen and their cristae are dilated and are often vesicular rather than flattened saccular structures as in control mitochondria (Figs. 10 and 11). These changes are of the same general type as those observed under the influence of ethidium bromide and chloramphenicol in various tissue culture cells (42, 46, 57, 75) and are consistent with the hypothesis that the products of mitochondrial protein synthesis are indispensable for the structural and functional organization of mitochondrial cristae (5, 44, 70). However, no reduction in the number of cristae was evident in the present study, in contrast to the depletion of mitochondrial cristae in *Paramecium* (1) and *Ochromonas* (74) after exposure to chloramphenicol.

Unlike ethidium bromide treatment, chloramphenicol had little effect on the synthesis of mitochondrial ribosomal RNA and the formation of mitochondrial ribosomes (Figs. 15 and 17*b*; Table IV). A lack of inhibition of mitochondrial ribosome formation by chloramphenicol was also observed in the alga *Ochromonas danica* (74). This is in agreement with the observation that mitochondrial protein synthesis is not required for the maintenance of mitochondrial nucleic acid synthesis in HeLa cells (77) and that probably all proteins of mitochondrial ribosomes are synthesized in the cytoplasm, for example, in *Neurospora* (48) and in HeLa cells (9).

In contrast to the mitochondria of expanded blastocysts, low concentrations of ethidium bromide and chloramphenicol had no effect on the fine structural changes of mitochondria from the four to eight cell stage to the early blastocyst. These changes involve a considerable enlargement of the mitochondria and loss of matrix density but no apparent increase in the number of cristae or mitochondrial ribosomes. The lack of inhibitor effect, together with the scarcity of mitochondrial ribosomes, suggests that mitochondrial RNA- and protein-synthesis does not play a role in these early transformations but rather that they are determined by cytoplasmic factors. A close coupling between the mitochondrial changes and cellular differentiation is indicated by the fact that when embryo development is arrested during cleavage by high concentrations of ethidium bromide and chloramphenicol, the fine structural transformation of the mitochondria stops at a level corresponding to the developmental stage (cell number) reached rather than to the age of the embryo. The same observation is true when embryo development is blocked by a low concentration (0.1 $\mu\text{g/ml}$) of actinomycin D (Pikó and Chase, unpublished observation). The mechanism of cytoplasmic control is obscure, but the fact that during early development the mitochondria are typically found in close apposition to rough endoplasmic reticulum suggests that the synthesis of new proteins by the cytoplasmic system is involved.

The effect of ethidium bromide and chloramphenicol on blastocyst mitochondria is fully reversible, since the treated blastocysts develop normally in uterine foster mothers in the same proportion as do control embryos (Table V). Tissue culture cells also show full recovery even after several weeks of exposure to these inhibitors (42). These findings suggest that the mitochondrial DNA is not lost or altered in its primary structure in these cells as a result of ethidium bromide and chloramphenicol treatment as it has been observed in cytoplasmic *petite* mutants of yeast induced by these drugs (37, 56, 62, 81). Physicochemical analysis also shows that the mitochondrial DNA is preserved intact in mammalian cells treated with ethidium bromide (73, 57). However, these studies do not exclude the possibility that treatment by ethidium bromide and chloramphenicol may be mutagenic in a small proportion of mitochondria. In the mouse em-

bryo there is evidence that only a few cells of the inner cell mass are needed for normal embryo development to occur (35, 47). Therefore, even if the mitochondrial population had been replaced by deficient mitochondria in some cells of the blastocyst, with the resultant death of these cells, normal embryogenesis could have occurred.

Role of the Mitochondrial Genome during Early Development

In the light of the above observations, the following model may be proposed on the function of the mitochondrial genome during early development in mice. The small, dense mitochondria of the one to four cell stage which have a low level of metabolic activity are also rather inactive genetically: mitochondrial RNA-, DNA- and protein-synthesis is either absent or of a low level. The fine structural and metabolic changes in the mitochondria from the late four cell stage to the early blastocyst appear to be controlled entirely by the nucleo-cytoplasmic system. During this period mitochondrial RNA synthesis becomes activated and contributes to the establishment of a mitochondrial protein synthesizing system which is very limited or absent at the early cleavage stages. In the blastocyst the growth of mitochondrial membranes begins and requires mitochondrial protein synthesis for the normal structural organization of mitochondrial cristae. The probable absence of mitochondrial DNA synthesis (66) and the progressive nature of the fine structural changes in the mitochondria up to this time suggest that mitochondrial differentiation is due to the transformation of pre-existing mitochondria. Mitochondrial DNA synthesis and mitochondrial replication are expected to begin at the time of implantation when the embryo begins to grow in mass. In the rat embryo oxidative metabolism becomes particularly active at 10-14 days of embryo development, at the time of organogenesis (50).

The results of the present study argue against the possibility that the mitochondrial genetic system has a direct role in normal development and cellular differentiation in the early mouse embryo, for example, by exporting specific morphogenetic substances in the form of RNA or proteins. A similar conclusion can be drawn from the relative lack of effect of moderate concentrations of ethidium bromide on sea urchin

embryogenesis (8, 21), and the lack of effect of chloramphenicol, except at very high doses, on the early development of amphibian embryos (45). The latter finding also agrees with the results of biochemical studies which detected mitochondrial genetic activity in *Xenopus* first at the time of gastrulation (19).

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