MITOCHONDRIA AND THE DEVELOPMENT OF SEA URCHIN EMBRYOS

Thesis by

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to Dr. Albert Tyler

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ABSTRACT

After artificial activation or fertilization of non-nucleate fragments or eggs of the sea urchin, the mitochondria actively synthesize RNA. The RNA made in non-nucleate fragments is shown to be mostly single stranded and to be associated primarily with the low speed pellet of centrifuged cellular homogenates.

Protein synthesis is observed in non-nucleate fragments in the presence or absence of the mitochondrial RNA synthesis: it is found to be qualitatively similar but quantitatively less in the absence of the RNA synthesis. The continued syntheses of proteins in the non-nucleate fragments in the absence of mitochondrial RNA synthesis provides additional evidence for the presence of a stable messenger RNA component in the unfertilized sea urchin egg.

Since the uptake of actinomycin D was found to be inhibited by the presence of a fertilization membrane, ethidium bromide, at 10 µgs/ml, is used as an effective inhibitor of RNA synthesis in non-nucleate fragments and in early cleavage stage embryos. However, this same concentration of ethidium bromide is found to be only partially effective in blocking RNA synthesis at the mesenchyme blastula stage of development.

Low concentrations of ethidium bromide (2 & 5 µgs/ml) are found not to be lethal but to be capable of producing moderate developmental defects. In the presence of

concentrations of ethidium bromide adequate to inhibit all the mitochondrial RNA synthesis (10 µgs/ml of ethidium bromide), from fertilization on, the embryos do not cleave beyond the 4-8 cell stages. When similar concentrations of ethidium bromide are added at an early mesenchyme blastula stage, the embryos do not gastrulate but continue to swim for more than 24 additional hours (adequate for control embryos to develop to a late prism stage). These results lead to the conclusion that mitochondrial RNA synthesis may be very essential for normal development to occur.

DNA is synthesized in the non-nucleate fragments of sea urchin eggs. None of the newly synthesized DNA is found in the closed circular form. When phenol extracted directly from the fragments, the DNA is found to sediment at approximately 38 and 27s in sucrose gradients but neither of these size classes could be found associated with the isolated mitochondria. The template for the synthesis of DNA in non-nucleate fragments remains unknown.

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INTRODUCTION

INTRODUCTION

In sea urchins, and in other animals, the mature eggs contain untranslated ("maternal" or "masked") messenger RNA (mRNA) and the RNA synthesized immediately after fertilization is believed to be unnecessary for the development of embryos to the blastula stage (1-7). This hypothesis has been partially dependent upon studies with actinomycin D (1,2) and partially upon studies with non-nucleate fragments of sea urchin eggs (8-10).

The presence of DNA in sea urchin mitochondria (11,12) and the possible existence of other forms of cytoplasmic DNA, lead one to inquire as to their contributions to biosynthetic processes within the cell, and in the case of the sea urchin, to their contributions to the developing embryo. RNA, homologous to cytoplasmic DNA, synthesized in blastula and gastrula stage embryos has been reported (13). The synthesis of RNA has been detected in non-nucleate fragments of sea urchin eggs (14-16).

However, the relationships of the RNA synthesized by mitochondria to the stable mRNA component of the eggs and/or to the processes of early development have not been reported.

This thesis deals with a study of the DNA, RNA and protein synthesis in non-nucleate fragments and embryos from sea urchin eggs. Part A of the introduction reviews the

present state of knowledge concerning RNA and protein synthesis in eggs, before and after fertilization. Part B reviews some of the present information concerning mitochondrial DNA, RNA and protein synthesis. Part C briefly describes the non-nucleate fragment as a system for the study of mitochondrial activity.

Part A - RNA and Protein Synthesis in Sea Urchin Eggs During Oogenesis and Immediately After Fertilization

Relative Inertness of the Mature Unfertilized Egg

Unlike the eggs of many other animals, the mature sea urchin egg possesses a spherical haploid nucleus rather than a swollen diploid germinal vessicle. In the absence of fertilization, sea urchin eggs may remain viable and fertilizable for several days (17). The incorporation of amino acids into protein in unfertilized sea urchin eggs is of very low magnitude (for references see 18-23).

RNA has also been reported to be synthesized in unfertilized eggs of the sea urchin (24). The newly synthesized RNA has been shown to sediment heterogeneously and similarly to the newly synthesized RNA obtained from fertilized eggs. The low values obtained for the incorporation of RNA precursors may be attributable primarily to very low uptake

(25, 26) rather than to low turnover rates, since practically all the precursor that enters is rapidly incorporated into RNA.

Fertilization

Fertilization is followed by an immediate increase in protein synthetic rates (27-30). A number of changes which occur immediately prior to this increase in protein synthesis have been observed. Among them are; 1) a breakdown of the cortical granules; 2) the elevation of a fertilization membrane; 3) a slight decrease of egg volume; 4) an increase in permeability of the surface membranes; 5) an increase in exchange of ^{32}P with the medium; 6) an increase in K^+ exchange; 7) a diffusion of Ca^{++} out of the eggs; 8) the production of acid during the first few minutes after fertilization; 9) an activation of NAD kinase; 10) increased respiration; 11) an increase in the activity of protealytic enzymes; 12) and an increased activity of the soluble transfer factors (for references see 20, & 31-33). Any one or more of these factors may be directly or indirectly related to the observed increase in protein synthesis.

Evidence for a Stable ("Masked") mRNA

The stimulation of protein biosynthesis following fertilization is further complicated by the evidence in favor of a pre-existing "masked" mRNA being the template for the majority of the early protein synthesis (1-10). Of primary concern to this thesis are the studies showing comparable levels of protein synthesis in artificially activated non-nucleate fragments and fertilized eggs (8-10, 34 & 35) and the studies showing continued protein synthesis in the presence of sufficient actinomycin D to inhibit most of the RNA synthesis (1, 2).

Unmasking the "Masked" mRNA

The unmasking of the "masked" mRNA in the cytoplasm of the mature eggs following fertilization has been suggested to be responsible for the observed increase in protein synthesis. At this time, the mechanism of masking and unmasking is a highly debated question.

Early work indicated that the inactivity of the mRNA might be a result of a translational inefficiency of the ribosomes which was eliminated by proteolytic activity (36-39). The proteolytic activity may have been stimulated by the ionic changes which occur upon fertilization (19, 21).

There is other evidence that the increased activity of the transfer factors associated with the ribosomes may be responsible for the differences in ribosomal activity before and after fertilization (33).

Spirin and Nemer (40) and Infante and Nemer (41) have suggested that the mRNA is released from subribosomal protein-RNA complexes, called informasomes, permitting their translation into proteins.

Others have suggested that the polysomes themselves are masked and that the unmasking permits immediate translation to follow (42, 43).

Annulate lamellae structures, containing ribosomal or polysomal appearing particles, have been observed in the cytoplasm of unfertilized eggs but disappear after fertilization (44).

Evidence has also been presented supporting the idea that the masking is accomplished via the control of chain initiation through changes in the available transfer RNA's (45-49).

RNA Synthesis Immediately After Fertilization

None of the RNA synthesized before the mesenchyme blastula stage of development seems to be of ribosomal types (50-54). Also, none of the RNA synthesized after

fertilization seems to be necessary for development to the blastula stage (1-7). Competition hybridization studies have shown that there is a change in the types of RNA's synthesized in sea urchin embryos before and after gastrulation (55, 56).

Part B - Mitochondrial DNA, RNA and Protein Synthesis

Mitochondrial DNA Synthesis

The mechanisms of DNA replication and mitochondrial biogenesis are not presently resolved. However, the synthesis of DNA has been reported in isolated mitochondria from slime molds (57) and in mitochondria from rat liver (58). Evidence for the semiconservative replication of the mitochondrial DNA has also been reported (59). However, whether mitochondrial biogenesis transpires via the fission of pre-existing mitochondria (60) or via the assemblage of "promitochondria" (61) or via some other mechanism is not known.

Mitochondrial RNA Synthesis

In neurospora (62-65) and in Hamster cells (66) evidence has been presented in favor of the existence of ribosomal

RNA's, in mitochondria, which are distinctly different from the 28 and 18s RNA's which are found in other cellular ribosomes.

The mitochondria in rat liver (67) and in neurospora (68, 69) have been shown to possess tRNA's differing from the analogous tRNA's utilized in the non-mitochondrial protein synthetic system.

RNA, with characteristics of messenger RNA, is synthesized on the mitochondrial DNA template in Hela cells (70-73), in sea urchins (13, 16) and in protozoans (74).

Mitochondrial Protein Synthesis

There is evidence that the cytoplasmically derived mutant "poky" of neurospora (75) produces a mutant membrane structural protein (76). Although, in yeast, the synthesis of cytochromes a and b appears to occur in the mitochondrial protein biosynthetic system (77), the source of the corresponding mRNA is not known.

Part C - The System

Since non-nucleate fragments of sea urchin eggs can be prepared in large numbers with relative ease, they have been utilized to examine cytoplasmic RNA synthesis in the absence

of nuclear activity (14-16).

In the following experiments the DNA, RNA and proteins synthesized in the non-nucleate fragments and embryos were localized, extracted and characterized by various means. The absence of nuclear activity helped to reduce the background activity otherwise contributed by the nuclear presence.

MATERIALS AND METHODS

MATERIALS

Sea Urchins

Urchins of the species Strongylocentrotus purpuratus were collected personally and commercially (Pacific Bio-Marine Supply Co.) off the coast of southern California.

Sea Water

Tyler's Artificial Sea Water was used as the incubation media in all experiments (78).

Antibiotics

Streptomycin sulfate and potassium penicillin were from Squibb.

Actinomycin D and ethidium bromide were from Cal-Biochem.

Chemicals

Acrylamide, methylene bis acrylamide, tetra-ethyl-methylene-diamine (teemed), and dimethyl sulfoxide (DMSO) were from Eastman Organic Chemical Co..

SeaKem Agarose, manufactured by Marine Colloid, Inc., was obtained through Bausch and Lomb.

Benzoylated-DEAE-cellulose (B-D cellulose) was obtained from Schwarz Bioresearch (sieve size 50-100).

Biosolve 3 was from Beckman Instruments.

Cesium Chloride was from the Harshaw Chemical Co..

Coomasie blue was from Mann Research Labs.

Fully deuterated DMSO (d_6 DMSO) was obtained from BioRad Labs.

2-Mercaptoethanol (2-Me) and sodium dodecyl sulfate (SDS) were from the Sigma Chemical Co..

RNAase free sucrose and ultra pure urea were obtained from Mann Research Laboratories.

Tri-chloro-acetic acid (TCA) was from Allied Chemicals.

Electrophoresis

The equipment for cylindrical SDS gels was from Canalco.

A vertical gel slab apparatus from E-C Apparatus was utilized for acrylamide electrophoresis of RNA's.

Enzymes

5x crystallized RNAase A from Bovine Pancrease was

from the Sigma Chemical Co.. The RNAase solutions were pre-heated to 100° C for 1 minute to destroy DNAase activity.

Filters

Bac-T-flex membrane filters were obtained from Schleicher and Schuell.

Whatman # 1 filter paper was used as a support for acid precipitation of macromolecules.

Isotopes

^{14}C and ^{3}H thymidine, uridine and valine were obtained from both Schwarz Bioresearch and Amersham Searle Corp.

Photography

Photos were obtained using a Zeiss microscope and in some cases with a Zeiss Planapo (400x) objective.

Panatomic X film was used.

Protein Markers

Trypsin was from Worthington.

Bovine Serum Albumin (BSA) and Ovalbumin (OVA) were obtained from the Sigma Chemical Co..

Scintillation Counting

Scintillation counting was done in a Beckman ambient temperature scintillation counter. For ^{3}H approximately a 7% counting efficiency was obtained using filter papers and a standard toluene, POP, POPOP cocktail. In a similar cocktail with Biosolve 3 as a solublizer approximately a 70% counting efficiency was obtained.

METHODS

Care and Handling of Sea Urchins and Eggs

Sea urchins were maintained in the laboratory and were periodically induced to shed (spawn) by the injection of 1 ml of 0.55 M KCl. The eggs were collected, washed, dejellied, fertilized and cultured according to the methods described in 21 and 78.

Dejellied eggs were fragmented into nucleate and non-nucleate fragments by centrifugation in buoyant, isotonic, sucrose-sea water gradients following the basic proceedures outlined in 10 and originally described by Harvey (79). The primary modification was to change the centrifugation from 5 minutes at 2500 rpm followed by 15 minutes at 10,000 rpm (in a Spinco SW 25.1 rotor) to 5 minutes at 2000 rpm followed by 30 minutes at 12,000 rpm. In this work the modification produced slightly better yields of non-nucleate fragments. See figure on next page.

After fragmentation, the non-nucleate fragments were collected by puncturing and draining from the bottom of the centrifuge tube. They were subsequently washed in ice cold sea water and were artificially activated in 0.005 M butyric acid at 18° C for 1.0 to 1.75 minutes (10). The treatment was stopped by the addition of 100 volumes of ice cold sea

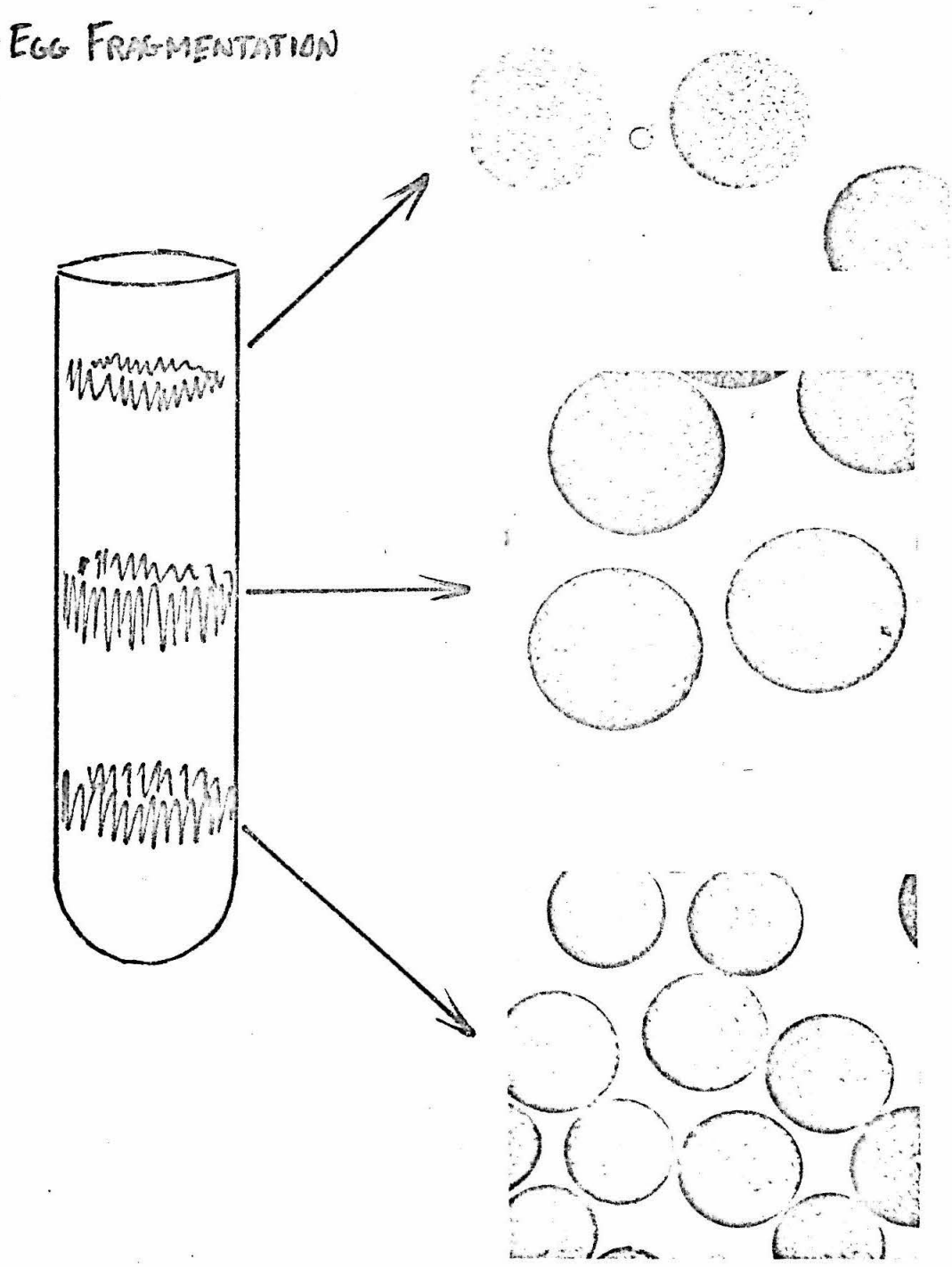

Figure 1

Figure 1. Fragmentation of sea urchin eggs in sucrose-sea water gradients.

Eggs of the sea urchin Lytechinus pictus were fragmented as specified in materials and methods. The upper band contains the nucleate fragments, the center band contains the unfragmented eggs, and the lower band contains the non-nucleate fragments.

water. The fragments were subsequently washed 3x in ice cold sea water before warming to incubation temperature and adding the antibiotics, inhibitors and/or radioactively labeled precursors.

Fertilization was according to the methods described by Tyler (78). The newly fertilized embryos were also maintained ice cold until the addition of the antibiotics, inhibitors and/or radioactively labeled precursors.

The embryos and fragments were cultured for up to 7 days in artificial sea water at 17-19° C. Incubation was terminated by reducing the temperature to 4° C and washing the cells 3x in ice cold artificial sea water with centrifugations (500 X g) between washes.

Cell Fractionation

In experiments where the fragments were homogenized but mitochondria were not subsequently isolated, the fragments were suspended in 4 volumes of a buffer containing 1 mg/ml of bentonite, 0.45 M KCl, 0.02 M MgAc and 0.05 M tris at pH 7.6. The fragments were homogenized with less than 10 strokes in a small Dounce homogenizer. For mitochondrial isolation, an homogenization buffer containing 0.3 M sucrose, 0.36 M KCl, 0.003 M EDTA, and 0.03 M tris (pH 7.6) was used. In all cases, the initial step in fractionation was to

centrifuge the homogenates at 15,000 x g for 20 minutes at 4° C. For mitochondrial isolation, the resulting pellet was resuspended in a volume of 0.8 M sucrose, 0.003 M tris and 0.0025 M EDTA (pH 7.6) equivalent to the initial homogenization volume. This suspension was layered on a 0.93 M to 1.88 M sucrose gradient in the same buffer. The gradients were spun for 2.5 hours at 4° C at 24,000 rpm in a S.W. 25.1 rotor to band the mitochondria isopycnically (11, 80). The bands of mitochondria were collected and diluted by adding 4 volumes of 0.005 M EDTA, 0.5 M KCl, and 0.05 M tris at pH 7.6. They were then centrifuged at 15,000 x g for 20 minutes. The resulting pellet was resuspended in a buffer containing 0.275 M KCl, 0.01 M MgAc, and 0.01 M tris at pH 7.5. The mitochondria were then lysed by adding sodium deoxycholate (DOC) to a concentration of 0.5%.

Nucleic Acid Extraction

DNA or RNA was extracted directly from the fragments by first suspending the washed, labeled fragments in 4 volumes of ice cold buffer (0.005 M EDTA, 0.275 M KCl, and 0.05 M tris at pH 7.5 for DNA or 0.1 M NaCl, 0.01 M NaAc at pH 5 for RNA). Bentonite was added to 1 mg/ml and Na DOC was added to a final concentration of 0.5%. After the suspension cleared, SDS was added to 2%, forming a precipitate which

dissolved after bringing to room temperature. The resulting solution was then extracted 2x with an equal volume of phenol containing 0.1% 8-Hydroxyquinoline. The extractions were conducted at room temperature, with constant mixing, for 30 minutes. After each extraction, the mixture was centrifuged at 5,000 rpm at 4° C in a Sorvall swinging bucket rotor and the aqueous phase was removed. After the second phenol extraction, the nucleic acid was precipitated from the aqueous phase, overnight at -20°C, after the addition of 2 volumes of ethanol. The precipitate was washed 3x in 100% ethanol, and 1x in 100% ether. Between washes the precipitate was repelleted by centrifuging at 5,000 x g for 10 minutes at 4° C. The pellet was dried from the ether and was then ready to be dissolved in the appropriate buffer for subsequent analyses.

SDS Protein Extraction

C^{14} valine labeled control fragments and H^{3} valine labeled fragments preincubated for 6 hours with ethidium bromide were mixed and homogenized in 4 volumes of a buffer containing 0.275 M KCl, 0.01 M MgAc and 0.01 M tris at pH 7.5. The homogenate was centrifuged at 15,000 x g for 20 minutes. The supernatant and the resulting pellet, resuspended in the same volume of the homogenization buffer were made to 2%

in SDS and 1% in 2-Me. The resulting solutions were incubated at 37° C for 3 hours and were then dialysed against 0.01 M phosphate at pH 7.1 with 0.1% SDS and 0.1% 2-Me (81). The extracts were centrifuged at 1000 x g for 10 minutes to remove the residue and aliquots were tested for hot TCA precipitability on filter papers.

B-D Cellulose Chromatography

The B-D cellulose chromatography followed the procedures of Sedat, Lyon & Sinsheimer (82) with the exception that a gradient of 0 to 4 M deionized urea was included in the increasing salt gradient in order to force the RNA to elute at a lower salt concentration than would otherwise occur.

DMSO Density Gradient Centrifugation

Purified RNA was analysed for molecular weight by sedimentation in gradients of DMSO to d_6 DMSO following the procedures described by Sedat et al. (82).

Sucrose Density Gradient Centrifugation

Dried, purified, RNA was dissolved in a buffer containing 0.1 M NaCl and 0.01 M NaAc at pH 5. 100 to

500λ was layered on 11 ml of a 5-20% (wt/vol) sucrose gradient with a 1 ml 60% sucrose pad. The gradients also contained 0.1 M NaCl and 0.01 M NaAc at pH 5. Centrifugations were for either 6 hours at 40,000 rpm or for 17 hours at 24,000 rpm at 2° C in a S.W. 41 rotor. 0.3 to 0.35 ml fractions were collected.

Mitochondrial lysates were layered onto 11 ml of a 15-30% (wt/vol) sucrose gradient with a 1 ml 60% sucrose pad. In this case the gradients contained 0.275 M KCl, 0.01 M MgAc and 0.01 M tris (pH 7.5)/ Centrifugation was at 30,000 rpm for 9 to 9.5 hours at 4° C in a S.W. 41 rotor. 0.4 to 0.5 ml fractions were collected.

Acrylamide Electrophoresis

The methods for preparing and staining agarose-acrylamide gels for the analysis of purified RNA are described by Peacock and Bunting (83). The method used required 1.75% acrylamide and 0.5% Agarose. The RNA was electrophoresed at 200 volts for 2 hours at 0° C using Peacock's Tris-EDTA-Borate buffer (83). 1 mm slices were treated with 0.2 ml of 0.5 M KOH for 24 hours at 25° C and were counted in a toluene-liquifluor-biosolve scintillation cocktail.

The SDS extracted proteins were analysed on SDS-acrylamide gels as described by Shapiro (81). Similarly

extracted known proteins were run in parallel as markers. Electrophoresis was at 7 milli amps per gel cylinder for 3.5 hours. 1 mm gel slices were dissolved overnight in 30% hydrogen peroxide. The dissolved gels were aired for several hours to reduce the peroxide concentration and were then counted in a toluene-liquifluor-biosolve scintillation cocktail. Some gels were stained for 3 hours in 0.25% Coomasie blue in methanol:HAc:H_2O (5:1:5) and destained electrophoretically in the same solvent for photography purposes.

Buoyant Density Centrifugation

In experiments where DNA was analysed by CsCl buoyant density centrifugation, the fragments were first dissolved in 4 volumes of a buffer containing 4% SDS, 9% ethanol and 0.08 M EDTA at pH 8 (11). The resulting cell lysate was adjusted to ρ=1.7 gms/cc with CsCl and centrifuged at 40,000 rpm for 48 hours at 20° C in a S.W. 65 rotor. 0.2 ml fractions were collected. In some experiments the material banding at 1.7 gms/cc was diluted to 1.55 gms/cc, made to 300 µgs/ml in ethidium bromide and recentrifuged in the S.W. 65 rotor at 40,000 rpm for 48 hours at 20° C. Fluorescence of the rebanded DNA in UV light permitted photography and localization of the bands of DNA. See figure on next page.

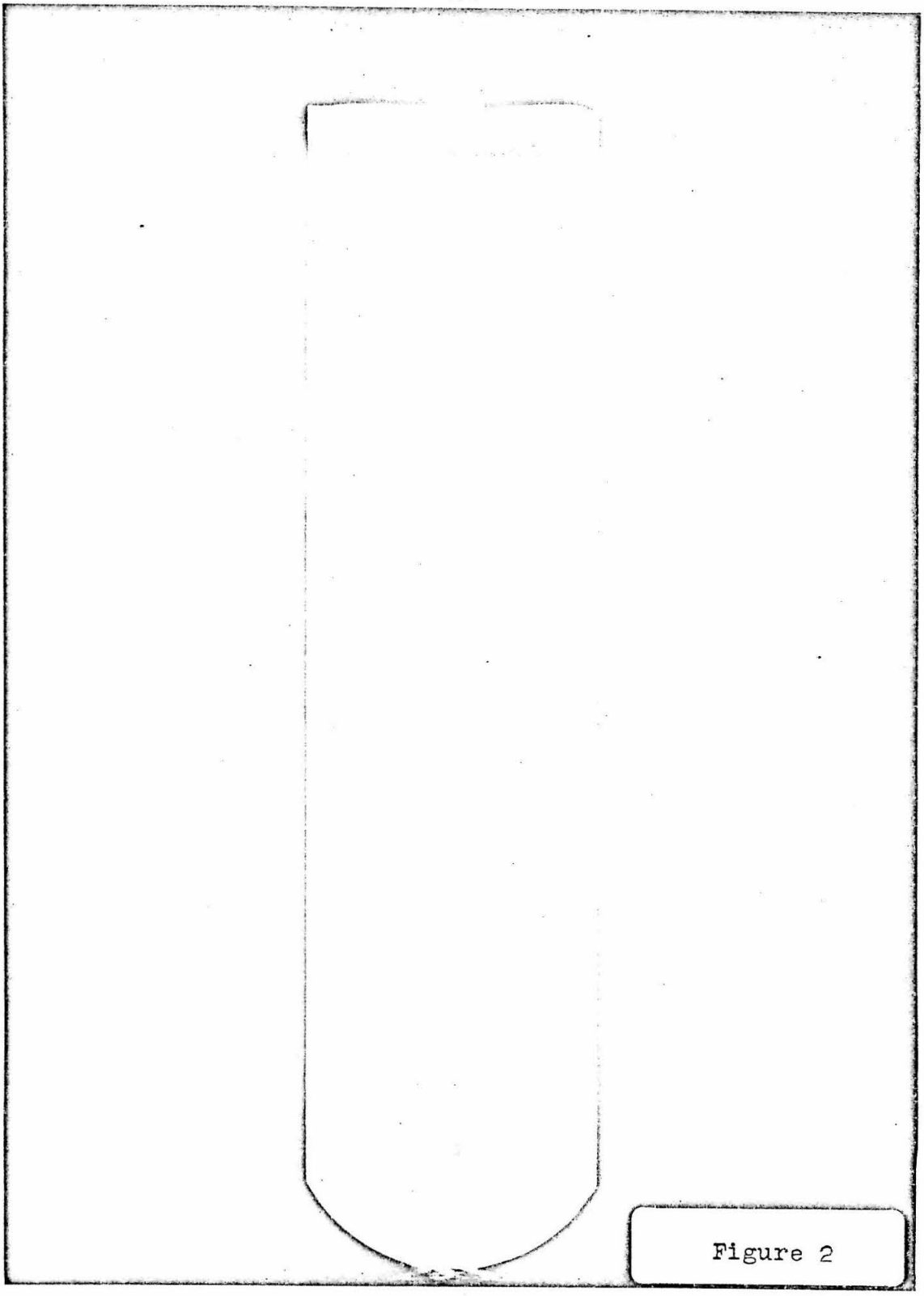

Figure 2

Figure 2. Preparation of closed circular mitochondrial DNA in ethidium bromide-CsCl buoyant density gradients.

Closed circular mitochondrial DNA was prepared as appears in methods. The upper band is the non-closed circular DNA. The lower band is the closed circular mitochondrial DNA. The fluorescence was photographed thru a yellow filter.

Hybridization

Closed circular mitochondrial DNA was isolated from sea urchin eggs by the ethidium bromide banding method (12). In this species, the broad separation between the non-closed circular (upper or less dense band) and the closed circular (lower or more dense band) bands of DNA is adequate to permit complete separation of the two types of DNA (see figure on previous page). The ethidium bromide was removed by chromatography through a bed of Dowex 50W-X4 resin (Cs^+). The DNA was prepared for hybridization by heat denaturation at 100° C for 20 minutes in standard sodium citrate/10 (0.1 x SSC) buffer and quick-cooled in a salt-water ice bath.

DNA from sea urchin sperm and Escherichia coli DNA were prepared by dodecyl sulfate-phenol extraction and were subsequently dialysed against 0.3 M NaOH for 24 hours at room temperature to hydrolyze the RNA and denature the DNA. The DNA was brought to a neutral pH by dialysis against 2 x SSC at 4° C.

RNA-DNA hybridizations were carried out in solution according to the method of Attardi, Huang & Kabat (84) with the exception that Sephadex G100 column chromatography of the RNAase-treated hybrids was found to yield no additional reduction in the level of background radioactivity sticking

to the membrane filter, and this step was therefore eliminated.

Inhibition Studies

Embryos and non-nucleate fragments were incubated in various concentrations of actinomycin D or ethidium bromide, in 1 ml of artificial sea water, for 30 minutes at 17° C. After this pre-incubation period, uridine-5-^{3}H was added to approximately 10 µc/ml and the cells were incubated for an additional 2 hours at 17° C. The cells were then washed 3 x in 100 volumes of ice cold artificial sea water. The cells were then suspended in 0.2 ml of 1% Na DOC. The cell lysates were spread on filter papers and dried. To provide a figure for the total uptake of the isotope the papers were counted in a toluene, POP, POPOP scintillation cocktail before rehydrating and washing 5 x with ice cold 5% TCA. After TCA washing the papers were dehydrated by washing 2 x in 95% ethanol, 2 x in 100% ethanol and 1 x in 100% ether. The papers were then dried and recounted to provide a figure for the amount of incorporation of the isotope.

RESULTS

PART I - RNA SYNTHESIS IN NON-NUCLEATE FRAGMENTS OF SEA URCHIN EGGS

(a publication)

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Synthesis of RNA in Non-nucleate Fragments of Sea Urchin Eggs

Non-nucleate fragments of eggs of the sea urchin *Strongylocentrotus purpuratus* were made by buoyant density centrifugation in sucrose–sea water gradients. After the fragments were artificially activated by butyric acid they were found to incorporate [5-^{3}H]uridine into RNA. The labeled RNA was extracted with phenol and examined by dimethyl sulfoxide density gradient centrifugation. The principal component of the labeled RNA sedimented with an average molecular weight of 5 to 6×10^5. The elution profile of the labeled RNA from benzoylated diethylaminoethyl-cellulose was consistent with that for messenger RNA rather than that for ribosomal RNA (Sedat, Lyon & Sinsheimer, 1969). Under non-saturating conditions, as much as 32% of the radioactivity in the RNA preparation hybridized to mitochondrial DNA, while virtually none hybridized to DNA extracted from spermatozoa.

The presence of DNA in sea urchin mitochondria (Piko, Tyler & Vinograd, 1967), and the possible existence of other forms of cytoplasmic DNA, lead one to inquire as to their contributions to biosynthetic processes within the cell, and in the case of the sea urchin, to their contributions to the developing embryo. The synthesis of RNA, homologous to cytoplasmic DNA, in blastula and gastrula stage embryos has recently been reported (Hartmann & Comb, 1969). Baltus, Quertier, Ficq & Brachet (1965) and Chamberlain (1968) have reported that RNA is synthesized in non-nucleate fragments of sea urchin eggs. The non-nucleate fragment of the sea urchin egg may therefore provide a system in which cytoplasmic biosynthetic processes can be studied independently of nuclear activity.

Nucleate and non-nucleate fragments of the sea urchin egg of *Strongylocentrotus purpuratus* were prepared by buoyant density centrifugation in sucrose–sea water gradients (Harvey, 1931). Non-nucleate fragments were parthenogenetically activated in 0·005 M-butyric acid in sea water. Nucleate fragments and unfragmented eggs were fertilized in a dilute suspension of sperm. The activated fragments and embryos were then incubated for from 30 minutes to 7 hours in [5-^{3}H]uridine.

In these experiments the RNA was extracted from the whole fragments and embryos by first dissolving the cells with sodium deoxycholate in the presence of bentonite, subsequently adding sodium dodecyl sulfate to 2% and finally extracting twice with phenol. The RNA was then precipitated from the aqueous phase by addition of two volumes of ethanol, pelleted by centrifugation, washed several times in 100% ethanol, and dried from ether.

The RNA was further purified on benzoylated DEAE-cellulose columns and analyzed for molecular weight by sedimentation in gradients of dimethyl sulfoxide to deuterated dimethyl sulfoxide according to the procedures described by Sedat *et al.* (1969).

Closed circular mitochondrial DNA was isolated from sea urchin eggs by the ethidium bromide banding method described by Piko, Blair, Tyler & Vinograd (1968). The DNA was prepared for hybridization by heat denaturation at 100°C for 20 minutes

in standard sodium citrate/10 (0·1 × SSC) buffer and quick-cooled in a salt–water ice bath. DNA from sea urchin sperm and *Escherichia coli* DNA were prepared by dodecyl sulfate–phenol extraction and subsequently dialyzed against 0·3 M-NaOH for 24 hours at room temperature to hydrolyze the RNA and denature the DNA. The DNA was brought to a neutral pH by dialysis against 2 × SSC at 4°C.

RNA–DNA hybridizations were carried out in solution according to the method of Attardi, Huang & Kabat (1965) with the exception that Sephadex G100 column chromatography of the RNase-treated hybrids was found to have no effect on the background radioactivity sticking to the membrane filter, and this step was therefore eliminated.

The results of the benzoylated DEAE-cellulose chromatography of the RNA from the non-nucleate fragments (Fig. 1) show that a large portion of the acid-precipitable [5-^{3}H]uridine counts are eluted on the trailing edge of the large optical density peak of ribosomal RNA in the 0·3 to 1·2 M-NaCl gradient and in the pH 3·5, 0 to 1 M-NH_4Cl gradient. This elution profile is consistent with that for messenger RNA as described by Sedat *et al.* (1969). Assuming that sea urchin mitochondrial ribosomal RNA may be similar to bacterial ribosomal RNA, the mitochondrial ribosomal RNA would be expected to elute from the benzoylated DEAE-cellulose coincidently or on the leading edge of the large optical density peak of ribosomal RNA. However, the results in Figure 1 suggest that very little if any ribosomal RNA is synthesized in the

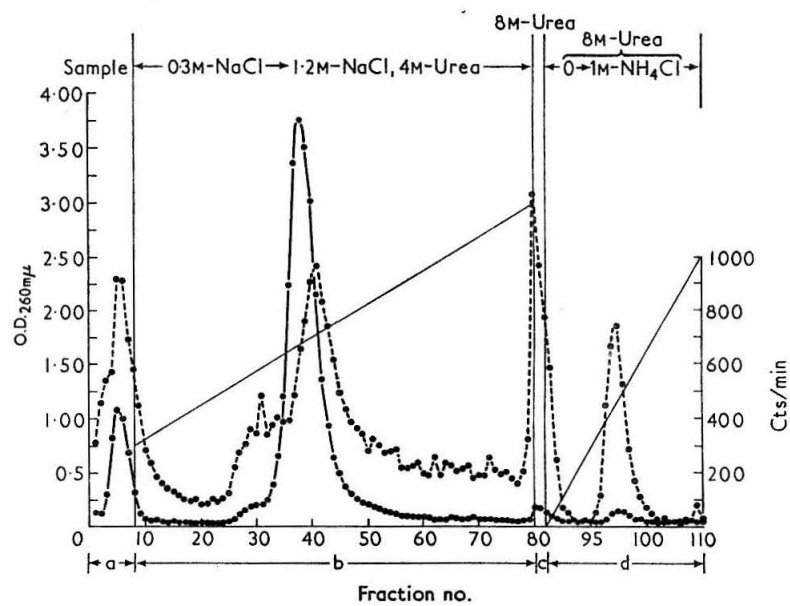

FIG. 1. Benzoylated DEAE-cellulose chromatography of 1-hr [5-^{3}H]uridine-labeled RNA from activated non-nucleate fragments of the sea urchin egg.

(a) Sample is being adsorbed to column in 0·3 M-NaCl, 0·01 M-Tris, 0·001 M-EDTA (pH 5·6).

(b) The sample is followed by a gradient of 0·3 M-NaCl to 1·2 M-NaCl and 0 to 4 M-urea in 0·01 M-Tris, 0·001 M-EDTA (pH 5·6).

(c) The column is washed in 8 M-urea (pH 3·5).

(d) The column is then washed with a gradient of 0 to 1 M-NH_4Cl in 8 M-urea at pH 3·5.

--●--●--, ^{3}H radioactivity; —●—●—, absorbance at 260 mμ.

non-nucleate fragments of sea urchin eggs. The peaks of ^3H radioactivity and optical density which eluted immediately before the ribosomal RNA are probably tRNA, DNA and RNA sticking to DNA (as evidenced by the alkaline sensitivity of the radioactivity associated with the DNA rebanded in CsCl).

The result of the sedimentation in dimethyl sulfoxide (Fig. 2) shows that the majority species of RNA has a molecular weight of 5 to 6×10^5. RNA may be present with a molecular weight as high as $1{\cdot}5 \times 10^6$. This same material when sedimented in

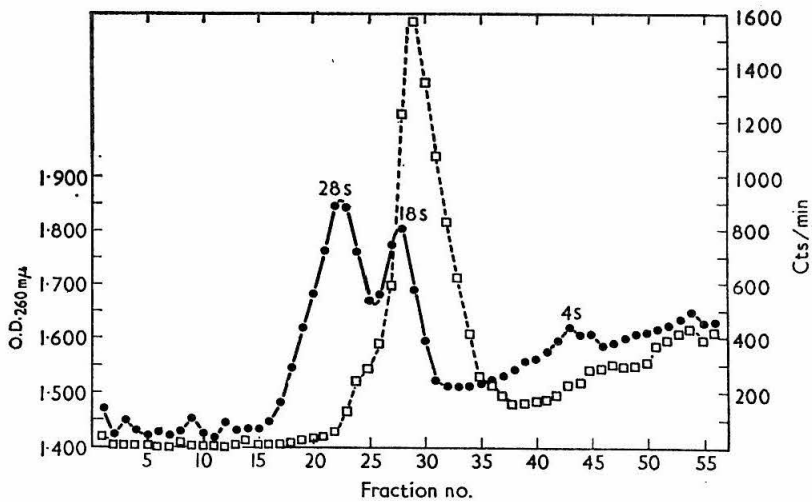

FIG. 2. Sedimentation of 1-hr [5-^3H]uridine-labeled RNA extracted from artificially activated non-nucleate fragments of sea urchin eggs in dimethylsulfoxide to deuterated dimethyl sulfoxide gradients.
--●--●--, ^3H radioactivity; —□—□—, absorbance at 260 mμ.

sucrose gradients peaks at 16 or 17 s and contains acid-precipitable material which may be as large as 25 s and as small as 10 s. Details will be described in a full report of this work.

Figure 3 shows the results of the RNA–DNA hybridization of embryo and non-nucleate fragment RNA's to mitochondrial DNA. Under non-saturating conditions, as much as 32% of the ^3H radioactivity in the non-nucleate fragment RNA and 4·5% in the embryo RNA was hybridized to mitochondrial DNA. The results of hybridization to sea urchin sperm DNA (considered equivalent to nuclear DNA) and *E. coli* DNA are not illustrated in this Letter since under non-saturating conditions no more than 0·3% of the radioactivity in the embryo or the non-nucleate fragment RNA hybridized to either of these DNA's. (Incubation of the mitochondrial DNA, sea urchin sperm DNA, and *E. coli* DNA with [5-^3H]uridine-labeled RNA for 20 hours at 37°C produced no reduction in the amount of acid-precipitable counts, indicating that all of the DNA preparations were free of ribonuclease activity.) These results suggest that very likely the majority of the labeled RNA in the non-nucleate fragments and at least some of the labeled RNA from embryos are synthesized on the mitochondrial DNA template.

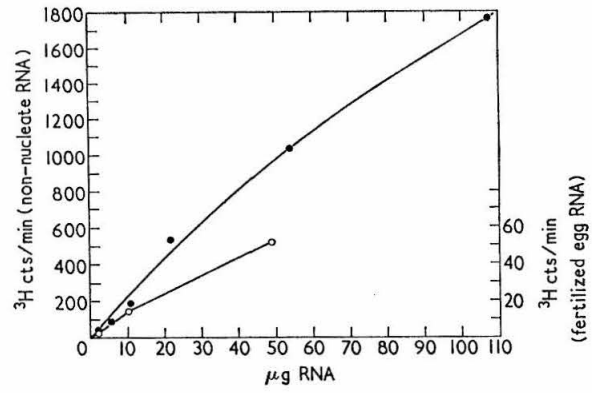

FIG. 3. Hybridization of RNA from fertilized eggs and from artificially activated non-nucleate fragments (purified on a pH 3·5 benzoylated DEAE-cellulose column) with mitochondrial DNA. Each hybridization mixture contained RNA and 4 μg of mitochondrial DNA in 1 ml. of 2 × SSC buffer at pH 7. Incubation was for 6 hr at 68°C. Each solution was cooled and treated with 40 μg of boiled RNase before increasing the KCl concentration and attachment of the DNA to filters. —●—●—, Non-nucleate fragment RNA; —○—○—, fertilized egg RNA.

In summary, my results demonstrate that mitochondria in the non-nucleate fragment are actively synthesizing RNA. The majority species has a molecular weight of from 5 to 6×10^5. The results of the benzoylated DEAE-cellulose chromatography indicate that little or no ribosomal RNA is synthesized.

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PART II - RNA AND PROTEIN SYNTHESIS IN NON-NUCLEATE FRAGMENTS OF SEA URCHIN EGGS

Part II is an extension of Part I in that the RNA synthesis occurring in non-nucleate fragments is further localized and characterized in the cells and cell homogenates. Part II also initiates the exploration of protein synthesis which may be a consequence of the mitochondrial RNA synthesis.

RNA Localization

Part I showed that the labeled RNA from non-nucleate fragments was primarily single stranded in nature. In order to localize the newly synthesized RNA, labeled non-nucleate fragments were homogenized and centrifuged at low speed in order to sediment the yolk, lysosomes, mitochondria and the membranes of the cell. The newly synthesized single stranded RNA was found associated exclusively with the sediment of this low speed centrifugation (figure II-1).

Further fractionation of the low speed sediment yielded the result that ^{3}H labeled RNA could be found in the mitochondria + membrane lysates. The sedimentation profile of these lysates appears in figure II-2. All of the radioactive label is sensitive to 1 µg/ml of RNAase at 2°

with the exception of a slight quantity of label associated with the Optical Density (O.D.) in fractions 21-23. This radioactivity, which sediments with the velocity of nicked mitochondrial DNA, 27s, appears to be increasing with the time of label.

Since uridine can be converted to cytidine (85) and since this conversion has been confirmed to be occurring in artificially activated non-nucleate fragments (see appendix), the question arises as to whether the 27s label is incorporated into the DNA or into RNA. Further analysis of the fractions in question by CsCl buoyant density centrifugation showed that while most of the radioactivity (approximately 75%) had the buoyant density properties of RNA, some radioactivity remained associated with the DNA banding at approximately 1.7 gms/cc. However, when the banded material was treated with 0.3 M KOH for 18 hours at 37°, more than 60% of this radioactivity became acid soluble. This indicates that at least 60% of the radioactivity that was associated with the DNA, in experiments of less than 2 hours duration, had ribose rather than deoxyribose for a molecular backbone. The conclusion from these tests is that most of the radioactivity associated with the DNA was possibly a product of recent RNA synthesis by the DNA.

The sedimentation profile of the phenol extracted, rapidly sedimenting material, from the mitochondrial and

membrane lysate appears in figure II-3. The resulting profile shows that the labeled RNA's are within the limits for size distribution for RNA from the whole fragment, fig. II-4.

Further Characterization of the RNA Made in Non-Nucleate Fragments of Sea Urchin Eggs

The sedimentation of phenol extracted RNA from non-nucleate fragments in a sucrose gradient, appears in figure II-4. The sedimentation profile of the radioactive label is similar to that obtained with RNA prepared similarly and sedimented in dimethyl sulfoxide, figure I-2. It is unlikely, therefore, that the rapidly sedimenting material is an artifactual aggregation of the slower moving material. The results show that most of the label sediments between 8 and 25s with a principal peak sedimenting just slightly slower than the 18s ribosomal RNA. Eighty two percent of the radioactivity in the RNA layered on the gradient became acid soluble after treatment with 10 µgs/ml of RNAase. Ninety two percent of the radioactivity became acid soluble after treatment with 0.3 M KOH for 18 hours at 37°.

Phenol extracted, ^{3}H-U labeled RNA, from non-nucleate fragments, figure II-5a, was compared with the newly synthesized RNA from early cleavage stage embryos, figure II-5b, and mesenchyme blastula stage embryos, figure II-5c,

in electrophoresis experiments in agarose acrylamide gels. The radioactive profiles of RNA from fertilized eggs, fig. II-5b, and mesenchyme blastula stage embryos, figure II-5c, are superimposed on the absorbance profile of the stained RNA in the gel of the non-nucleate fragment RNA, figure II-5a. The low levels of radioactivity make it difficult to compare the electrophoretic profile of the radioactive RNA from non-nucleate fragments, figure II-5a, to the sedimentation profile in DMSO, figure I-2, and in sucrose, figure II-4, of the radioactivity in similarly prepared RNA. It should be noted that the observed radioactivity, associated with the 4s peak (fractions 96-99), may be a product of CCA turnover in the transfer RNA (86).

Several observations concerning the results in figures II-5 & 6 follow. Figure II-6 is a plot of the molecular weights versus the fraction numbers for the slices of the agarose acrylamide gels appearing in figure II-5.

1) The synthesis of mature rRNA can not be detected in fragments or in embryos as late as the mesenchyme blastula stage of development.

2) No predominance of species of RNA's appear at 12, 13 or 21s among the relatively heterogeneous population of RNA's made in the non-nucleate fragments (figure II-5a).

3) The amount of RNA snthesis at the mesenchyme blastula stage of development is considerably higher than

that in non-nucleate fragments and early cleavage stage embryos. There is also a greater proportional synthesis of high molecular weight RNA's at the mesenchyme blastula stage of development.

Protein Synthesis in Non-Nucleate Fragments in the Presence and Absence of Mitochondrial RNA Synthesis

The observed levels of protein synthesis and uptake of precursor for embryos with and without the presence of ethidium bromide, at 10 µgs/ml, are relatively equivalent during the first 3 hours of development (table 1). (This concentration of ethidium bromide is shown to be adequate to inhibit all mitochondrial RNA synthesis in Part III.) After 3 hours, however, the rate of precursor uptake, in the presence of mitochondrial RNA synthesis, declines while that for embryos in the absence of mitochondrial RNA synthesis is sustained. Assuming that the pool size for valine is large relative to the precursor uptake and equal for the 2 cases, protein synthesis declines by 25-30% in the absence of mitochondrial RNA synthesis.

The proteins synthesized in the presence and absence of mitochondrial RNA synthesis seem to be qualitatively similar (figure II-7). In both cases the proteins are of a relatively broad span of molecular weights (9-40 thousand for low speed

supernatant proteins; 6-60 thousand for proteins pelleted at low speed, fig. II-7 & 8). The proteins made in the non-nucleate fragments differ qualitatively in the 15,000 x g pellet, figure II-7a, and the 15,000 x g supernatant, figure II-7b.

The results of this section can be summarized as:

1) RNA synthesized in artificially activated non-nucleate fragments in the duration of 1 hour is found exclusively associated with the membranes or organelles of the cell.
2) The synthesis of mature rRNA is not detected in fragments or in embryos at the mesenchyme blastula stage of development.
3) A larger proportion of high molecular weight RNA's are synthesized at the mesenchyme blastula stage than are synthesized during early cleavage.
4) The uptake of valine is sustained in the presence of a concentration of ethidium bromide adequate to inhibit all the mitochondrial RNA synthesis.
5) Assuming that the pool size of valine is large relative to the amount of precursor uptake and that the pool sizes are equal in experiments with and without ethidium bromide, the amount of protein synthesis drops 25-30% after 3 hours in concentrations of ethidium bromide adequate to inhibit mitochondrial RNA synthesis.

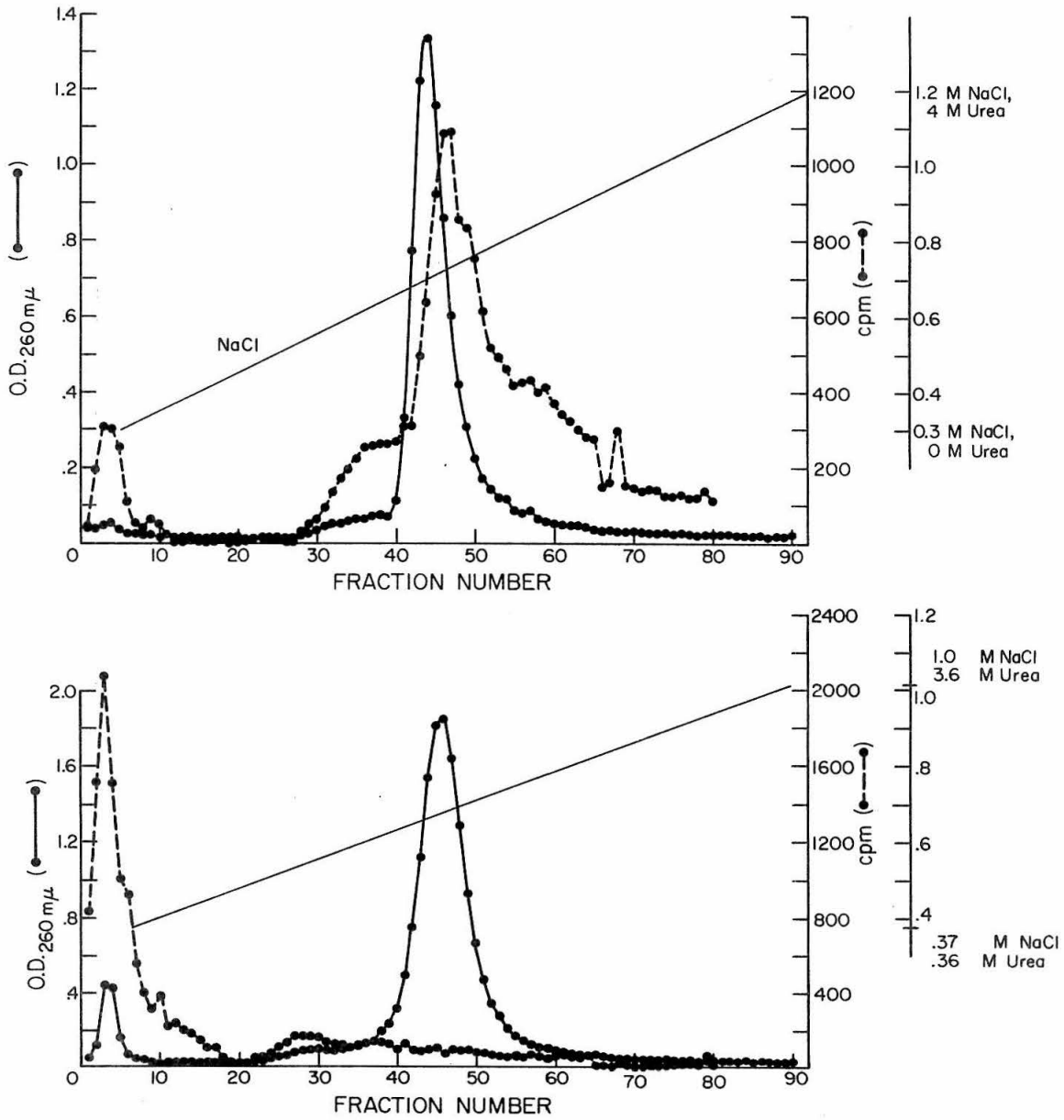

O.D.260 mμ
NaCl
cpm
1.2 M NaCl,
4 M Urea
0.3 M NaCl,
0 M Urea
FRACTION NUMBER
1.0 M NaCl
3.6 M Urea
.37 M NaCl
.36 M Urea
FRACTION NUMBER

Figure II-1

Figure II-1. Benzoylated DEAE cellulose chromatography of the nucleic acid extracted from the 15,000 x g pellet and the 15,000 x g supernatant of a homogenate of non-nucleate fragments.

Non-nucleate fragments of sea urchin eggs were incubated for 1 hour in a total volume of 30 ml with 15 μc of U-5-H^3/ml. The fragments were then washed, homogenized, centrifuged and phenol extracted as specified in methods. The dried nucleic acid precipitates were dissolved in a buffer of 0.3 M NaCl, 0.01 M tris and 0.001 M EDTA (pH 5.6). The nucleic acid was then adsorbed to B-D cellulose equilibrated in the same buffer. The nucleic acid solution was followed by a gradient of 0.3 M NaCl to 1.2 NaCl plus 4 M urea. The gradient also contained a constant concentration of buffer of 0.01 M tris and 0.001 M EDTA (pH 5.6). The straight lines slanting up to the right in the graphs represents the salt gradients. Graph a is the result of chromatography of the nucleic acid from the 15,000 x g pellet. Graph b is the nucleic acid from the 15,000 x g supernatant. DNA and tRNA elute on the leading edge and mRNA elutes on the trailing edge of the large O.D. peak of rRNA in fractions 40-50. O.D.$_{260\ m\mu}$ (•———•), cpms (•------•).

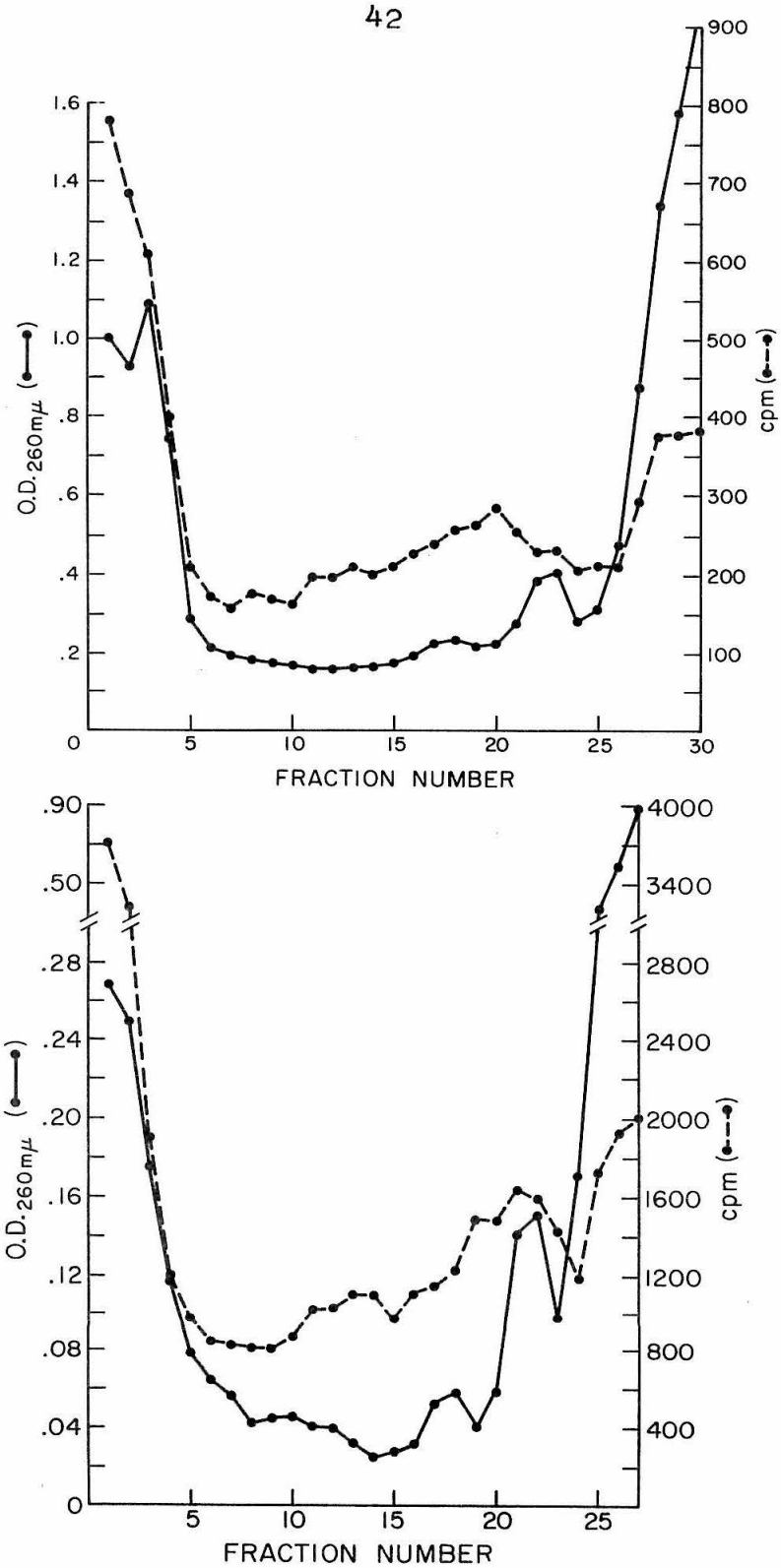


Figure II-2

Figure II-2. Sucrose sedimentation of the lysate of isolated mitochondria from U-5-H^3 labeled non-nucleate fragments.

Artificially activated non-nucleate fragments were incubated for 30 minutes (a) and 2 hours (b) in a total volume of 20 ml with 20 µc U-5-H^3/ml. The mitochondria were isolated and lysed as specified in materials and methods. Centrifugation was at 30,000 rpm for 9.5 hours (a) and 9 hours (b) at 4° C in a S.W. 41 rotor. The optical density peaks in tubes 17 and 18 are presumed to be the closed circular mitochondrial DNA. The peaks in tubes 22 and 23 in a and in tubes 21 and 22 in b are presumed to be the 27s nicked mitochondrial DNA. In graph a, 0.4 ml fractions were collected and 0.1 ml aliquots were acid precipitated for radioactive counting. In graph b, 0.5 ml fractions were collected and 0.25 ml fractions were acid precipitated for radioactive counting. Optical density (•———•), cpms (•-----•).

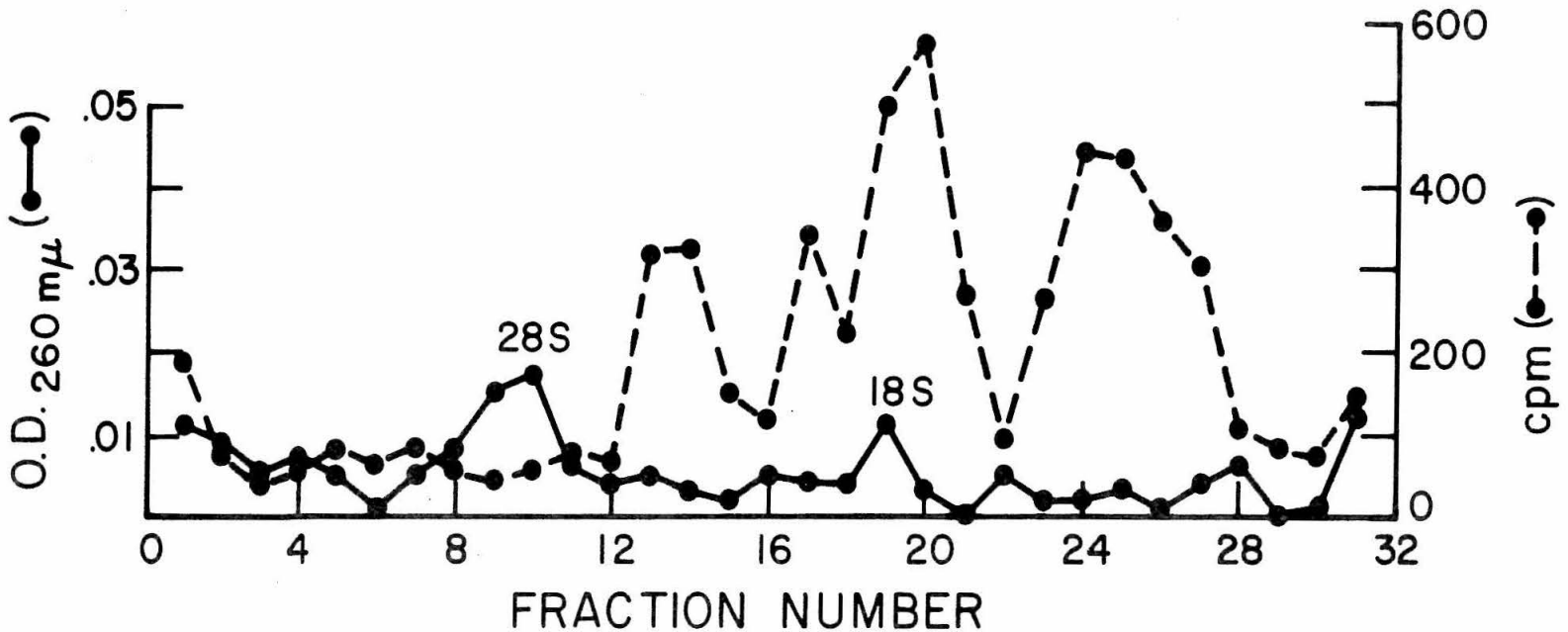

O.D. 260 mμ
.05
.03
.01
28S
18S
600
400
200
0
cpm
0
4
8
12
16
20
24
28
32
FRACTION NUMBER

Figure II-3

Figure II-3. Sucrose sedimentation of RNA extracted from rapidly sedimenting material in the U-5-H^3 labeled mitochondrial lysate.

The RNA from fractions 1 through 4, appearing in graph a of figure II-2, was phenol extracted as specified in methods. This RNA was then suspended in 0.1 M NaCl, 0.01 M NaAc at pH 5, and was layered on 12 ml of a 5-20% (wt/vol) sucrose gradient in the same buffer. Centrifugation was for 5 hours at 40,000 rpm at 4^{o} C in a S.W. 41 rotor. 0.4 ml fractions were collected and 0.3 ml fractions were TCA precipitated on papers for radioactive counting. Optical density at 260 mμ (•———•), cpms (•-----•).

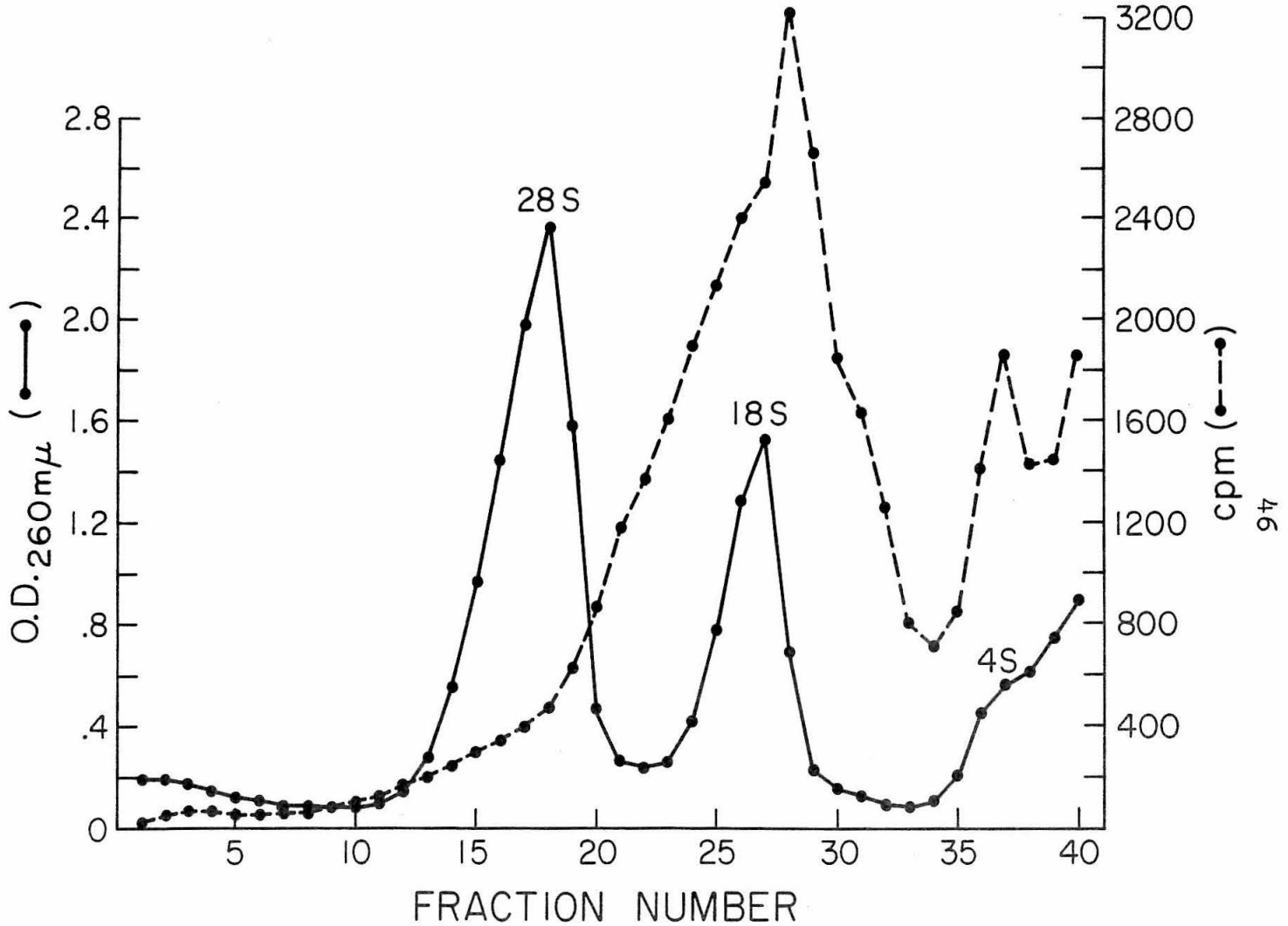

O.D.260mμ
cpm
28S
18S
4S
FRACTION NUMBER
0
.4
.8
1.2
1.6
2.0
2.4
2.8
400
800
1200
1600
2000
2400
2800
3200
5
10
15
20
25
30
35
40

Figure II-4

Figure II-4. The sedimentation in sucrose of RNA extracted from non-nucleate fragments of sea urchin eggs.

Artificially activated non-nucleate fragments were incubated for 1 hour in a total volume of 25 ml containing 20 μc of U-5-H^3/ml. The nucleic acid was extracted as specified in materials and methods. A portion of the dried nucleic acid was dissolved in a buffer containing 0.1 M NaCl and 0.01 M NaAc at pH 5. 0.01 ml was layered on 11 ml of a 5-20% (wt/vol) sucrose gradient plus a 1 ml 60% sucrose pad. Centrifugation was for 6 hours at 40,000 rpm at 2° C in a S.W. 41 rotor. 0.3 ml fractions were collected. The radioactivity represents 0.01 ml aliquots of each fraction after TCA precipitation. Optical density at 260 mμ (•——•), cpm (•-----•).

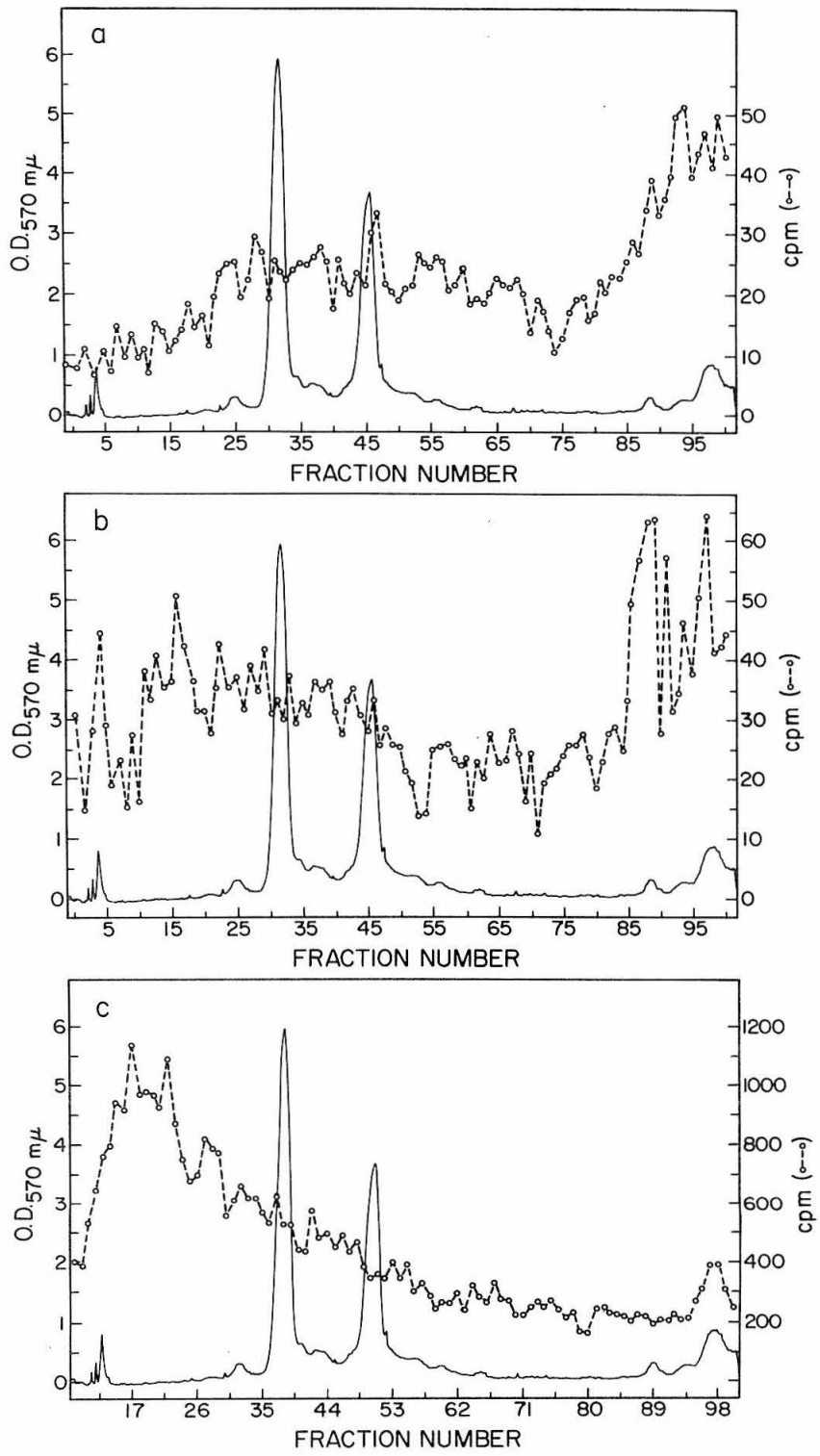

a
O.D.570 mμ
cpm (o—o)
FRACTION NUMBER
b
O.D.570 mμ
cpm (o—o)
FRACTION NUMBER
c
O.D.570 mμ
cpm (o—o)
FRACTION NUMBER

Figure II-5

Figure II-5. Agarose acrylamide gel electrophoresis of U-5-H^3 labeled RNA's from non-nucleate fragments and embryos

Ten micro liters, at 10 O.D.'s/ml, of RNA from non-nucleate fragments (a), early cleavage stage embryos (b), and mesenchyme blastula stage embryos (c), incubated for 1 hour in 10 ml of sea water plus 50 µcs U-5-H^3, were made to 8% in sucrose and were layered into preformed slots in a slab gel. Electrophoresis and slicing were as specified in methods. Since the stain pattern for the RNA's were similar for all three cases the radioactive profiles of the RNA from early cleavage stage embryos (b) and mesenchyme blastula stage embryos (c) are superimposed on the absorbance profile of the stained RNA in the gel of the non-nucleate fragment RNA (a). The fraction numbers are equivalent to the milli meters migration from the origin. The 26s rRNA appears in fractions 30-33 in a & b and in 36-39 in c; the 18s rRNA appears in fractions 44-46 in a & b and in 48-51 in c; and the 4s tRNA appears in fractions 96-99. cpms (o-----o).

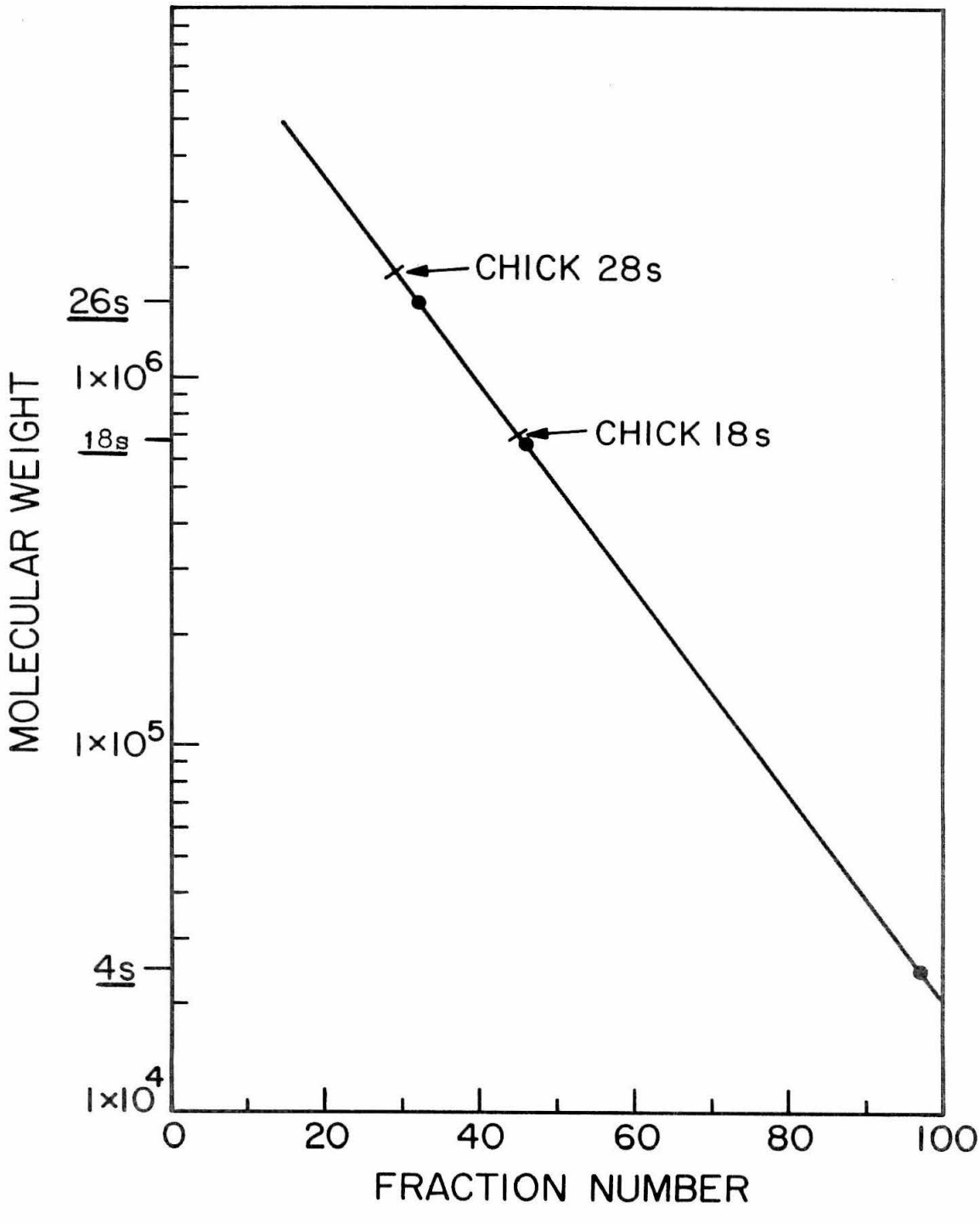

MOLECULAR WEIGHT
26s
1×10^6
18s
1×10^5
4s
1×10^4
CHICK 28s
CHICK 18s
0
20
40
60
80
100
FRACTION NUMBER

Figure II-6

Figure II-6. Molecular weight approximations from agarose-acrylamide gel electrophoresis.

The approximate molecular weights for chick and sea urchin rRNA's and 4s RNA were plotted with respect to the mm of migration or fraction number. These values appear in logarithmically linear relationship to one another. Points mark the locations of the sea urchin RNA's. The mms of migration for chick RNA's, electrophoresed in a parallel slot. are represented by ticks.

TABLE I

Bulk protein synthesis in the presence and absence of mitochondrial RNA synthesis.

Experiment	Uptake	Incorporation	I/U x 100
Control			
0-0.5 hours	216,830	66,751	30.78
1-1.5 "	313,413	106,849	34.09
2-2.5 "	339,694	137,908	40.60
3.5-4 "	285,959	105,059	36.74
6-6.5 "	184,910	56,603	30.61
10-10.5 "	128,016	25,317	19.62
+ ethidium			
0-0.5 hours	200,344	51,416	25.66
1-1.5 "	242,171	70,308	29.03
2-2.5 "	283,615	112,754	39.76
3.5-4 "	336,952	115,415	34.25
6-6.5 "	265,119	56,817	21.43
10-10.5 "	261,739	38,917	14.87

Table I. Bulk protein synthesis in the presence and absence of mitochondrial RNA synthesis.

Embryos were sellected and fertilized following the procedures appearing in methods. Control animals were incubated in sea water. Plus ethidium animals were incubated in sea water plus 10 µgs/ml ethidium bromide. The time period, during which the embryos were incubated with ^{14}C valine (numbers are in hours), appear in the left column. The total counts/minute taken up into the washed embryos appear under Uptake. The same radioactive material, after washing with hot TCA, was recounted. The TCA precipitable counts appear in the column headed Incorporation. The percent of Incorporation is calculated as Incorporation/Uptake x 100 (I/U x 100) and appears in the last column.

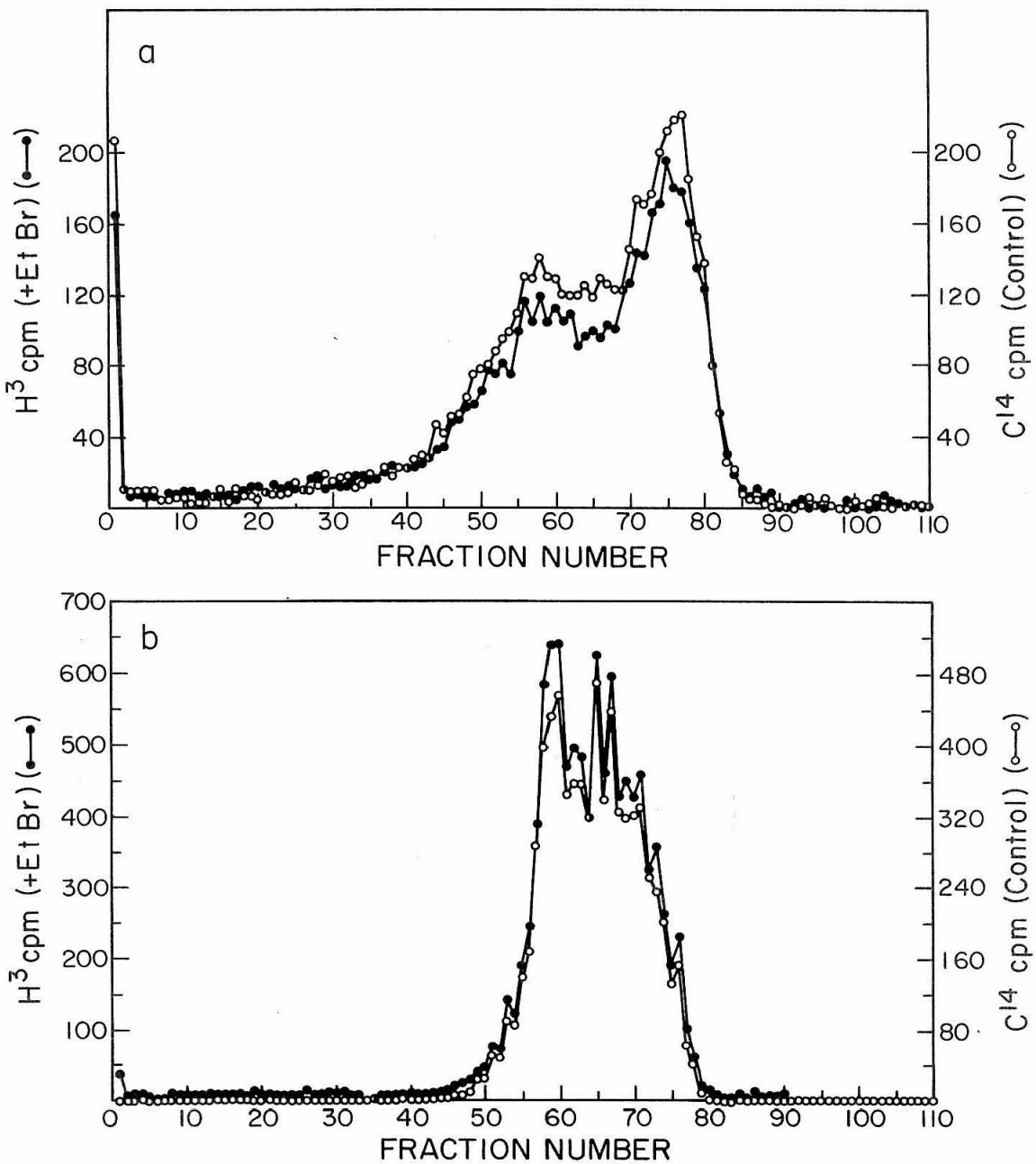

a
H^3 cpm (+Et Br) (●—●)
C^{14} cpm (Control) (○—○)
FRACTION NUMBER
b
H^3 cpm (+Et Br) (●—●)
C^{14} cpm (Control) (○—○)
FRACTION NUMBER

Figure II-7

Figure II-7. Electrophoresis of SDS extracted proteins from non-nucleate fragments.

Artificially activated non-nucleate fragments were incubated at 17° C in 10 ml of sea water (o——o) or in 10 ml of sea water plus 10 µgs/ml of ethidium bromide (•——•). After six hours, 100 µcs of H^3-valine was added to the fragments in sea water plus ethidium, and 2.5 µc of C^{14}-valine was added to the fragments in sea water. After 30 minutes, the fragments were washed, and homogenized as described in methods. The homogenate was centrifuged for 20 minutes at 15,000 x g and the proteins were extracted from the supernatant and the resuspended pellet as described in methods. Graph a is the results of electrophoresis of the pellet proteins, graph b is the results from the supernatant proteins.

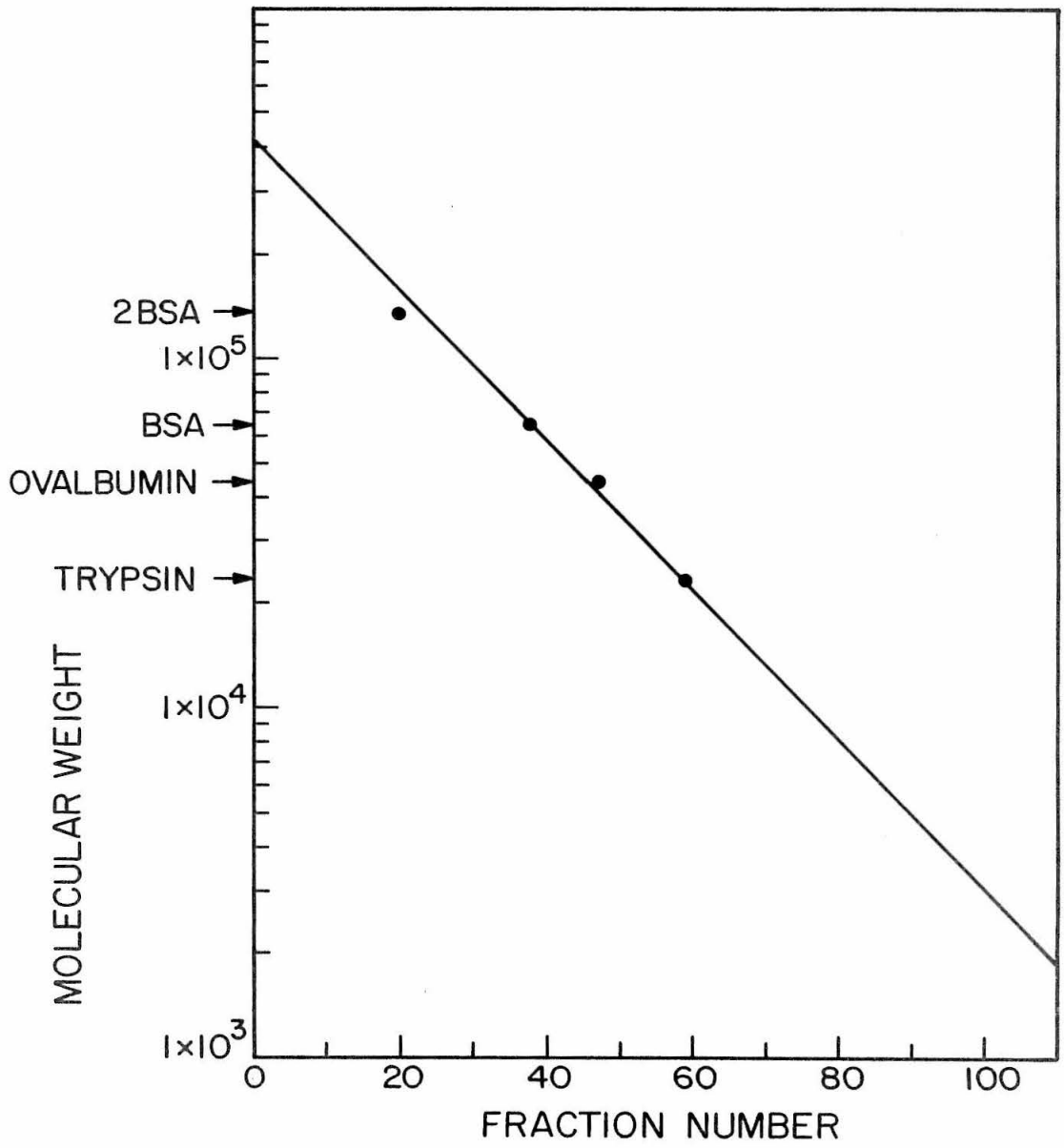

2BSA
1×10^5
BSA
OVALBUMIN
TRYPSIN
1×10^4
1×10^3
MOLECULAR WEIGHT
0
20
40
60
80
100
FRACTION NUMBER

Figure II-8

Figure II-8. Molecular weight approximations for proteins in the SDS gel electrophoresis.

Marker proteins (see ordinate) are nearly logarithmically linear with respect to their molecular weights. The marker proteins were run in gels parallel to the sea urchin proteins. Fraction numbers correspond to mms of migration.

PART III - PARTIAL AND COMPLETE INHIBITION OF MITOCHONDRIAL RNA SYNTHESIS AND ITS EFFECTS ON DEVELOPMENT

In Part III the effects of inhibitors upon RNA synthesis in embryos and non-nucleate fragments are explored. Since ethidium bromide was found to be an effective inhibitor of mitochondrial RNA synthesis, its morphological effects on development are also explored.

Actinomycin D and Ethidium Bromide Inhibition of RNA Synthesis

The effects of actinomycin D and ethidium bromide on ^{3}H-U incorporation into RNA are illustrated in figure III-1. The rates of precursor uptake are relatively constant in experiments at differing concentrations of actinomycin D and ethidium bromide (see appendix). As indicated by the normalized incorporation in embryos, figure III-1a, actinomycin D inhibits RNA synthesis linearly with respect to its concentration. This linear inhibition curve may be the result of only a small fraction of the actinomycin D entering the embryos, with the small amounts of actinomycin being completely effective in inhibiting RNA synthesis.

Actinomycin D appears to be partially effective in inhibiting RNA synthesis in non-nucleate fragments, figure III-1b. Linearity is reached in this inhibition curve at

approximately 2 µgs/ml of actinomycin D. The failure of actinomycin D to inhibit RNA synthesis as well in sea urchins as in other systems may indicate that the fertilization membrane acts as a barrier to the uptake of actinomycin D. This failure of the apparent barrier to uptake of actinomycin D, which occurs in embryos, may be explained by the fact that some of the fertilization membranes of non-nucleate fragments have been disrupted.

On the other hand, ethidium bromide inhibits RNA synthesis in both non-nucleate fragments and early cleavage stage embryos, figure III-1. The curves of inhibition reach a plateau at 10 µgs of ethidium bromide per ml. The base line for the apparent levels of RNA synthesis in 10 µgs/ml of ethidium bromide will be discussed with figure III-2. Compared to actinomycin D, ethidium bromide is a more effective inhibitor of RNA synthesis, at the concentrations tested.

The sedimentation of the RNA from non-nucleate fragments and early cleavage stage embryos, in sucrose gradients, appears in figure III-2. The results show that in the presence of 10 µgs/ml of ethidium bromide, all of the high molecular weight RNA synthesis is stopped in the non-nucleate fragments, figure III-2b, and most of it is stopped in the cleavage stage embryos, figure III-2d. These results indicate that the base line, at least for non-nucleate fragments, for the synthesis of high molecular weight RNA's

in the presence of 10 µgs of ethidium bromide per ml, is actually zero. No label sediments faster than about 10s.

Figure III-3 shows the sedimentation of RNA extracted from mesenchyme blastula stage embryos, pre-incubated for 1 hour in sea water (o----o) or in sea water plus 10 µgs/ml of ethidium bromide (▲----▲) and subsequently incubated for an additional hour in ^{3}H-U. The results show that although the total level of synthesis of high molecular weight RNA molecules is reduced in the presence of ethidium bromide, the reduced level is still at approximately 50% of that in the control embryos.

The Morphological Effects of Ethidium Bromide on Development

Plate 1 illustrates normal development of sea urchin embryos through the completion of gastrulation (Plate 1-d).

The effects of 2 µgs/ml of ethidium bromide on the development of sea urchins is shown in plate 2. This concentration of ethidium bromide is adequate to inhibit more than 50% of the mitochondrial RNA synthesis (figure III-1). Although the embryos develop slightly more slowly, they hatch, gastrulate, form spicules (Plate 2-b, arrow 2), and develop to an early prism stage. However, the resulting gut is only about half the normal length (Plate 2-c versus Plate 1-d). These embryos were actually more viable than

the control embryos and survivived at this stage of development for more than a week. Under the same culture conditions, untreated embryos, normally do not survive beyond 5 days. This increased viability is assumed to result from an antibiotic effect of the ethidium bromide.

Plate 3 illustrates the effects of 5 µgs/ml of ethidium bromide on development. This concentration of ethidium is adequate to produce more than 80% inhibition of the mitochondrial synthesis in non-nucleate fragments (figure III-1b).

Normal embryos reach the mesenchyme blastula stage after 24 hours of development, Plate 1-c. In 5 µgs/ml of ethidium bromide, the embryos reach a comparable stage after almost 48 hours of development. They, however, have a very thick blastoderm wall, Plate 3-c (arrow). Approximately 30% of these embryos are alive and continue to swim after three full days of development. Although they do not gastrulate, the thickness of the blastoderm wall over the "animal" pole of the embryo appears to thin considerably (Plate 3-d, arrow 1) while cells appear to pile up in the vegetal region of the embryo (Plate 3-d, arrow 2).

Plate 4 shows the effect of 10 µgs/ml of ethidium bromide, added immediately after fertilization. In no case does an embryo cleave beyond the 8 cell stage. Most embryos were arrested at the 4 cell stage (after approximately 4

hours of development). This concentration of ethidium is adequate to inhibit all of the mitochondrial RNA synthesis. After cleavage stopped, the nuclei became visible and appear swollen, Plate 4-b & c. After 24 hours, most of the arrested embryos appeared to lose cellular definitions, Plate 4-d. Plate 4-e shows an unusual embryo which was fixed after 24 hours incubation. Eight swollen bodies are visible. None of the arrested embryos hatched, even if cultured for up to a week at 17°.

Plate 5 shows embryos that have been exposed to 10 µgs per ml of ethidium bromide for 1 hour (a) and for 24 hours (b) starting at the mesenchyme blastula stage of development (Plate 1-c). Although many of the embryos continue to swim for up to 30 hours in the presence of the ethidium bromide, gastrulation does not occur. Under normal conditions the embryos would have been fully gastrulated within 22 additional hours of development, Plate 1-d.

The results of this section can be summarized as:

1) Actinomycin D appears to penetrate the fertilization membrane of sea urchin embryos slowly but once inside it appears to inhibit RNA synthesis as effectively as in other systems.
2) Ethidium bromide effectively inhibits all of the RNA

synthesis in non-nucleate fragments, most of the RNA synthesis in early cleavage embryos, and approximately 50% of the RNA synthesis in mesenchyme blastula stage embryos.

3) Embryos do not develop beyond the 4-8 cell stage in the absence of mitochondrial RNA synthesis.

4) Development is arrested at the mesenchyme blastula stage in the absence of more than 80% of the mitochondrial RNA synthesis.

5) Development proceeds to a modified early prism stage in the absence of more than 50% of the mitochondrial RNA synthesis.

6) Ten µgs/ml of ethidium bromide, added at the mesenchyme blastula stage of develpoment, prevents gastrulation although the embryo may remain viable and swimming for up to thirty hours.

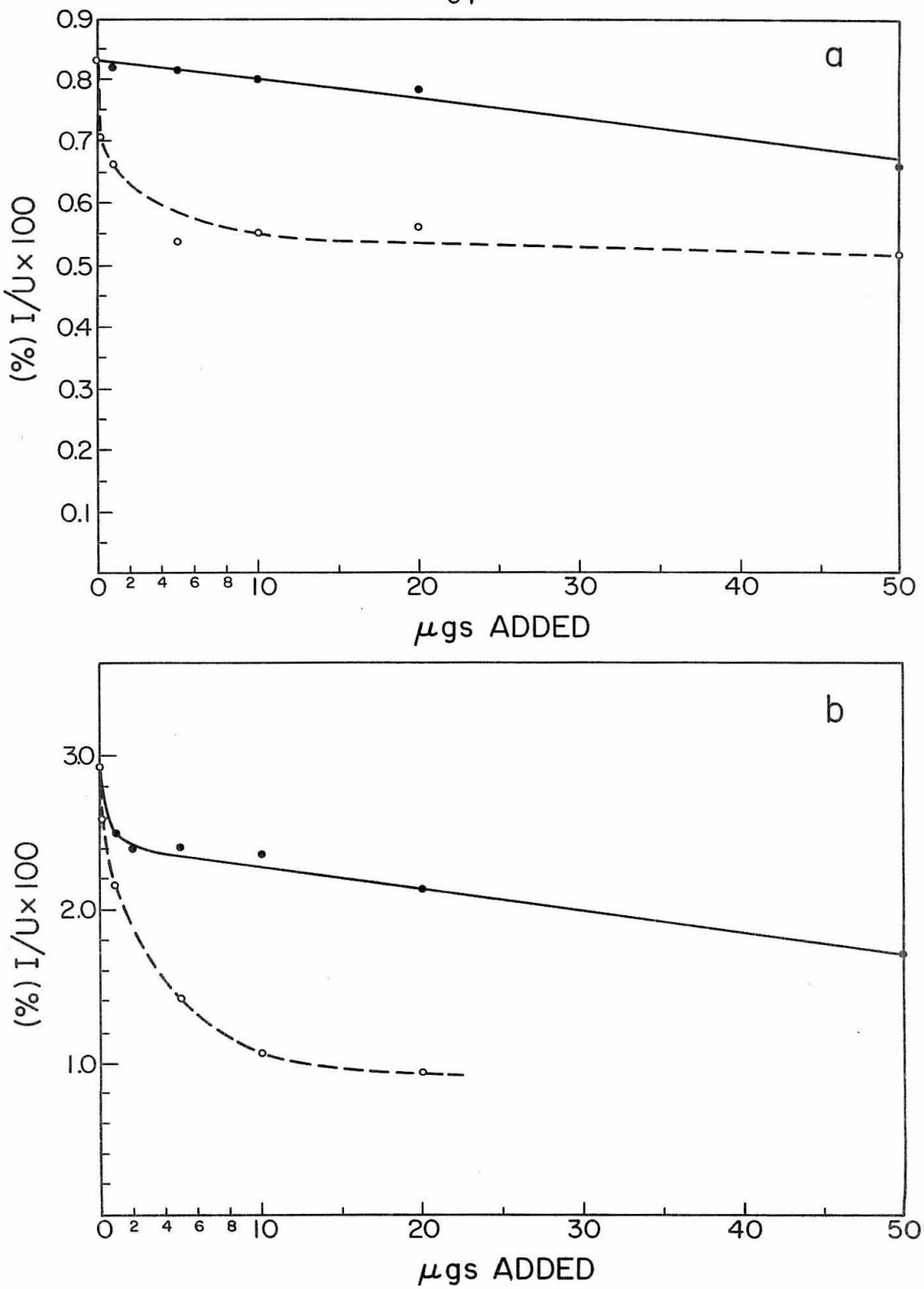

a
(%) I/U x 100
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0 2 4 6 8 10 20 30 40 50
μgs ADDED
b
(%) I/U x 100
3.0
2.0
1.0
0 2 4 6 8 10 20 30 40 50
μgs ADDED

Figure III-1

Figure III-1. Actinomycin D and ethidium bromide inhibition of RNA synthesis.

Fertilized embryos (a) and artificially activated non-nucleate fragments (b) were pre-incubated in 1 ml of sea water plus various concentrations (abscissa) of actinomycin D (•———•) and ethidium bromide (o----o). After 30 minutes, at 17°, 5 µcs of ^{3}H-5-U were added and the cells were incubated for 2 additional hours at 17° after which they were washed 3x with ice cold sea water. The cells were then lysed in 0.5% DOC and were spread on filter papers to dry. Procedures for determining the uptake (U) and incorporation (I) are described in methods. The % incorporation is calculated as I (cpm)/U (cpm) x 100.

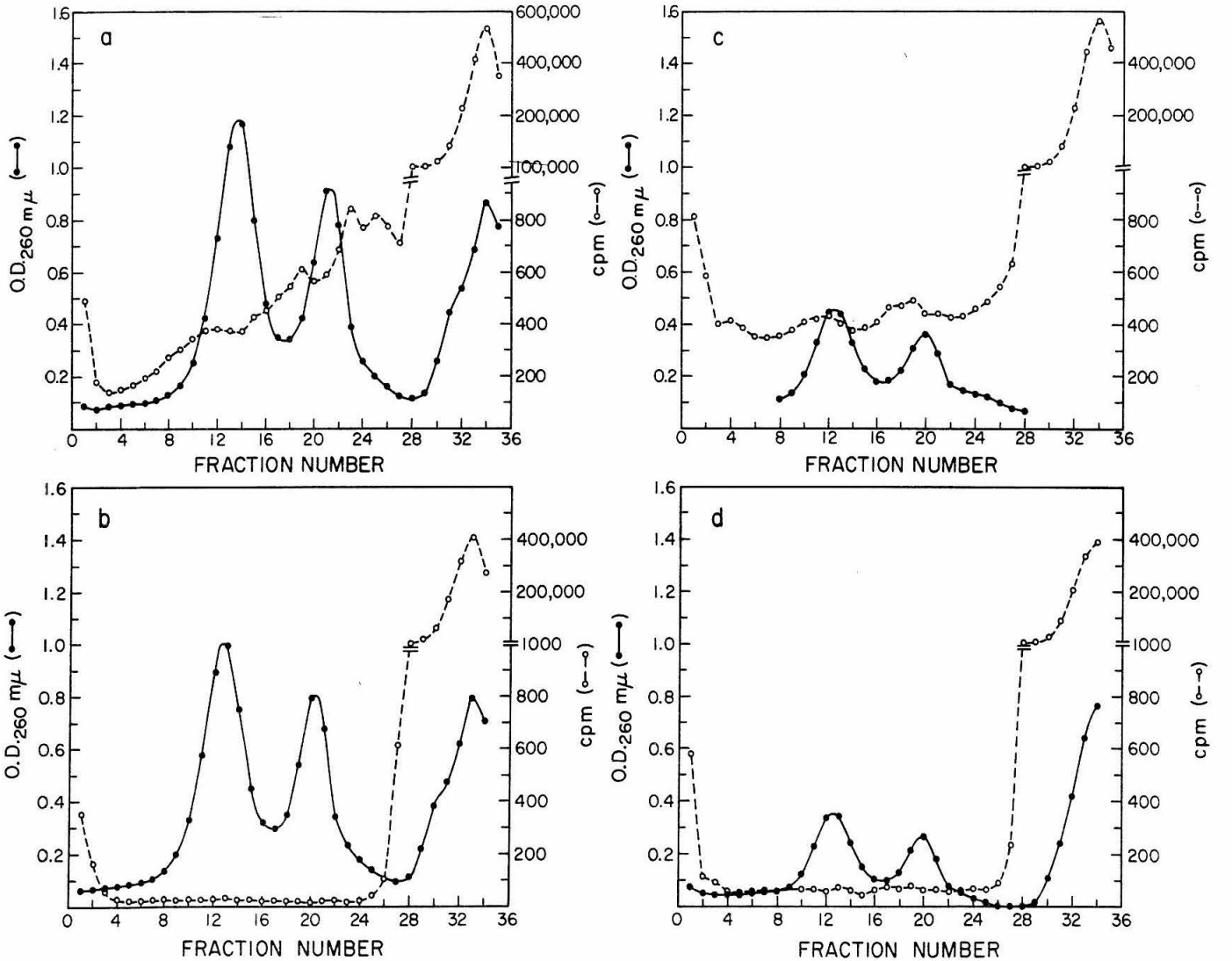


Figure III-2

Figure III-2. Ethidium bromide inhibition of RNA synthesis

Non-nucleate fragments and cleavage stage embryos were pre-incubated for 6 hours in 10 ml of sea water (a & c) or in 10 ml of sea water plus 10 µgs of ethidium bromide per ml (b & d) after which 50 µcs of ^{3}H-5-U were added. The cells were then incubated for 1 hour at 17° before washing and phenol extracting the RNA as described in methods. Sedimentation analysis of the redissolved RNA was in a 5 to 20% sucrose gradient at 2°, at 40,000 rpm in a S.W. 41 rotor for 6 hours. 0.35 ml fractions were collected. (a), non-nucleate fragment RNA; (b), RNA from non-nucleate fragments with ethidium bromide; (c), RNA from early cleavage embryos; (d), RNA from early cleavage embryos in ethidium bromide. $C.D._{260\ m\mu}$ (•———•); cpm, not TCA precipitated (o----o). Sedimentation is towards the left.

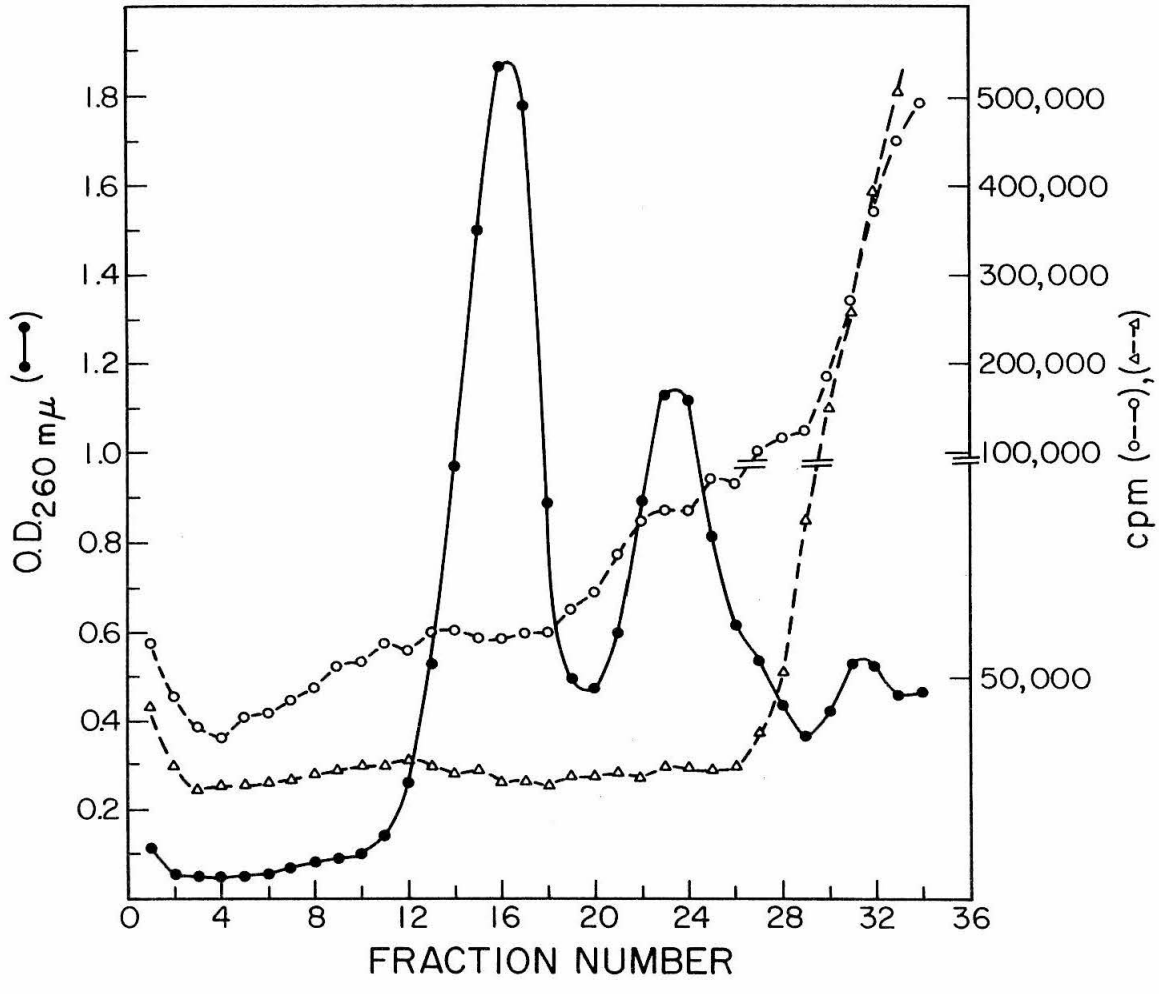

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
O.D.260 mμ (—●—)
500,000
400,000
300,000
200,000
100,000
50,000
cpm (o—o), (Δ—Δ)
0
4
8
12
16
20
24
28
32
36
FRACTION NUMBER

Figure III-3

Figure III-3. Ethidium bromide inhibition of RNA synthesis in mesenchyme blastula stage embryos.

Mesenchyme blastula stage embryos were incubated for 1 hour in 10 ml of sea water (o----o) or in 10 ml of sea water plus 10 µgs of ethidium bromide per ml (----) after which 50 µcs of ^{3}H-5-U were added. The embryos were then incubated for 1 hour at 17° before washing and phenol extracting the RNA as described in methods. The redissolved RNA was layered onto a 5-20% sucrose gradient and was centrifuged for 17 hours at 23,000 rpm at 2° in a S.W. 41 rotor. 0.35 ml fractions were collected. The O.D.'s were read and the liquid samples were counted directly without TCA precipitation. $O.D._{260\ m\mu}$ (•———•), cpms plus ethidium (Δ----Δ), cpms without ethidium (o----o).

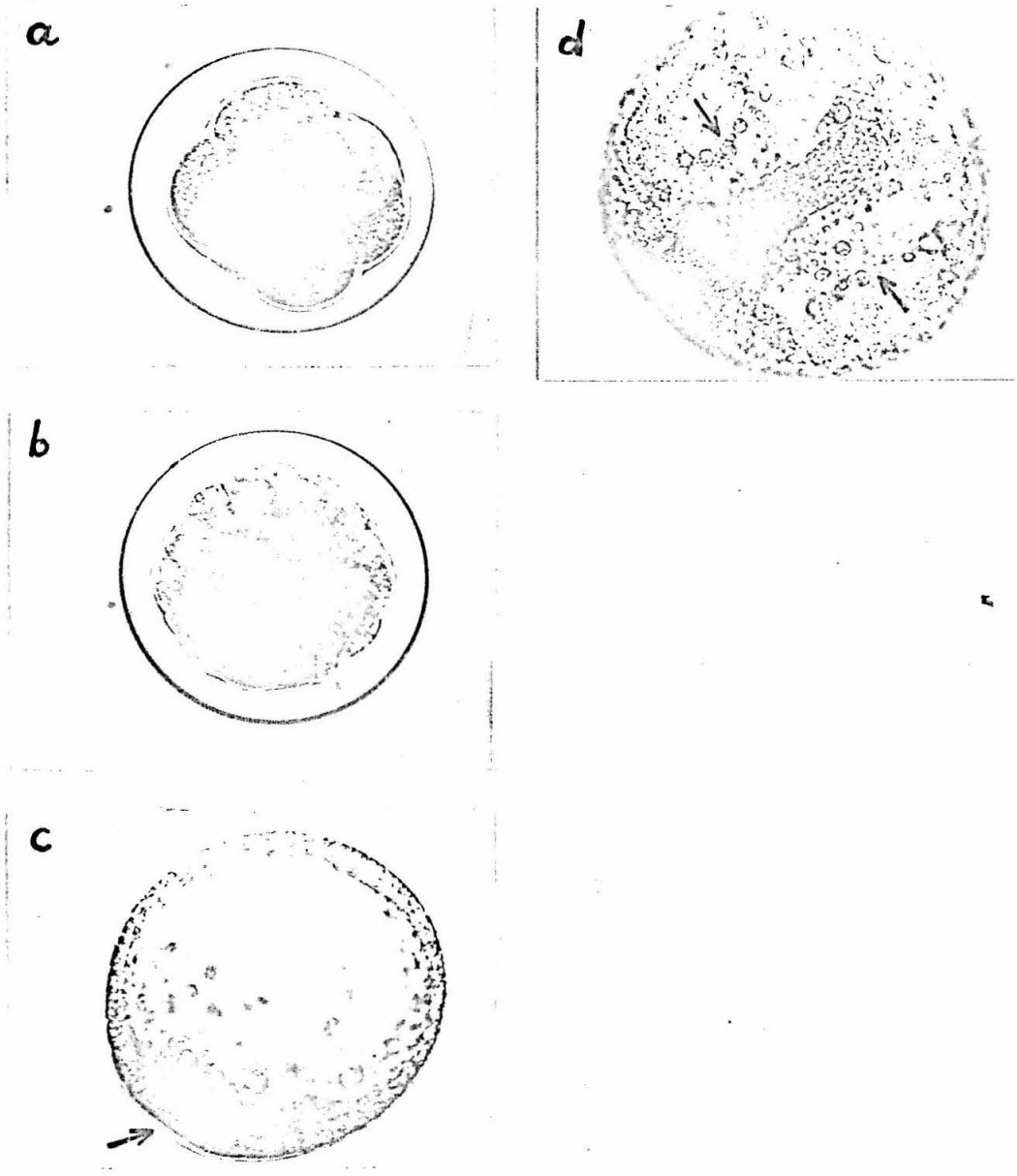


Plate - 1

Plate 1. The normal development of sea urchin embryos in artificial sea water at 17°.

a - 3.5 hours after fertilization (8 cell stage).

b - 6.25 hours after fertilization (morula stage).

c - 25.5 hours after fertilization (mesenchyme blastula stage); arrow points to the vegetal or presumptive gastropore region.

d - 46 hours after fertilization (gastrulation complete); arrows point to cells synthesizing the spicules.

magnification is 400 x normal size.

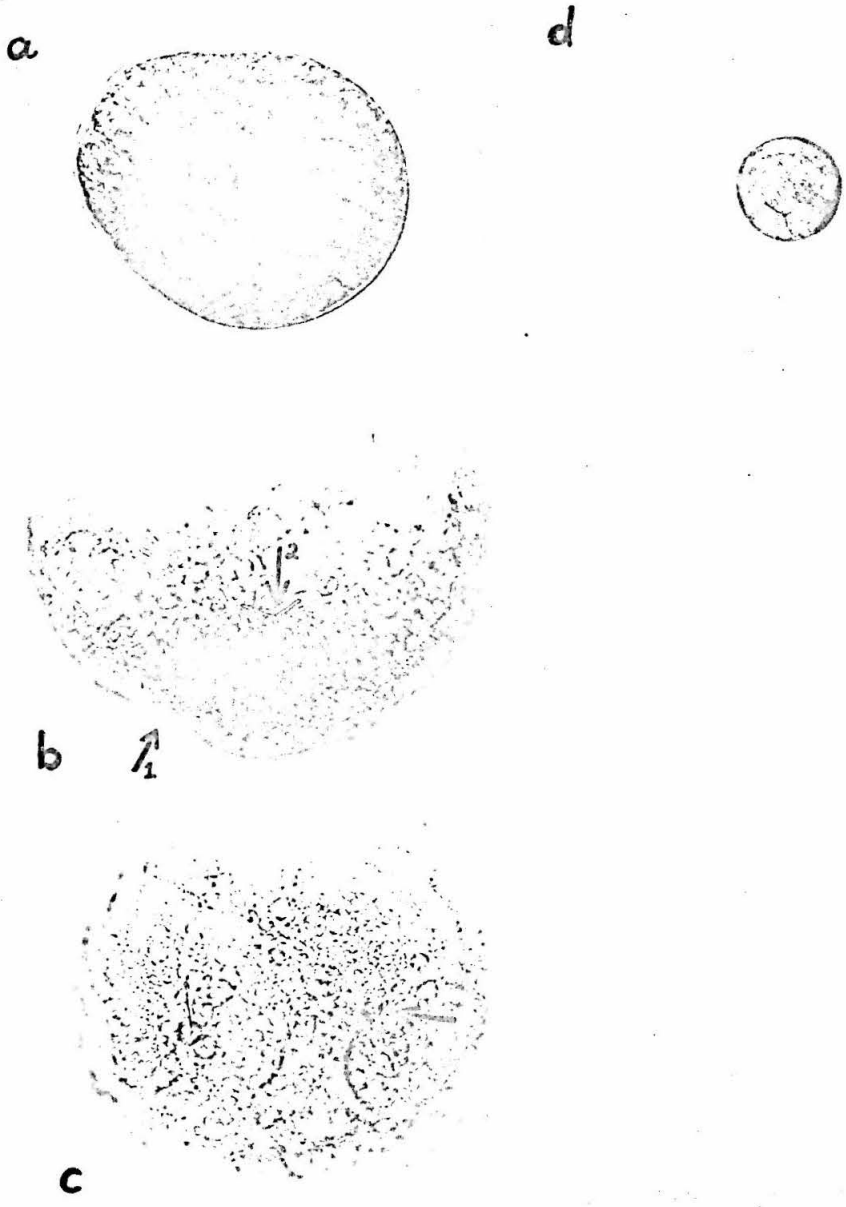


Plate - 2

Plate 2. The development of sea urchin embryos in artificial sea water plus 2 µgs/ml of ethidium bromide at 17°.

a - 26 hours after fertilization (mesenchyme blastula stage).

b - 47 hours after fertilization (gastrula stage); arrow 1 points to the gastropore; arrow 2 points to a newly forming spicule.

c - 72 hours after fertilization (gastrulation complete); arrow points to the mouth end of the gut.

d - also 72 hours after fertilization; note spicules.

a,b & c are magnified 400 x, d is magnified 100 x.

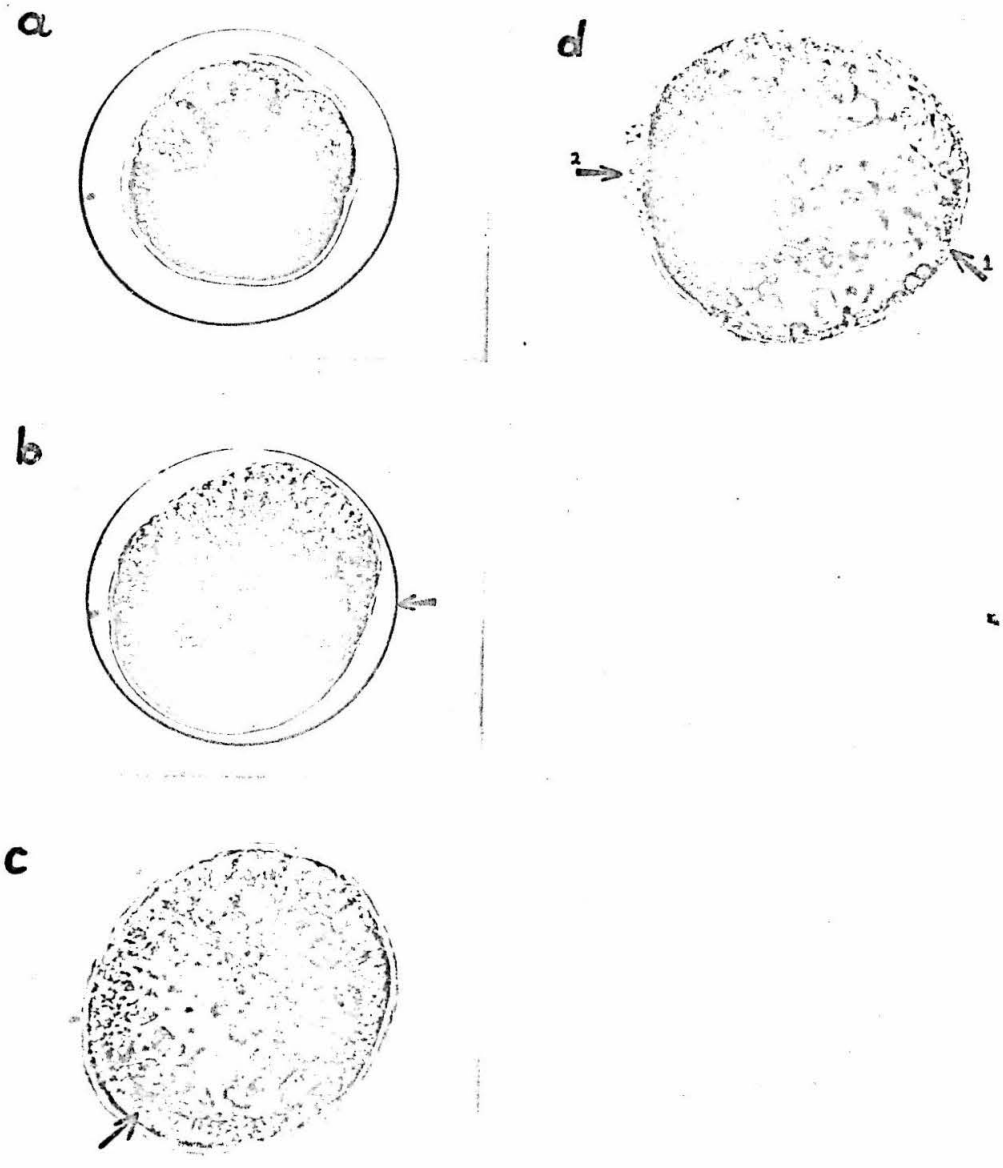


Plate - 3

Plate 3. The development of sea urchin embryos at 17° in artificial sea water plus 5 µgs/ml of ethidium bromide.

a - 6.5 hours after fertilization (16 cell stage).

b - 26 hours after fertilization (immediately before hatching) note arrow pointing to fertilization membrane.

c - 47 hours after fertilization (mesenchyme blastula stage); arrow points to thickened blastoderm wall over animal hemisphere.

d - 72 hours after fertilization; arrow 1 points to the animal hemisphere of the embryo; arrow 2 points to the presumptive gastropore of the embryo.

magnification is 400 x

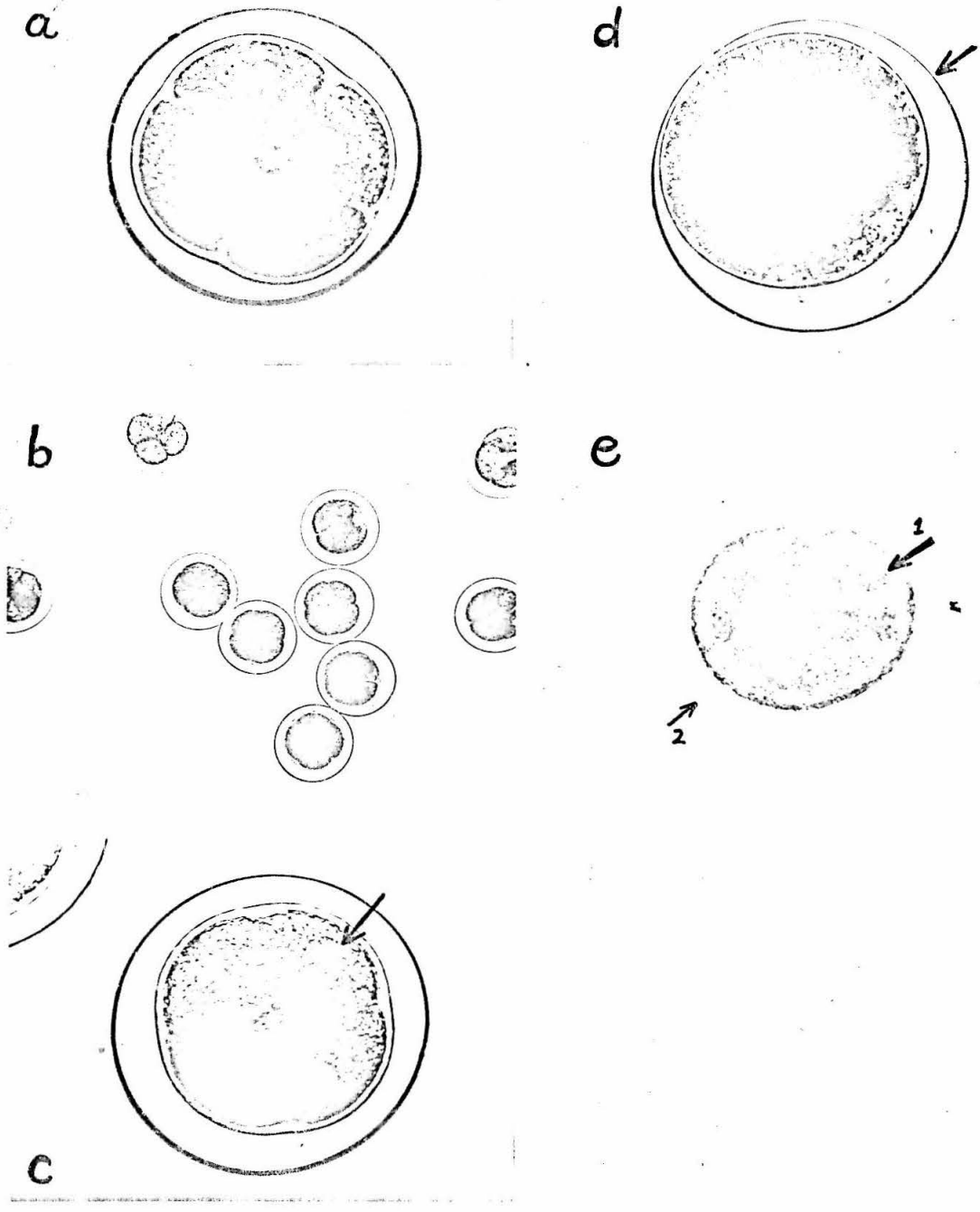


Plate - 4

Plate 4. The development of sea urchin embryos at 17° in artificial sea water with 10 µgs of ethidium bromide per ml.

a - 3.5 hours after fertilization (four cell stage).

b - 6.25 hours after fertilization (retarded 4 cell stage).

c - 6.25 hours after fertilization; arrow points to swollen body.

d - 26 hours after fertilization; the fertilization membrane remains intact, arrow.

e - 26 hours after fertilization, fixed in bouins fixative (an infrequent case); arrow 1 points to a swollen body, arrow 2 shows the position of the fertilization membrane which is invisible in this reproduction.

a, c, d & e are magnified 400 x; b is magnified 100 x.

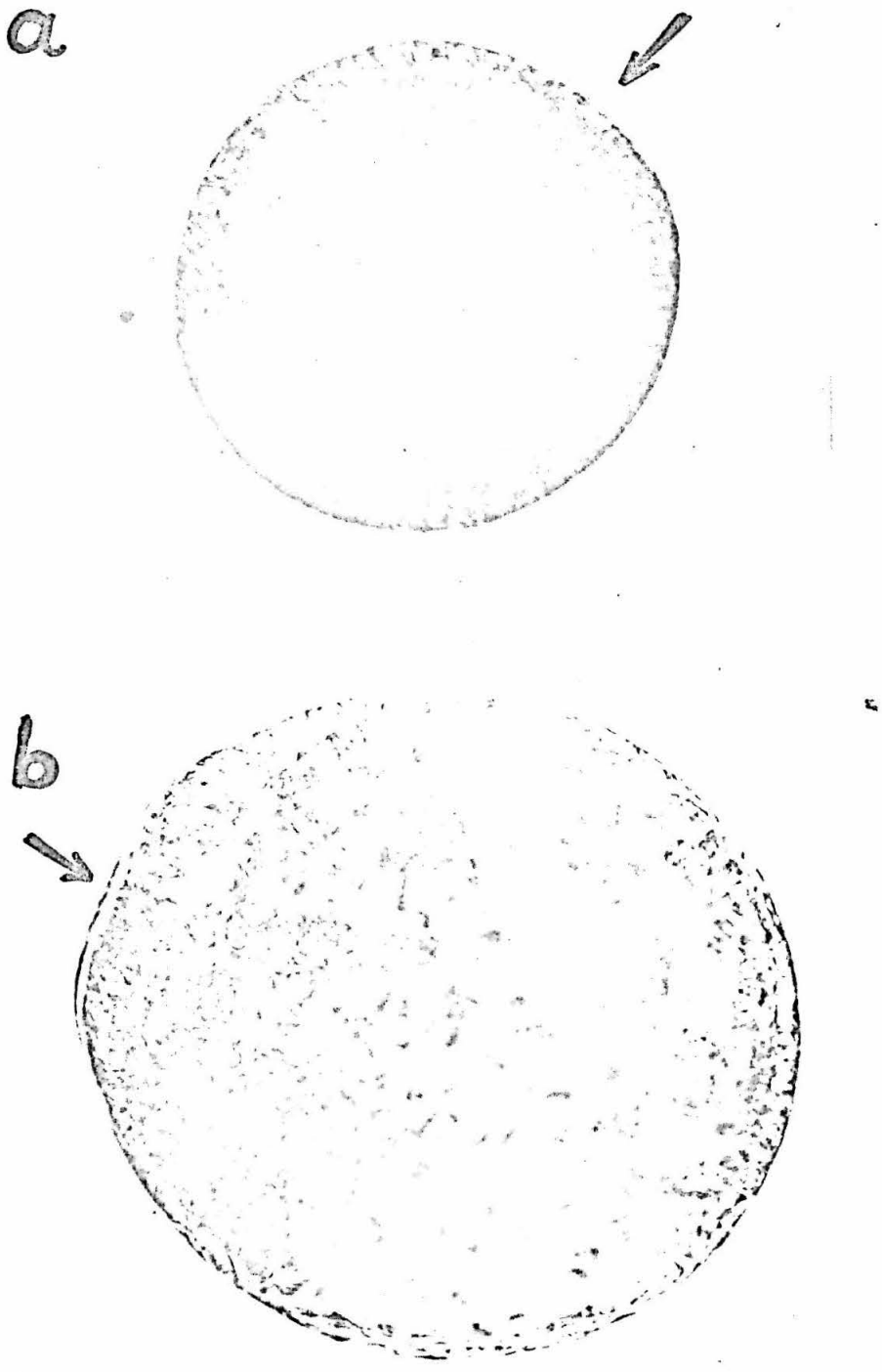


Plate - 5

Plate 5. The development of sea urchin embryos after the addition of 10 µgs of ethidium bromide at the mesenchyme blastula stage (after 24 hours at 17°)

a - 7 hours after the addition of the ethidium bromide, 31 hours after fertilization; arrow points towards the presumptive gastropore region; the embryos were killed in bouins fixative to slow them for taking pictures.

b - 22 hours after the addition of the ethidium bromide, 46 hours after fertilization; arrow points to the presumptive gastropore region of the embryo.

both photos are 400 x magnifications, the fixative caused the shrinkage of the embryo in a.

PART IV - DNA SYNTHESIS IN NON-NUCLEATE FRAGMENTS OF SEA URCHIN EGGS

In Strongylocentrotus purpuratus an unfertilized egg contains about 4.3 times the haploid amount of DNA (11). As much as 3.3 times the haploid amount may be in the form of mitochondrial DNA. These experiments used artificially activated non-nucleate fragments for the study of cytoplasmic DNA synthesis in the absence of the overwhelming nuclear DNA synthesis of fertilized eggs (figure IV-3b).

The results of CsCl buoyant density centrifugation of lysates of ^{3}H-Thymidine (^{3}H-T) labeled non-nucleate fragments appear in figure IV-1. The upper graph gives the counts per minute contained in every fraction collected after equilibrium and hence all of the radioactive precursor that was taken up into the activated and non-activated fragments. The upper graph illustrates that although considerably more precursor has gotten into the activated fragments, both the non-activated and the activated fragments were able to take up the labeled precursor. The lower graph illustrated the radioactivity remaining after TCA precipitation of the incorporated isotope. The band of acid precipitable counts at approximately 1.7 gms/cc is present in the activated non-nucleate fragments but is totally absent in the non-activated fragments. The profile of the band of radioactivity from the

activated fragments can be correlated to the homogeneity of the bucyant densities of the molecules and to the apparent molecular weight of the labeled DNA (87). The downward concavity of the leading and trailing edges of the band of radioactivity is evidence for homogeneity in buoyant density whereas the asymmetric distribution of counts around the band center tend to indicate heterogeniety. Utilizing the equation for molecular weight ($M = RT/ \bar{v}(d\rho/dr)_{r_o} w^2 r_o \sigma^2$) given by Meselson et al. (87) and the density at band center as an approximation of the partial specific volume in addition to using the calculated statistical deviation for the band width (σ = 0.677 cm), the apparent molecular weight of the radioactive band is 7.6×10^5. The molecular weight for sea urchin mitochondrial DNA is 1.4×10^7 (11). The molecular weight for the DNA synthesized in non-nucleate fragments appears to be too low to be mitochondrial. However, the above formula was based on the measurement of band width at equilibrium. In the preparative ultracentrifuge the band width may be affected by roter de-acceleration and fraction collecting procedures.

The DNA was rebanded in CsCl after intercalation with ethidium bromide in order to test for ^3H label in the closed circular mitochondrial DNA, figure IV-2. After equilibrium, the DNA bands fluoresced strongly in UV light and the polysaccharide, contaminating the prep, was visible

to the naked eye. Fractions labeled a, b and c designate the positions for the elution of polysaccharide, closed circular mitochondrial DNA and non-closed DNA respectively.

After 2 hours (figure IV-2B) and 8 hours (figure IV-2C) incubation with ^{3}H-T, radioactively labeled closed circular mitochondrial DNA is found in neither embryos nor non-nucleate fragments, figure IV-2C.

Also of note is the fact that in non-nucleate fragments, but not in embryos, a considerable proportion of radioactivity was incorporated into the polysaccharide after 8 hours incubation with ^{3}H-T.

An attempt to localize the template for the DNA synthesis, occurring in non-nucleate fragments, led to the analysis of the 15,000 x g pellet lysate by sucrose sedimentation, figure IV-3a. No appreciable amount of label was found in these lysates, sedimenting with the small 27s O.D. peak (found to be reproducible in several experiments). Since the pellet lysate contains nearly all of the mitochondria, it is concluded that none of the DNA synthesis is in the mitochondria.

When the sedimentation from the non-nucleate fragment pellet lysate (figure IV-3a) is compared to a similarly prepared pellet lysate from embryos (figure IV-3b) it is obvious that the non-nucleate fragment is essentially devoid of nuclear DNA synthesis (several hundred versus 50,000

cpms in high molecular weight material from comparable volumes of cells).

Conflict arises, however, when the whole non-nucleate fragments are phenol extracted and the nucleic acid is analysed by sucrose sedimentation, figure IV-3c. Although ^{3}H-T labeled material is found to sediment around 38 and 27s (corresponding to closed and nicked circular mitochondrial DNA), none of the label is found to band with the closed circular mitochondrial DNA in CsCl after intercalation with ethidium bromide (figure IV-3d). The possibility exists that the 37s material is composed of doubly nicked catenane dimers of mitochondrial DNA as described elsewhere (98).

A brief summary of the results of this section follows:

1) DNA is synthesized in artificially activated non-nucleate fragments of sea urchin eggs.
2) None of the incorporated radioactivity was found to cosediment with the 27s, nicked mitochondrial DNA, in 15,000 x g pellet lysates or to coband with the closed circular mitochondrial DNA, in CsCl buoyant gradients, after intercalation with ethidium bromide.
3) When phenol-extracted from the whole fragments, the DNA sedimented in sucrose at s values close to those for

nicked monomers and doubly nicked catenane dimers of the mitochondrial DNA.

4) During extended incubation with ^{3}H-T, acid precipitable radioactivity was incorporated into the polysaccharide fraction of the artificially activated non-nucleate fragments. In cleaving embryos, however, no radioactivity was similarly incorporated.

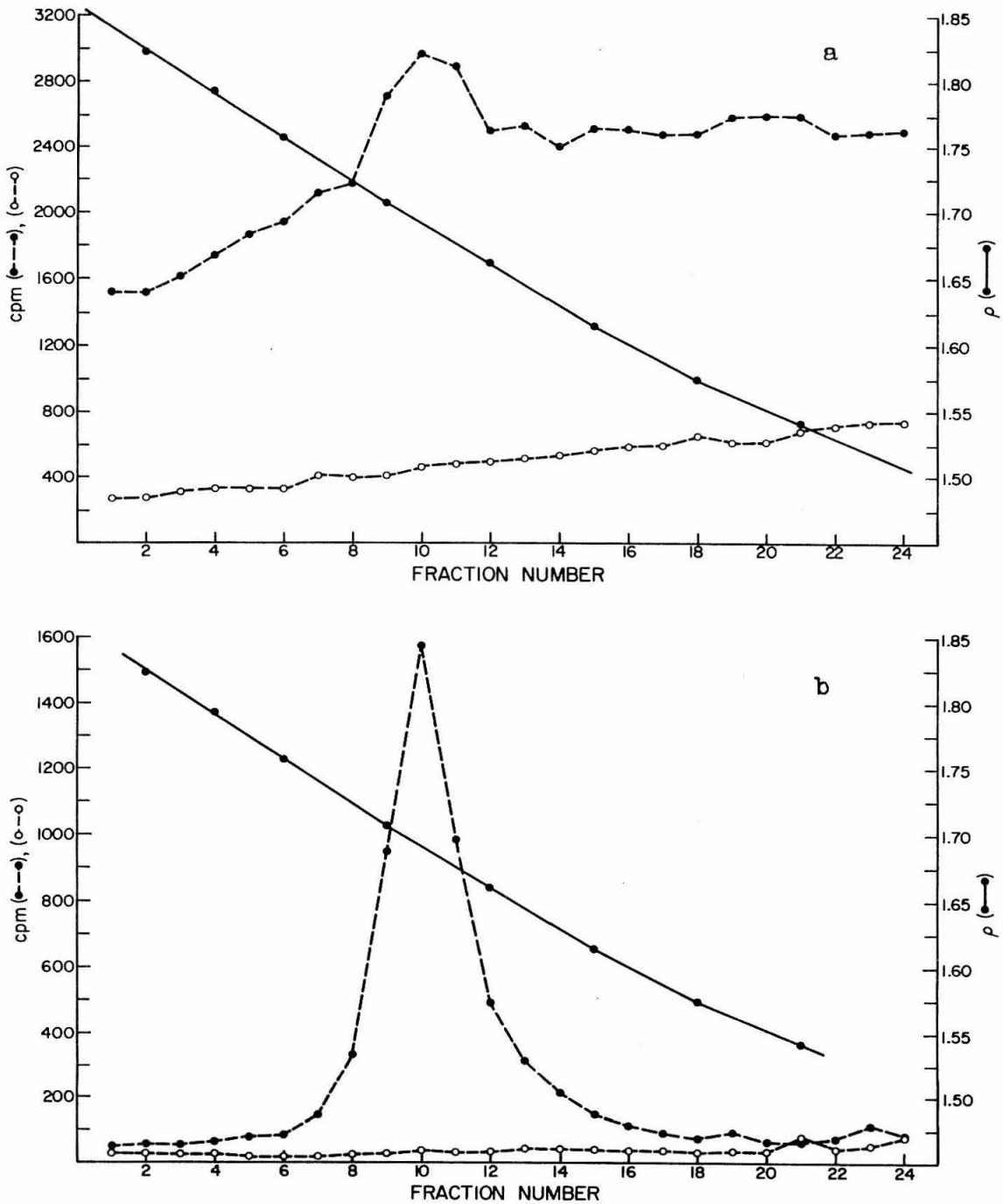
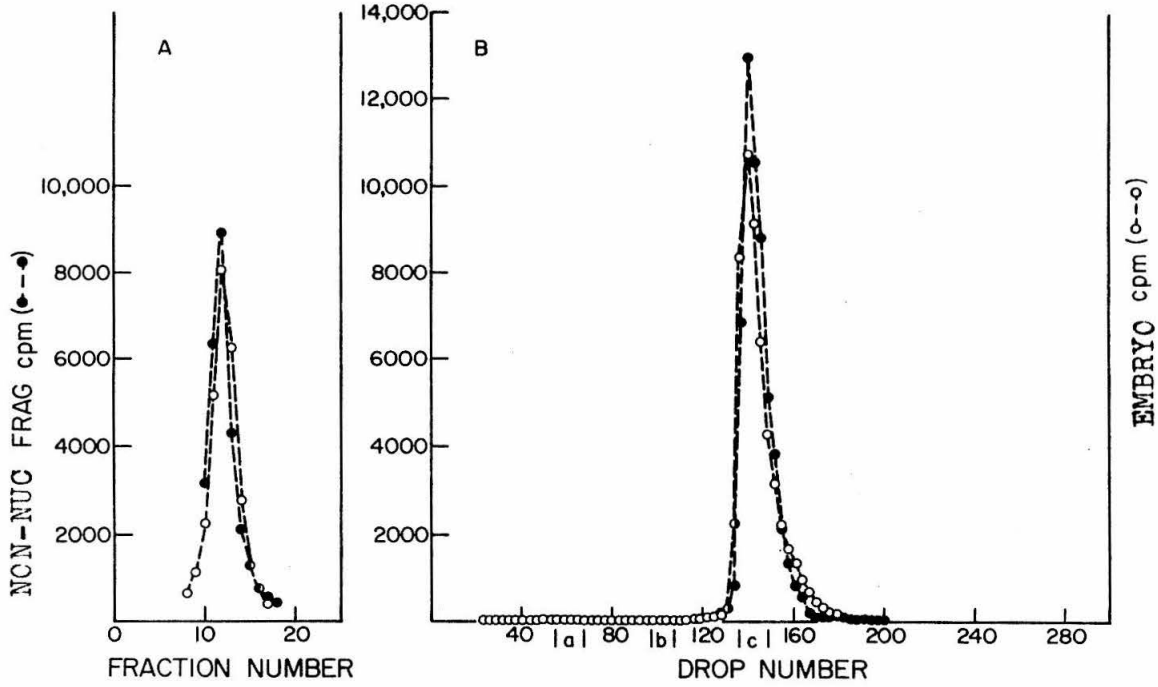

a
b
cpm (●—●), (o—o)
ρ (●—●)
FRACTION NUMBER
3200
2800
2400
2000
1600
1200
800
400
1600
1400
1200
1000
800
600
400
200
1.85
1.80
1.75
1.70
1.65
1.60
1.55
1.50
2
4
6
8
10
12
14
16
18
20
22
24

Figure IV-1

Figure IV-1. DNA synthesis in activated and non-activated non-nucleate fragments of sea urchin eggs.

Equal volumes (0.3 ml) of activated and non-activated non-nucleate fragments were incubated for 2 hours in a total volume of 15 ml with 13 μc ^{3}H-T/ml. In this experiment the washed labeled fragments were suspended in 3 volumes of 0.275 M KCl, 0.01 M MgAc and 0.01 M tris (pH 7.5) and were dissolved with 0.5% Na-DOC. The resulting solution was made to 1.7 gms/cc with CsCl and centrifuged for 60 hours at 40,000 rpm at 20° in a S.W. 65 rotor. 0.2 ml fractions were collected. Solution density was calculated from the refractive index (99). (a), the total radioactivity per fraction. (b), the acid precipitable radioactivity per fraction. Non-activated fragments (o----o), activated fragments (•-----•).


A
B
NON-NUC FRAG cpm (●--●)
EMBRYO cpm (o--o)
FRACTION NUMBER
DROP NUMBER
|a|
|b|
|c|

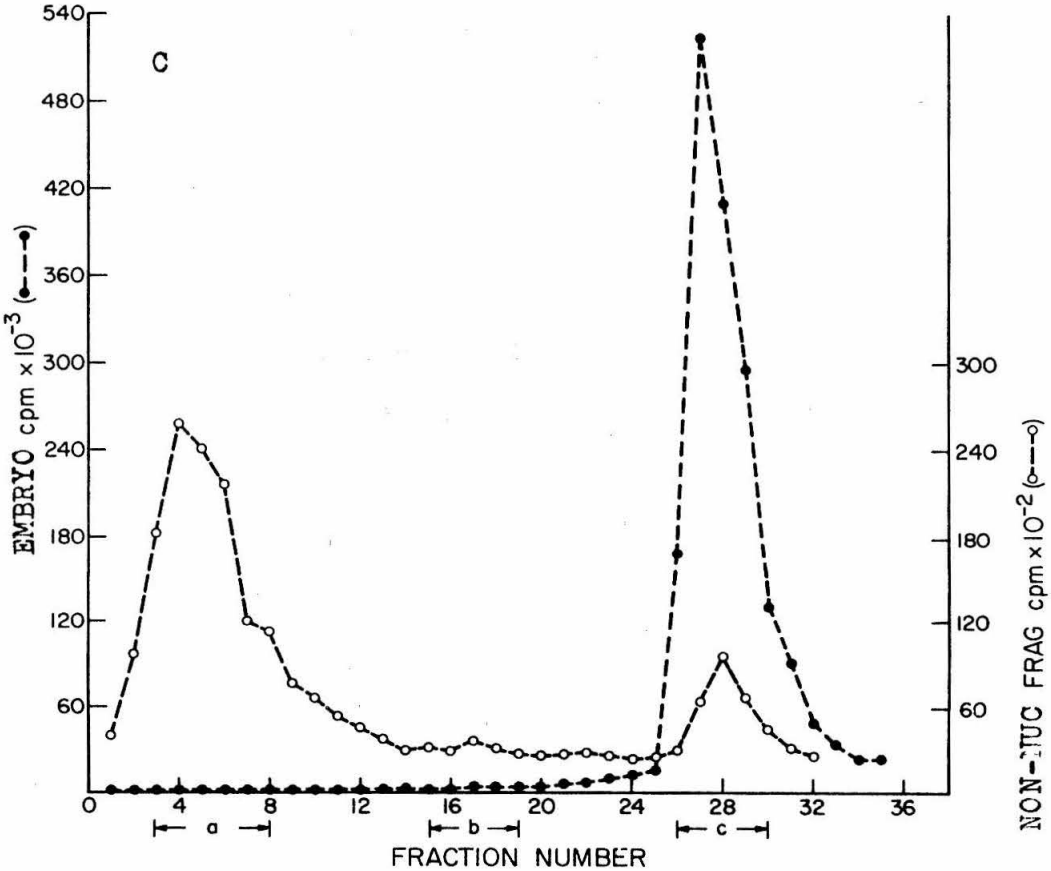

C
EMBRYO cpm x10⁻³ (●--●)
NON-NUC FRAG cpm x10⁻² (o--o)
FRACTION NUMBER
a
b
c

Figure IV-2

Figure IV-2. CsCl buoyant density centrifugation of ^{3}H-T labeled DNA intercalated with ethidium bromide.

In graphs IV-2A & B, fertilized eggs (o----o) and artificially activated non-nucleate fragments (•-----•) were incubated for 2 hours at 18° in 20 ml of artificial sea water containing 100 units of penicillin/ml and 20 µc ^{3}H-T/ml. In graph C, fertilized nucleated fragments (•-----•) and artificially activated non-nucleated fragments (o----o) were incubated for 8 hours at 18° in 40 ml of artificial sea water containing 100 units of penicillin per ml, 50 µgs of streptomycin sulfate per ml and 12.5 µcs of ^{3}H-T/ml. At the end of the incubation periods the fertilized eggs (upper graphs) were at the two cell stage and the fertilized nucleate fragments (lower graph) were at 4, 8, 16 and 32 cell stages. The upper left graph is the results of preliminary banding of the DNA in CsCl. The upper right and lower graphs are the results of rebanding after adding ethidium bromide to 300 µgs/ml. The fraction numbers in B & C, designated by a, b and c, are the fractions containing polysaccharide, closed circular mitochondrial DNA and non-closed DNA respectively.

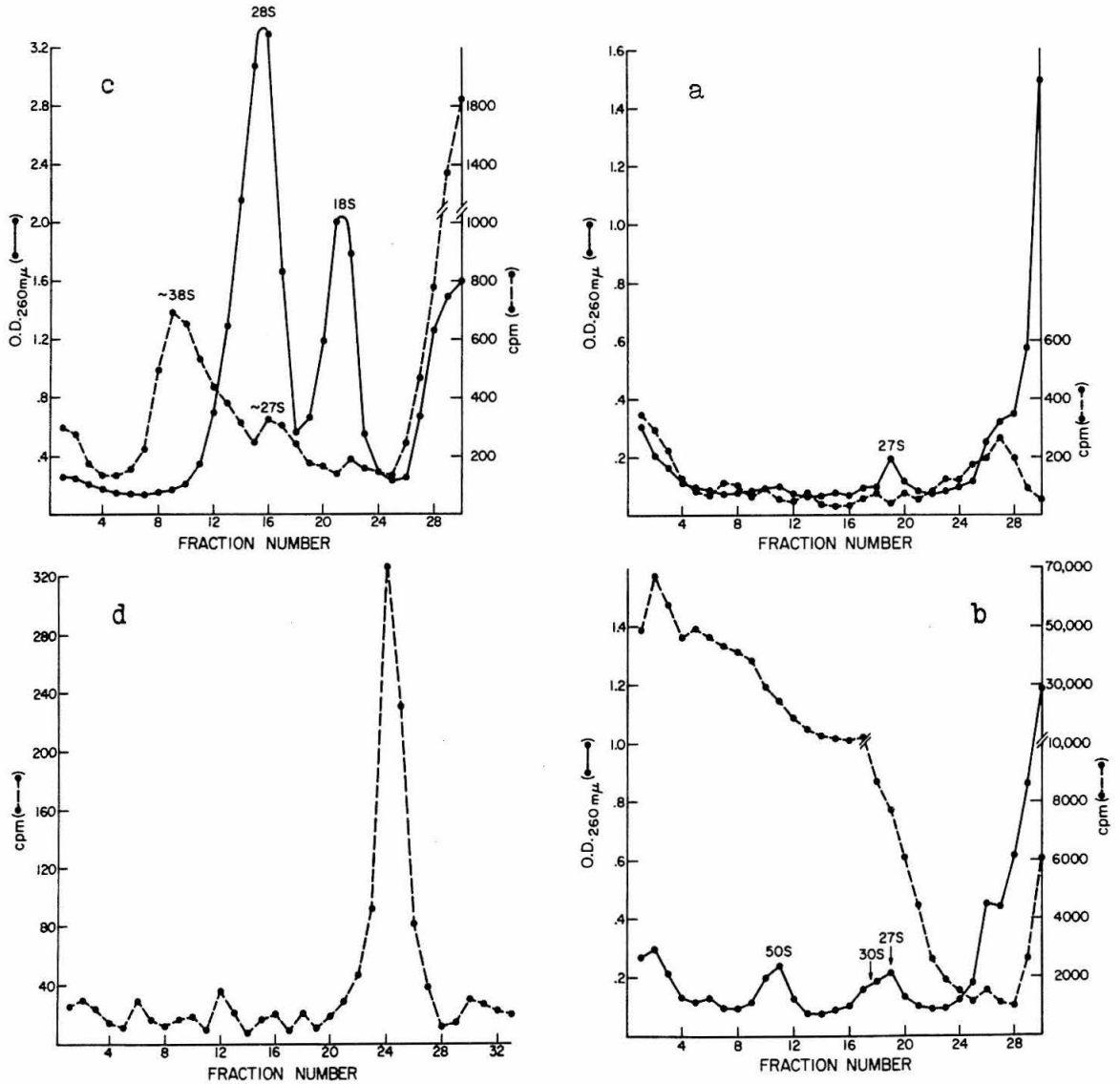

c
28S
18S
~38S
~27S
O.D.260mμ
cpm
FRACTION NUMBER
a
27S
O.D.260mμ
cpm
FRACTION NUMBER
d
cpm
FRACTION NUMBER
b
50S
30S
27S
O.D.260 mμ
cpm
FRACTION NUMBER

Figure IV-3

Figure IV-3. DNA synthesis in non-nucleate fragments

Artificially activated non-nucleate fragments, figure IV-3a, and fertilized nucleated fragments, figure IV-3b, were incubated for 2 hours in a total volume of 25 ml with 10 µcs of ^{3}H-T/ml. The cells were washed, homogenized and then centrifuged at 15,000 x g for 20 minutes. The sediment or pellet of this centrifugation was resuspended and lysed in 0.5% Na-DOC, 0.275 M KCl, 0.005 M EDTA and 0.05 M tris at pH 7.5. The lysates were layered onto 11 ml of a 15-30% (wt/wt) sucrose gradient with 0.275 M KCl, o.005 M EDTA and o.o5 M tris (pH 7.5) plus a 1 ml 60% sucrose pad and centrifuged at 40,000 rpm for 13 hours at 2^{o} in a S.W. 41 rotor. The 27s O.D. peak in figure IV-3a is assumed to be that of the nicked mitochondrial DNA. O.D. peaks at 50 and 30s in figure IV-3b are the ribosomal subunits.
Artificially activated non-nucleate fragments were incubated for 1 hour in a total volume of 30 ml with 10 µcs ^{3}H-T/ml. The fragments were washed and phenol extracted as specified in methods. A portion of the dried nucleic acid was suspended in 0.005 M EDTA, 0.275 M KCl and 0.05 M tris at pH 7.5. Four tenths ml of the solution was layered on 11 ml of a 5-20% (wt/vol) sucrose gradient plus a 1 ml 60% sucrose pad all in the same buffer as the nucleic acid. The gradient was spun at 40,000 rpm for 4 hours at 2^{o} in a S.W.

41 rotor. Four tenths ml fractions were collected. Three tenths of each fraction was dried on papers and washed with ice cold TCA as specified in methods. The results of the scintillation counting appear in figure IV-3c. Figure IV-3d is the results of banding the DNA in figure IV-3c in CsCl after intercalation with ethidium bromide. The closed circular mitochondrial DNA elutes between fractions 12 and 20. The non-closed DNA elutes between fractions 20 and 28. O.D. 260 mu (•———•); cpm (•------•).

DISCUSSION

DISCUSSION

Mitochondrial RNA Synthesis

Since the mitochondria of Neurospora make "bacteria-like" ribosomes for their own protein synthetic machinery, many investigators have assumed that the mitochondria of all organisms possess "bacteria-like" ribosomal RNA (rRNA). Dawid (89), in his search for mitochondrial rRNA, has concluded that the 13 and 21s species of mitochondrially associated RNA's, in Xenopus eggs, are mitochondrial rRNA's, unlike either bacterial rRNA or the normal cytoplasmic rRNA's. I believe, however, that there has never been any sound evidence for ribosomes, unique to the mitochondria, in sea urchins, vertebrates or in vertebrate tissue cultures.

There is evidence against the synthesis of rRNA by the mitochondria of sea urchins and HeLa cells. The RNA's made in non-nucleate fragments of sea urchin eggs have little secondary structure, see Part I. Attardi and Attardi (90) do not find rRNA made by the mitochondria of HeLa cells and they also find that the 21s RNA, associated with crude mitochondrial preparations, is not even transcribed from the mitochondrial DNA template.

The length of the circular mitochondrial DNA from yeast is approximately 27 microns (91) while that of sea urchins

and higher organisms is approximately 5 microns. To assume that redundant functions of the mitochondrial genome (such as the synthesis of its own ribosomes) may be lost during evolution would be reasonable. Mitochondrial control of its own protein syntheses could be maintained through the retention of genes for a few specialized tRNA molecules (67-69).

Mitochondrially derived RNA is present in the membrane fraction of cellular homogenates (70) but the absence of newly synthesized mitochondrial RNA from the soluble portion of the cell homogenates has not been previously shown. In Part II the newly synthesized RNA from the 1 hour labeled non-nucleate fragments is found only in the 15,000 x g pellet of the fragment homogenate. Although some of the RNA was shown to be associated with the isolated mitochondria, it is still not known whether the RNA is inside the mitochondria or on polysomes attached to membranes contaminating mitochondrial preparations or otherwise.

The evidence for "masked" mRNA in sea urchin eggs is partially dependent upon work with high concentrations of actinomycin D (20 µgs/ml) as an inhibitor of RNA synthesis (1, 2). However, autoradiographic studies of sea urchin embryos incubated in ^{14}C actinomycin D show that no actinomycin gets into the eggs until after hatching (92, 93). There is a recent report that abnormal cleavage results from

the presence of 20 μgs/ml of actinomycin D (94). Still another laboratory has reported that embryos develop normally in the presence of actinomycin D, even after the removal of the fertilization membranes (95). The fact that the presence of the fertilization membrane seems to slow the uptake of actinomycin D and therefore retard its potential for RNA synthesis inhibition (Part III) can now be added to this presently raging controversy regarding actinomycin D effects. The results of Part III agree with the autoradiographic data (92) in that the fertilization membrane appears to retard the uptake of actinomycin D. It should be noted, however, that some of the discrepencies, regarding the effects of actinomycin D, may be partially a difference between the fertilization membranes of different species, or even of animals of the same species collected in different areas or during different seasons. The relative impenetratibility of the fertilization membranes could depend on the diet of the urchins or the salt concentrations of the water they are maintained in.

Protein Synthesis in Sea Urchin Eggs

Protein synthesis, in artificially activated non-nucleate fragments, is not seriously affected by concentrations of ethidium bromide, adequate to inhibit mitochondrial

RNA synthesis, Part II, figure II-7. Earlier studies of the protein synthesis in non-nucleate fragments did not take into account possibilities for extranuclear RNA synthesis (8-10). The fact that, in Part II, protein synthesis continues in the absence of RNA synthesis, substantiates the evidence, in the earlier investigations, for a "masked" mRNA template in sea urchin embryos. Also, since the separation of proteins in Part II is on the basis of molecular weight, one can make an estimate for the range of size classes of proteins made from the "masked" mRNA template. The soluble proteins range from 9 to 40 thousand while the low speed pelleted proteins range from 6 to 60 thousand in molecular weight.

The possibility exists that pleiotropic effects from ethidium bromide cause the retardation of embryos as seen in Part III. Evidence in favor of ethidium bromide inhibiting primarily mitochondrial transcription and against deleterious pleiotropic effects is: 1) Protein synthesis is not seriously affected by concentrations of ethidium bromide, adequate to inhibit mitochondrial RNA synthesis, Part II. 2) There is a decrease in the percent inhibition of RNA synthesis by the ethidium bromide as development proceeds and as the proportional amount of mitochondrial DNA present diminishes with respect to the amount of nuclear DNA in the cell, Part III, figures III-2 & 3. 3) Although embryo

development stops before gastrulation, after the addition of 10 µgs/ml of ethidium bromide at the mesenchyme blastula stage, the embryos continue to swim for 24-30 hours, indicating that the presence of the ethidium is not immediately toxic to many of the life functions of the embryo.

Brachet (96), in a brief note to Nature, describes the effects of ethidium bromide on the development of embryos of sea urchins. He reported that high concentrations (20-30 µgs/ml) of ethidium bromide would stop development during cleavage. He also found that 10 µgs/ml of ethidium bromide retarded spicule formation. The higher concentration of ethidium bromide required for inhibition of cleavage and the effects of ethidium bromide on spicule formation differ slightly from the results discussed in Part III.

A likely candidate for the cause behind the retardation of embryo development in the absence of mitochondrial RNA synthesis is a requirement for membrane structural components during cleavage and gastrulation. There is evidence, in Neurospora, for membrane protein synthesis by RNA from the mitochondrial DNA template (76). If a similar condition for membrane synthesis exists in sea urchins, the cell requirements for membrane synthesis, during cleavage and gastrulation, may not be met in the absence of mitochondrial RNA synthesis. As a result cleavage may be stopped. In Part III, increasing concentrations of ethidium bromide actually

produced a gradual increase in the extents of retardation of the embryos. At 2 µgs/ml of ethidium bromide, enough membranes may still be synthesized to permit a short gut to be formed during gastrulation.

Also, in Part III, embryos developing in 5 µgs/ml of ethidium bromide do not gastrulate. However, the cells on the blastoderm seem to be capable of moving to the presumptive gastropore in the absence of gastropore formation. The observed effect was the thinning of the blastoderm over the animal hemisphere and an apparent piling up of cells at the vegetal hemisphere.

DNA Synthesis in Non-Nucleate Fragments of Sea Urchin Eggs

The study of DNA synthesis in non-nucleate fragments was undertaken to investigate the synthesis of cytoplasmic DNA in the absence of nuclear DNA synthesis.

Although there is considerable DNA synthesis in artificially activated non-nucleate fragments, it does not appear to be associated with the intact mitochondria, in the 15,000 x g pellet lysates (figure IV-3a), and it doesn't become closed circular DNA within 8 hours after synthesis. This evidence against the DNA synthesis being mitochondrial in origin may be contrasted to the fact that phenol extracts of whole non-nucleate fragments contain labeled DNA which

sediments at 38 and 27s, corresponding to closed circular and nicked mitochondrial DNA, during sucrose sedimentation. It has recently been reported that a doubly nicked catenane dimer sediments slightly slower than the closed monomeric form (97). The labeled DNA in non-nucleate fragment phenol extracts could therefore be mitochondrial DNA's entirely in the nicked form. At this point however the discussion becomes entirely conjecture. Further investigations are necessary before definite conclusions can be drawn.

APPENDIX

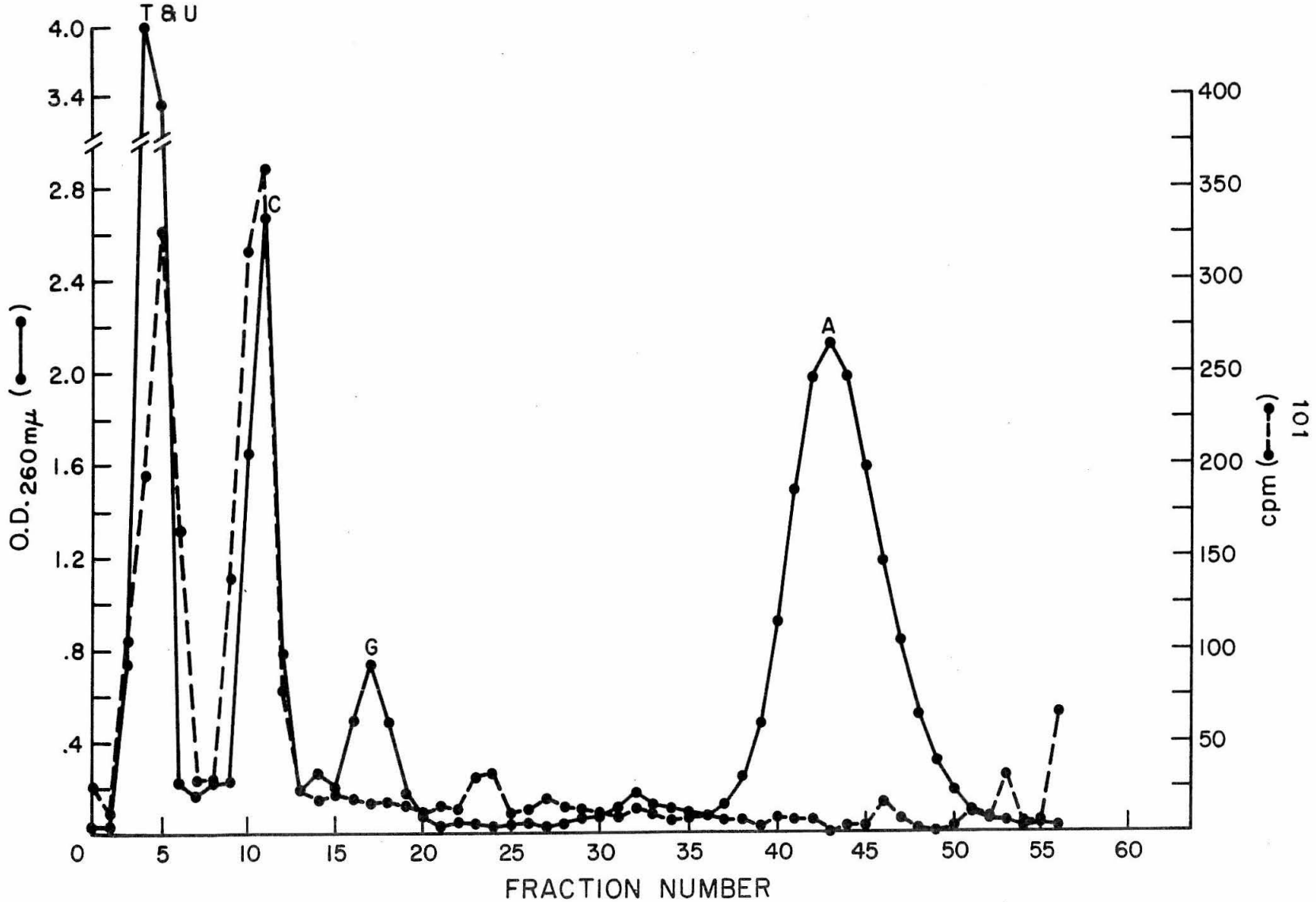

T & U
C
G
A
4.0
3.4
2.8
2.4
2.0
1.6
1.2
.8
.4
O.D. 260mμ
400
350
300
250
200
150
100
50
101 cpm
0
5
10
15
20
25
30
35
40
45
50
55
60
FRACTION NUMBER

Figure A-1

Figure A-1. ^{3}H-5-U conversion to ^{3}H-C in non-nucleate fragments of sea urchin eggs.

Artificially activated non-nucleate fragments were incubated for 6 hours at 19° in 10 ml of artificial sea water with 15 µcs of ^{3}H-5-U per ml, after which they were washed and phenol extracted as specified in methods. The dried nucleic acid was dissolved in 1 ml of 0.275 M KCl, 0.005 M EDTA and 0.05 M tris at pH 7.5 and treated with 10 µgs of preheated RNAase for 1 hour at 25°. The remaining nucleic acid was precipitated in 2 volumes of ethanol at -20° for 4 hours and subsequently washed 2 times in ice cold 5% TCA before redehydrating and drying. The remaining nucleic acid was hydrolysed and column chromatographed through a Dowex 50-X8 column following the procedures of Brown and Attardi (98). The bases come off in the order U & T, C, G, and A (from left to right in figure A-1). O.D.$_{260\ m\mu}$ (•———•); cpm (•------•).

TABLE A - 1

Actinomycin D and Ethidium Bromide Inhibition of RNA Synthesis in Embryos of the Sea Urchin

Experiment (# of cases)	Average Uptake	Incorporation	I/U x 100
Controls (4)	235,744	1,963	0.833
Actinomycin D			
1 µg/ml (2)	243,801	2,003	0.822
5 µgs/ml (2)	230,762	1,884	0.816
10 µgs/ml (2)	235,758	1,894	0.803
20 µgs/ml (2)	229,360	1,801	0.785
50 µgs/ml (2)	211,358	1,398	0.662
Ethidium Bromide			
0.1 µg/ml (1)	290,291	2,049	0.706
1.0 µg/ml (1)	297,861	1,974	0.663
5 µgs/ml (1)	272,145	1,465	0.538
10 µgs/ml (1)	244,394	1,347	0.551
20 µgs/ml (1)	226,839	1,276	0.562
50 µgs/ml (1)	187,596	969	0.517

Table A-1. Actinomycin D and ethidium bromide inhibition of RNA synthesis in embryos of sea urchins.

See legend to figure III-1.

TABLE A - 2

Actinomycin D and Ethidium Bromide Inhibition of RNA Synthesis in Non-Nucleate Fragments of Sea Urchin Eggs

Experiment	Uptake	Incorporation	I/U x 100
Controls (3 cases)	211,498	6,187	2.93
Actinomycin D (µgs/ml)			
1	222,944	5,582	2.50
2	212,365	5,097	2.40
5	214,593	5,167	2.41
10	222,200	5,239	2.36
20	225,098	4,800	2.13
50	228,039	3,900	1.71
Ethidium Bromide (µgs/ml)			
0.1	215,999	5,592	2.59
1.0	222,708	4,816	2.16
5	213,333	3,023	1.42
10	179,577	1,918	1.07
20	212,555	2,006	0.94

Table A-2. Actinomycin D and ethidium bromide inhibition of RNA syntheisi in non-nucleate fragments of sea urchin eggs.

See legend to figure III-1.

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