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EXPERIMENTAL OBSERVATIONS ON RIBONUCLEIC ACID SYNTHESIS DURING DEVELOPMENT OF THE MARINE MUD SNAIL *ILYANASSA OBSOLETA* SAY

by

Richard B. Koser

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1974

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

May 24, 1974
Date

~~Chairman of Examining Committee~~
Professor J. Collier

May 28, 1974
Date

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ABSTRACT

Distributions of nascent RNAs from normal and lobeless embryos of the marine mud snail Ilyanassa obsoleta were examined to determine whether morphogenetic factors, localized in the polar lobe at first cleavage and differentially segregated in the course of determination, affect development through the control of RNA transcription. Electrophoretic profiles revealed that nascent RMAs differed significantly between normal and lobeless embryos during organogenesis but not before gastrulation and that these differences originated in non-ribosomal RNAs. It was concluded that cytoplasmic localization involves factors that control transcription and possibly translation.

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> "That a single cell can carry the total heritage of the complex adult, that it can in the course of a few days or weeks give rise to a mollusk or a man, is one of the great marvels of nature."
>
> (Wilson, 1925 p. 1035)

INTRODUCTION

Cell lineage studies on invertebrate eggs, beginning with Whitman's (1878) observations on Clepsine, revealed a remarkably precise cleavage pattern which implied that egg materials were being exactly packaged and distributed in a manner somehow related to differentiation. Gross cytoplasmic movements occurring before or after fertilization in several precisely cleaving (mosaic, or spiralean) embryos (Conklin, 1905, 1910, 1912, 1931; Lillie, 1906) organized the visible (and therefore the invisible) egg components in a manner suggestive of a role in development. This was tested in ctenophores (Driesch and Morgan, 1895; Yatsu, 1911, 1912a,b), nemertines (Wilson, 1903; Yatsu, 1904) and molluscs (Wilson, 1904a,b) by following the development of egg fragments; in general, normal or abnormal development resulted according to when the egg was fragmented in relation to germinal vesicle breakdown and the maturation divisions. Similar results obtained when the development of isolated blastomeres was examined (Boveri, 1901; Fischel, 1898; Farfaglic, 1963; Zeleny, 1904; Wilson, 1904a,b,c; see Morgan, 1927 pp. 307-380) and it became apparent that the distinction between mosaic (parts of the embryo developing as though part of the whole) and regulative (parts of the embryo compensating to

develop as the whole) embryos concerned only the time at which the egg materials became differentially distributed.

Applying pressure to cleaving molluscan embryos altered the position of cleavage planes, thus changing the allocation of cytoplasm among cells. The abnormal development resulting (e.g., Conklin, 1912; Styron, 1967) was additional evidence for the localization of morphogenetically important cytoplasms. Indeed, special cytoplasms were shown to control the formation of germ cells in insects (Hegner, 1911; Reith, 1925; Seidel, 1926), nematodes (Boveri, 1909) and frogs (Smith, 1966).

A paradigm of the localization phenomenon is found in the polar lobe, a cytoplasmic protuberance, of the molluscs Ilyanassa and Dentalium. When the lobe was removed at the first cleavage, Ilyanassa embryos displayed a modified cleavage pattern and no mesoblast bands developed (Crampton, 1896). Similarly treated Dentalium embryos lacked the apical organ, the post-trochal region and also mesoblast bands (Wilson, 1904a,b). "The conclusion is irresistible that prelocalization of the structures in question exists in the ooplasm of the unsegmented egg, and that to this extent at least, the specification of the blastomeres is due to the nature of the specific cytoplasmic materials which they receive during cleavage. These materials we may conveniently designate as organ-forming substances or formative stuffs." (Wilson, 1925 p. 1065).

Ilyanassa obsoleta (Say, 1821; Stimpson, 1865) is an ideal organism for the study of cytoplasmic localization

for its polar lobe is a container of organ-forming substances whose experimental removal leads to well-defined morphological consequences. These substances are precisely distributed through spiral cleavage during which time determination occurs temporally removed from morphorgenesis.

Clement (1952), through the use of pasteurized sea water, was able to obtain extended development of Ilyanassa embryos well beyond the 2-day, ciliated larvae observed by Crampton. He confirmed Crampton's observations on the lobeless embryo: the cleavage pattern of the derivatives of the D quadrant was no longer different from that of the other quadrants and the 4d cell arose neither precociously nor free of yolk. Developmental features lacking in the lobeless embryo are (Clement, 1952, 1962; Atkinson, 1968) the eyes, foot, statocysts, operculum, external shell, heart and intestine. Non-lobe-dependent features include velar cilia, stomadaeum, pigment, and partial differentiation of endoderm, muscle and shell. The polar lobe thus has a selective effect upon organogenetic processes; furthermore, the lobeless embryo develops at a rate similar to controls, remains alive after controls have formed veliger larvae and displays some structural organization.

Experiments with isolated blastomeres (Clement, 1956) and selective deletion of micromeres (Clement, 1963, 1967) and macromeres (Clement, 1962) have revealed the morphological values of the cleavage cells and the fact that the polar lobe's developmental influence segregates to the D quadrant derivatives in the 2nd, 3rd and 4th quartets. Induction by

the 3D macromere was also inferred in the formation of eyes, velum, foot and shell. Wilson (1904b) showed that the polar lobe's influence was progressive in Dentalium also.

Centrifugation experiments (Morgan, 1933, 1935; Clement, 1968) have shown that the visible components of the polar lobe (or the egg cytoplasm) are not responsible for the localization; this is true also in Chaetopterus (Lillie, 1906), Arbacia (Lyon, 1907) and ascidians (Conklin, 1931).

The biochemical nature of organ-forming substances is of prime interest to embryologists. Boveri (1902) demonstrated the chromosomal control of development (confirmed and extended by Fankhauser, 1934) which was supposed to involve differential gene expression (Stedman and Stedman, 1950). Temporal modulation of the genome during development was confirmed in plants (Walbot *et al*., 1972), slime mold (Firtel, 1972), echinoderms (Glišin *et al*., 1968), mammals (Brown and Church, 1972) and was inferred from studies of the mollusc Ilyanassa (Collier and Yuyama, 1969; Collier, 1966). Regional specificity of transcription in developing embryos was shown in plants (Walbot *et al*., 1972), echinoderms (Markman, 1961), amphibians (Flickinger *et al*., 1966); Flickinger and Daniel, 1972; Bachvarova and Davidson, 1966) and mammals (Brown and Church, 1972). These phenomena were presumably a result of the differential derepression of embryonic genes as evidenced by changes in the *in vitro* transcribability of embryonic chromatin (Flickinger *et al*., 1965; Kohl *et al*.,

1969; Georgiev et al., 1966).

One step removed from the above, the translation process allows the additional control step of selection from new and preformed messages. Selective translation processes were disclosed in echinoderms (Spiegel et al., 1965; Terman and Gross, 1965; Terman, 1970; Gustavson and Hasselberg, 1951; Ellis, 1966), ascidians (Smith, 1967), amphibians (Spiegel, 1960) and in Ilyanassa (Teitelman, 1973; Freeman, 1971; Morrill and Norris, 1965; Koser, 1968). This process also showed regional specificity (Denis, 1961, amphibia; Donohoo and Kafatos, 1973, Ilyanassa).

The Ilyanassa polar lobe has a high concentration of acid-soluble phosphorous compounds (Collier, 1960b) and ultra-violet-absorbing materials suggestive of nucleic acid precursors (Berg and Kato, 1959) and it is possible that the lobe functions by controlling RNA transcription. This would explain the depression of RNA synthesis observed in lobeless embryos (Davidson et al., 1965) and their reduction (Collier, 1961) and altered patterns (Teitelman, 1973) of protein synthesis during embryogenesis (Morrill and Norris, 1965; Koser, 1968, Freeman, 1971). Evidence for the control of RNA transcription by cytoplasmic factors already exists in amphibians (Merriam, 1969), avian and mammalian erythrocytes (Harris, 1967) and various mammalian cells (Thompson and McCarthy, 1968).

Accordingly, this dissertation was undertaken to examine whether the Ilyanassa polar lobe influences development by affecting genomic transcription. One approach, RNA/DNA

hybridization, was unfeasible because the difficulty in obtaining biological material would limit the accumulation of sufficient competitor RNA and the results obtained from low RNA concentrations would apply only to the redundant portion of the genome (Church and McCarthy, 1968; McCarthy and Church, 1970) that comprises only 30-40 % of the DNA in Ilyanassa (Davidson et al., 1971). The genetic information responsible for the polar lobe's organ-specific effects, however, is presumed (Britten and Davidson, 1969) to be in the unique portion of the genome which comprises most of the genetic complexity in Ilyanassa (Davidson et al., 1971) and which transcribes differentially during development (Firtel, 1972; Church and Brown, 1972; Brown and Church, 1972).

Estimation of genomic activity through quantitation of RNA synthesis was also impossible both for lack of information about the Ilyanassa RNA precursor pool and for the unattainability of complete RNA extraction.

Consequently, an electrophoretic analysis was chosen for its inherent advantages: sensitivity, allowing the economical utilization of small numbers of embryos; high resolution, allowing consideration of distinct RNA categories; no requirement for RNA precursor pool measurement and quantitative RNA recovery since electrophoretic patterns could be expressed and compared as percentage distributions; selectivity, eliminating concern for the terminal addition to transfer RNA (Glišin and Glišin, 1964) by appropriate choice of gel formulations and, thereby, molecular weight ranges of the RNA distributions. Firstly, ribosomal RNA and its precursor

were characterized (see appendix A). Then dual-label experiments were designed to allow an exact comparison of normal and lobeless RNA profiles by means of internal controls (Terman, 1970) but this design disclosed a serious disruption of RNA metabolism at precursor concentrations high enough for adequate ^{14}C-uridine incorporation (see results). Thus, statistical procedures for profile analysis were employed to compare embryonic RNA distributions labeled with the same precursor.

The analysis brough to light two prominent differences between nascent RNAs from normal and lobeless Ilyanassa embryos, namely, that dissimilarities exist in discrete size-classes, in the non-ribosomal, non-transfer RNAs, and that they emerged not during the early developmental period encompassing determination and gastrulation, but during the later stages of organogenesis. The influence of the polar lobe in Ilyanassa, then, is manifest in newly transcribed RNAs, presumably through control of transcription but also, possibly, through control of translation.

MATERIALS AND METHODS

Biological Material

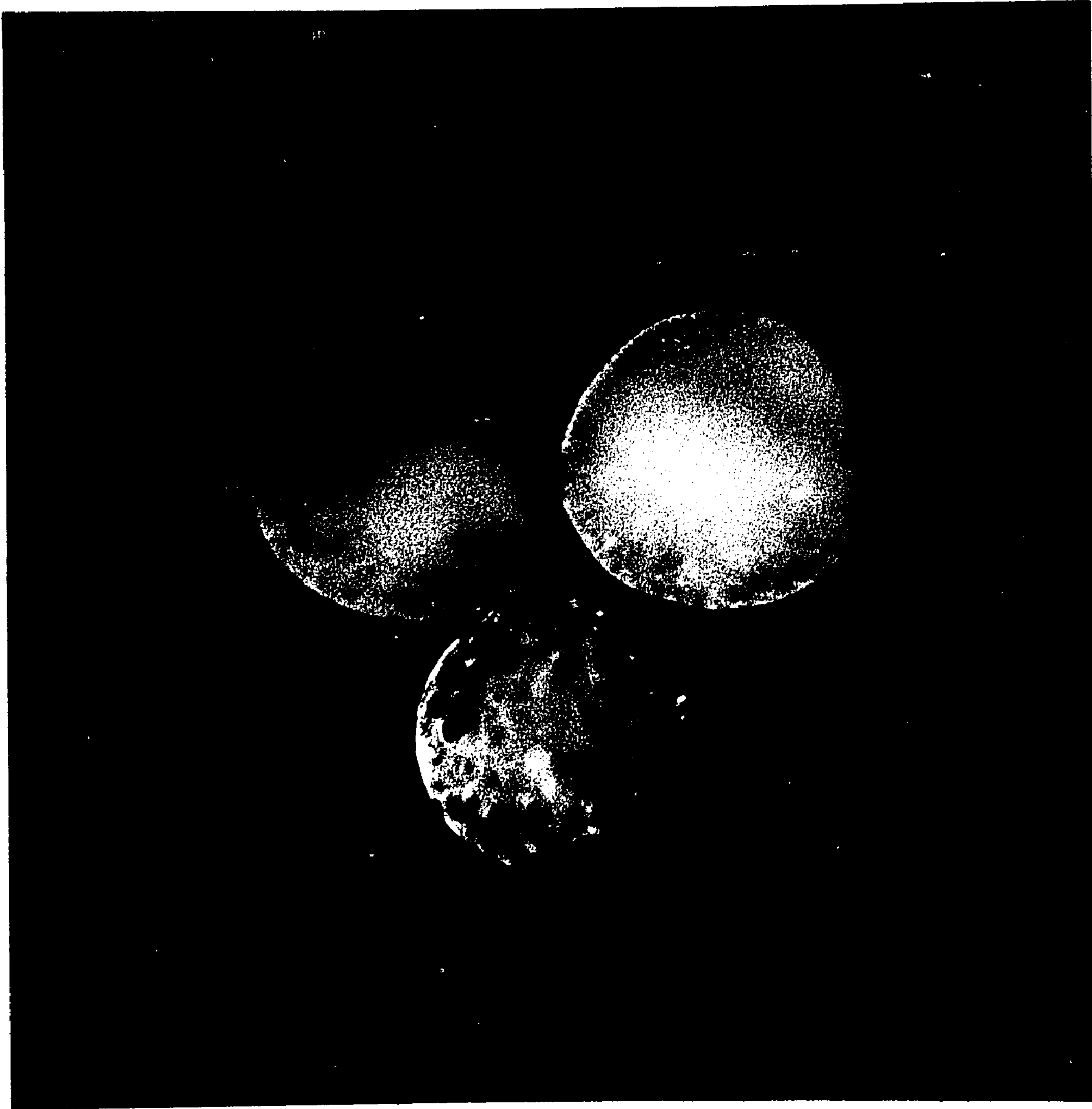
Specimens of the marine mud snail Ilyanassa obsoleta were collected near Woods Hole and Barnstable, Massachusetts and were maintained in groups of 75-100 in glass aquaria with a recirculating sea water system. Although the normal breeding season occurs during the summer, snails in the laboratory lay eggs from November through July; freshly collected snails

provide eggs for a period ranging from several weeks to several months. The sexes are separate; eggs are fertilized within the female and deposited in capsules containing approximately 80-100 embryos which undergo more-or-less synchronous development.

Egg capsules or isolated embryos were sorted according to their age and cultured in filtered sea water containing the antibiotics penicillin and streptomycin (100 µg/ml each) at 20°C until the desired stage of development (see results).

Lobeless embryos were produced by a procedure similar to that of Collier (1957). The third polar lobe is a vegetal, cytoplasmic protrusion formed at the first cleavage: when the two blastomeres and the lobe are about equal in size, forming a trefoil, attachment of the lobe is most tenuous and it can be removed. Freshly deposited capsules containing uncleaved eggs were cleaned and placed in iced sea water containing the antibiotics (herinafter referred to as sea water) until time permitted the removal of the eggs from each capsule to an individual, small dish of artificial sea water (Van't Hoff's (1903) alpha formulation) which lacked calcium and magnesium; the solution had been adjusted to pH 8 with sodium bicarbonate and was used as an 80-90 % mixture with sea water. When embryos reached the trefoil stage of first cleavage (figure 1) they were sucked into a finely-drawn glass pipette and delobed by expelling them against the bottom of the dish. Lobeless embryos and controls were then removed to separate dishes of sea water and cultured at 20°C until the desired stage of development.

FIGURE 1-Ilyanassa embryo at first cleavage. The egg has cleaved to the characteristic "trefoil" of two cells (upper right, AB blastomere; upper left, CD blastomere) and the yolky, vegetal polar lobe which is narrowly attached to, and will be resorbed into, the CD blastomere. Each sphere is approximately 125 μ in diameter.



Ilyanassa embryo RNA was radioactively labeled by incubating lots of approximately 300 embryos in 0.2 ml of sea water and 10-250 μC/ml of uridine-5-H^3 (New England Nuclear, 25.6C/mM), 25-50 μC/ml uridine-C^{14} (New England Nuclear, 55.6 μC/μM) or 30-250 μC/ml of L-methionine-methyl-H^3 (New England Nuclear, 3.25 C/mM; the radioactive pulse was terminated by rinsing the embryos with sea water and quickly freezing them with dry ice.

RNA Preparation

RNA was extracted from Ilyanassa embryos, veliger larvae, adult digestive glands and from E. coli and rat liver. Fifteen milliliters of homogenizing solution (1 % triisopropylnapthalenedisulfonate (TNS) and/or 1% sodium dodecyl sulfate (SDS), 0.1 % Macaloid, 10^{-2} M magnesium acetate, 10 μg/ml polyvinylsulfonate (PVS), in 0.1 M sodium acetate buffer-pH 5.0) and 30 ml phenol (distilled, saturated with buffer and containing 0.1 % 8-hydroxyquinoline) were combined with each gram of material in a high-shear mixer. After homogenization and stirring at 4°C the homogenate was centrifuged and the aquenous phase was extracted two to three times with phenol until no gel-interphase was present after centrifugation. The final aqueous phase was made 0.1 M with NaCl and precipitated with two volumes of 95 % ethanol.

For convenience, and because only small numbers of lobeless embryos could be obtained in a reasonable period of time, the procedure was scaled down to extract radioactive RNA from only 300 embryos. To the embryos, which had been frozen in Beckman Microfuge tubes (approximately 400 μl capacity),

were added 100 µl of homogenizing solution and 200 µl of phenol; the tube was immersed in ice water and the contents were homogenized with a glass needle rotating at about 27,000 rpm. In some cases the homgenate was then incubated at 55°C for 2½ min (Scherrer and Darnell, 1962) to extract all of the RNA (Collier and Yuyama, 1969). The homogenate was periodically mixed thoroughly with a Beckman Micro-mixer and then was centrifuged at approximately 30,000 x g. The aqueous phase was conserved and the gel-interphase was washed twice with 50 µl aliquots of homogenizing solution by breaking it up with the glass needle. The aqueous phases were combined and twice more extracted by thorough mixing with 100 µl aliquots of phenol. The resulting aqueous phase was brought to 0.1 M with NaCl and the RNA was precipitated by the addition of two volumes of 95% ethanol. In later experiments, the buffer system was changed to 0.1 M Tris-HCl, pH 8.3 (Hadjavassiliou and Braverman, 1965) whereby nearly 70 % of the acid-precipitable radioactivity was ultimately accounted for after electrophoresis (DPM distributed on electrophoretic gel/gel DPM + non-recovered, acid precipitable DPM in the extraction residue).

RNA was also prepared from ribosomal pellets of Ilyanassa digestive gland and rat liver. Ribosomes were isolated at 4°C in 0.05 M Tris-HCl, pH 7.6, containing 0.4 M KCl, 0.018 M magnesium acetate and 10 µg/ml PVS (Cohen and Iverson, 1967). Bentonite (0.1 %) and a 1:2 mixture of deoxycholate:Triton X-100 (0.5 %) were added for homogenization. The tissue was broken with a loose-fitting pestle in a Dounce homogenizer

in four to five times the tissue-volume of homogenizing solution. After five strokes the homogenate was centrifuged at 12,000 x g for 10 min and the supernatant was layered over 0.5 M sucrose in buffer. The ribosomes were sedimented at 4°C for 2 hr in a Spinco 30 rotor at 30,000 rpm (105,000 x g). RNA was extracted from the ribosomal pelled by incubation with 1 % SDS (45 min at 25°C) in gradient buffer (see below) for subsequent purification on sucrose gradients or by repeated extraction of the pellet with phenol as above.

Density Gradient Centrifugation

Gradients of 8-30 % or 9-30 % sucrose were made with a Beckman Density Gradient Former from ribonuclease-free sucrose (Mann Research Laboratories) in gradient buffer (0.1 M sodium acetate, pH 5.0, 0.5 % SDS, 10^{-2} M magnesium acetate, 10 µg/ml PVS). Five to fifteen optical density units of RNA dissolved in 0.5 ml of gradient buffer were layered over the gradient and centrifuged at 15°C at 24,000 rpm for 16 hr (SW 25.1 rotor, 90,000 x g) or 17 hr (SW 41 rotor, 98,000 x g) or for equivalent times and speeds. Optical density profiles of the gradients were recorded at 254 mµ by displacing them through a flow cell with a 2, 5 or 10 mm optical path; as little as 25 µg of rRNA could be resolved using the SW 41 rotor. Areas under the resulting optical density peaks were measured with a planimeter.

Melting Profiles

RNA was dissolved in 0.15 M sodium acetate, pH 5.0 and placed in a stoppered, water-jacketed cuvette with a 1 cm light path. The absorbance at 260 mµ was measured after

equilibration at various temperatures which were monitored by a thermistor fitted to the water jacket; the absorbance was corrected for volume changes (Mandel and Marmur, 1968).

Electrophoresis

RNA was electrophoresed by the method of Loening (1967). Acrylamide and bisacrylamide were recrystallized from chloroform and acetone and stored as a 15 % acrylamide solution (bisacrylamide concentration was 0.75 %) in distilled water at 4°C. Gels (6 cm x 0.5 cm) at 2.2-2.4 % concentration were cast in 7 cm quartz tubes (Amersil, Inc.) and stored in distilled water at 4°C until used (usually overnight). The electrophoresis buffer contained 0.04 M Tris-acetic acid, pH 7.4, and 0.02 M sodium acetate (Loening, 1967); 0.2 % SDS was added before use. All gels were pre-run for at least 60 min before applying the sample. RNA was dissolved in electrophoresis buffer (1-10 ug/20 µl) containing sucrose and layered over the gel surface beneath the upper (cathode) buffer. The gels were run at 10 v for 5 min and then at constant current (5 ma per gel, 11 v/cm) for 50-60 min in a Canalco Model 12 apparatus; heating was minimized by surrounding the gels with the lower buffer bath. After electrophoresis the gels were left in the quartz tubes and scanned at 260 mµ in a Gilford linear transport. Ribosomal RNA was resolved from as little as 1 µg of sample when the gels were also scanned at 310 mµ to determine the background absorbance introduced by imperfections in the quartz tubes. However, a concentration of 4-5 µg per sample gave better results and was used routinely.

Ilyanassa embryos incorporate substantial amounts of

uridine into DNA (Collier, 1963); therefore, for analysis of radioactive RNA, the ethanol precipitate was washed twice with 80 % ethanol, dried in vacuo and dissolved in 30 µl of buffer (0.01 M Tris-HCL, pH 7.4, 0.005 M $MgCl_2$) containing 50 µg/ml of electrophoretically pure deoxyribonuclease (DNase) (Worthington Biochemical Co.). After 30 min incubation at 25°C, 20 µl of electrophoresis buffer-sucrose was added, the sample was layered over the gel surface and current was applied. To increase resolution and facilitate handling and slicing, these gels were made 9 cm long and contained 0.75 % agarose (lyophilized Sepharose) and 2.0 % acrylamide (Peacock and Dingman, 1968). They were run at constant voltage (9 v/cm, about 4 ma/gel) until the 18S rRNA had migrated one-half the length of the gel (about 140 min) and, after optical scanning, they were chilled on ice and divided into approximately 1.25 mm slices with a Canalco transverse gel slicer. When slices (n=59) were sampled from two gels cast on different occasions, the mean slice weight was 24.5 ± 0.26 mg and the coefficient of variation was 8.1 % which was similar to values reported by others (Neiman and Henry, 1971; Sheu and Ries, 1972; Helleiner and Wunner, 1971). There was no significant difference in slice size between the two gels or between slices in either half of the first gel. Prior to the adoption of 9 cm gels containing agarose, acrylamide gels were frozen and cut to 1.0 or 1.5 mm slices with a razor blade fitted to a modified microtome. Each slice was incubated with 250 µl of NCS (Nuclear Chicago Solubilizer) in a sealed vial at 60°C for 8 hr after which a toluene-based counting solution was

added. The counting efficiency was 40-50 % for tritium and 30-40 % for carbon^{14} (background was 10-30 cpm) in a Nuclear Chicago Unilux II scintillation counter.

All of the radioactivity distributed on gels represented incorporation into RNA. After routine DNase treatment (see above), ribonuclease (pancreatic, 100 µg/ml in 0.15 M NaCl, 0.015 M sodium citrate) removed all of the high molecular weight readioactivity but left fragments that migrated to the low molecular weight region of the gel (slightly larger then transfer RNA). The incomplete digestion probably resulted from the salt concentration used (J. R. Collier, personal communication). No radioactivity remained on the gel after alkaline treatment, assuring that no DNA radioactivity was present and supporting the inference that all radioactivity originated in RNA. Elements from the RNA precursor pool would not be present in gel profiles for nucleotides had a higher mobility than transfer RNA which was usually allowed to migrate off the gel during electrophoresis.

RESULTS

Embryonic Development

The descriptive and experimental embryology of _Ilyanassa_ have been reviewed by Clement (1971) and Collier (1965b, 1966). Briefly, the egg is approximately 160 µ in diameter and has been fertilized when laid. The first and second polar lobes arise during the respective maturation divisions and the third polar lobe forms a trefoil with the first two cleavage blastomeres (AB and CD). A fourth polar lobe forms at the second cleavage and all lobe contents are passed on

to the D macromere and its descendents, most prominent of which, by virtue of its unique timing and contents, is the 4d cell, or mesentoblast, whose derivatives give rise to the mesodermal bands and establish bilaterality in the embryo. These distinguishing features of the 4d cell and its progeny are lost in the lobeless embryo. Gastrulation is by epiboly after about two days at 20°C, stomadaeal invagination begins during the third day and shell gland tissue appears during the fourth day as do other organ anlagen. Organogenesis proceeds from the fourth to seventh days (heart and intestine are the last organs to differentiate) resulting in the veliger larva by the seventh day of development.

Ribosomal RNA

Studies on Ilyanassa ribosomal RNA (Koser and Collier, 1971) and the ribosomal RNA precursor (Koser and Collier, 1972) are detailed in appendix A.

RNA Transcription during Embryogeny

To obtain maximum informationfor the comparison of normal and lobeless embryos, the RNA extraction procedures were re-examined. Low-temperature RNA extraction at pH 5 yielded mainly ribosomal RNA (rRna) and heating at pH 5 was required to liberate theDNA-like RNAs (dRNA) (Collier and Yuyama, 1969) likely to play an important role in embryogenesis. However, since heating degraded 26S rRna (see appendix A) it was also possible that some dRNAs might degrade and confuse the analysis. Since higher pH also extracts more dRNA (Hadjavassiliou and Braverman, 1965), combinations of pH and temperature were used sequentially to obtain RNA from samples

of the same batch of radioactively labeled, stage 3½-4½ embryos. Extracts were made in the cold (4°C) at (a) pH 5 and at pH 8.3. The gel-interphase, or residue, from (a) was re-extracted at 4°C at pH 8.3 (b) and the residue from the cold pH 8.3 extraction was re-extracted at 55°C, pH 8.3 (c). The results are shown in Fig. 2; the cold pH 8.3 re-extraction (b) recovered considerable radioactivity that was not released by the cold pH 5 extraction (a) while only a small amount of additional radioactivity was recovered in the hot pH 8.3 re-extraction (c) after a cold pH 8.3 extraction and this was almost exclusively rRNA broken down to 18S material by the elevated temperature. In another experiment, no significant radioactivity was recovered by a hot pH 5 re-extraction after a hot pH 8.3 extraction. The cold, pH 8.3 procedure was adopted for all subsequent extractions as it provided a greater yield of RNA (nearly 70 %) while minimizing thermal breakdown.

In Fig. 3 are shown representative electrophoretic distributions of nascent RNAs extracted from normal and lobeless embryos during the first six days of development. The somewhat long (24 hr) pulse time was chosen both to allow sufficient incorporation of radioactive precursor for analysis and to focus upon the longer-lived RNA species since differentiation in this organism does not immediately follow the underlying transcriptive events (Collier, 1966). The data were converted to distributions to allow comparison of all electrophoregrams despite variation in pool specific activities and rates of incorporation and, consequently, an increase in one region of the gel profile could have resulted from

FIGURE 2-Electrophoregrams of Ilyanassa RNA. A single batch of stage 3½-4½ embryos was incubated for 24 hr with ^{3}H-uridine at 20 µC/ml. RNA was extracted with phenol from 250-embryo samples under the conditions: (a ____) 4°C, pH 5.0; (b ----) 4°C, pH 8.3 re-extraction of the residue (gel-interphase) from (a); (c ····) 55°C, pH 8.3 re-extraction of the residue from a 4°C, pH 8.3 extraction.

Electrophoresis (migration to the right) and analysis as described in Materials and Methods.

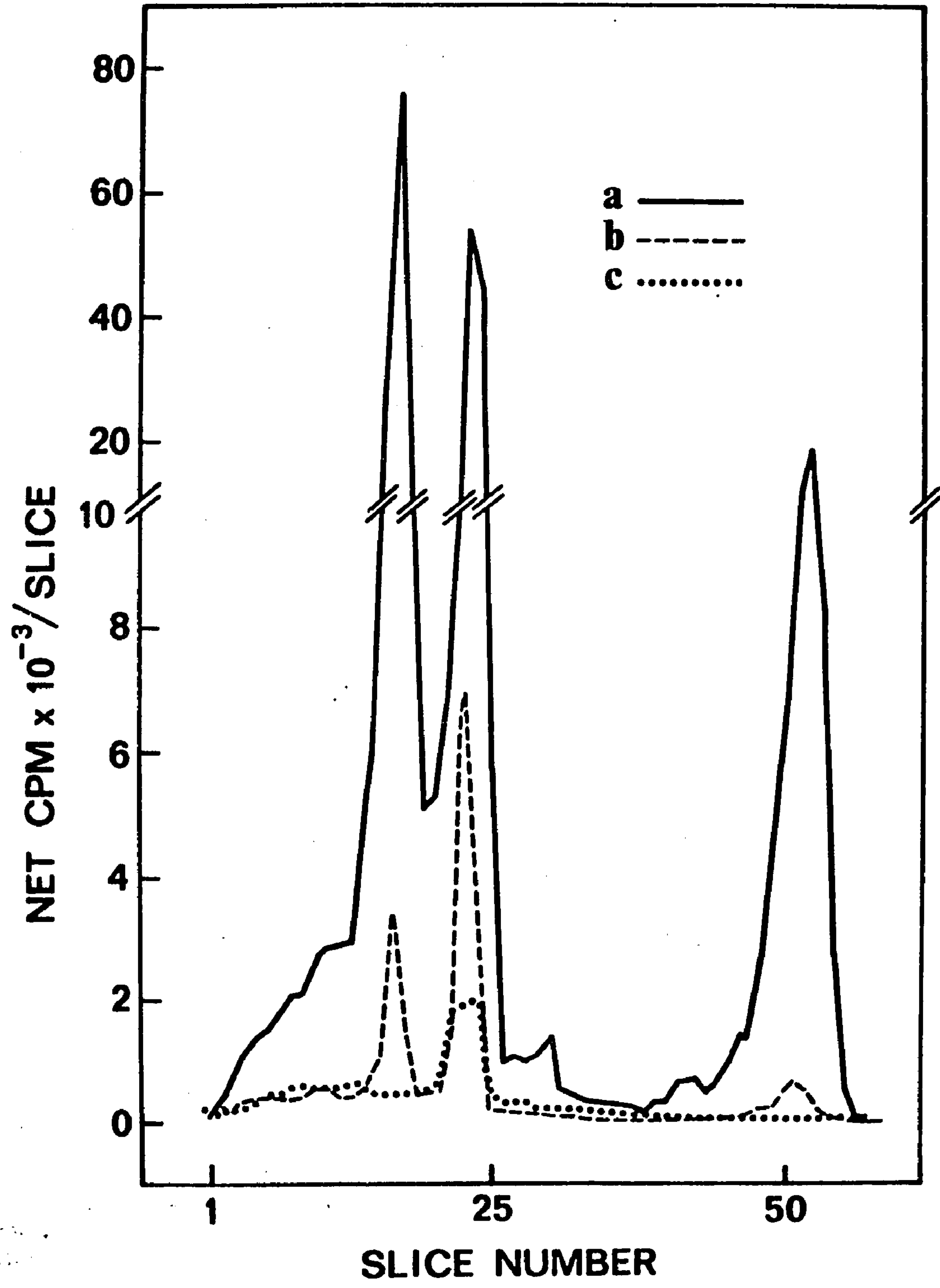
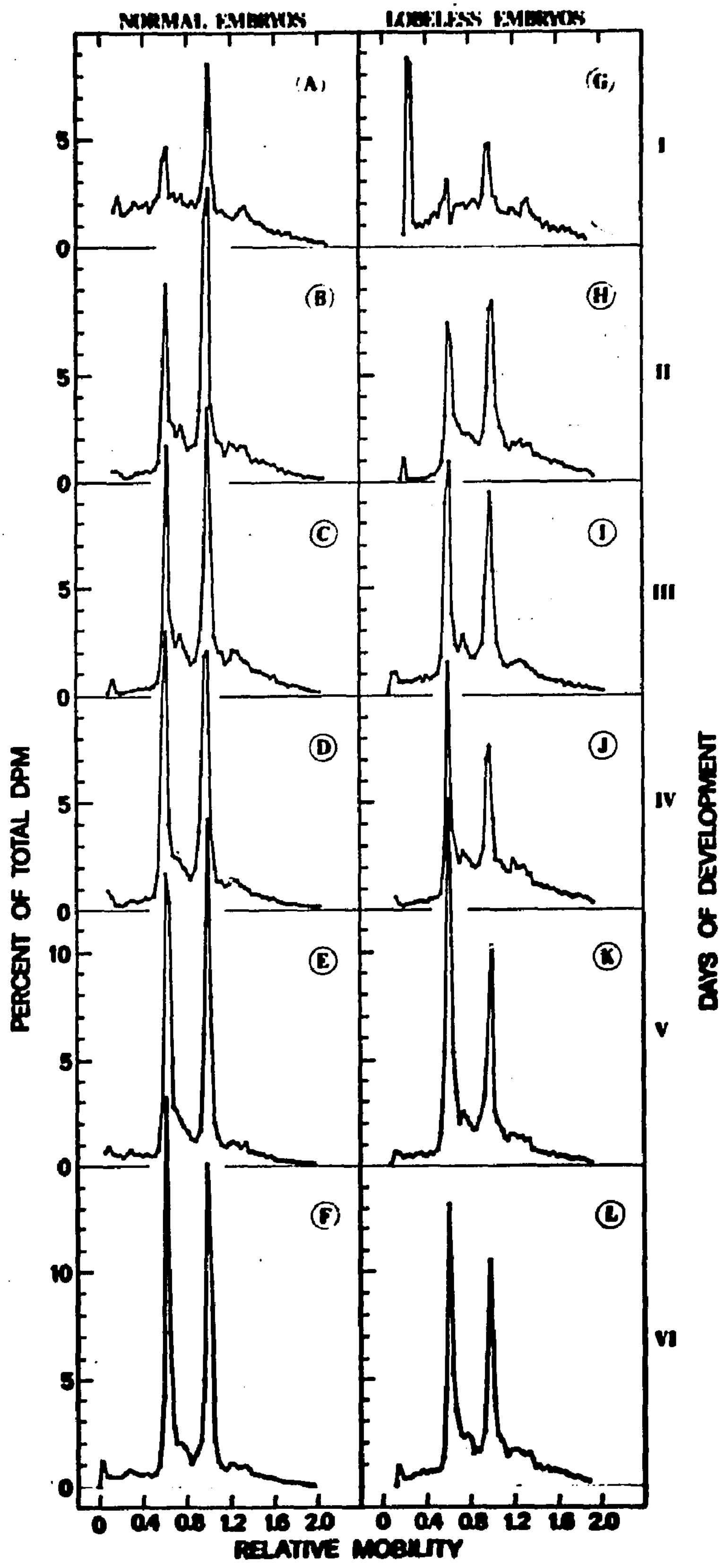

NET CPM x 10^{-3}/SLICE
80
60
40
20
10
8
6
4
2
0
a
b
c
1
25
50
SLICE NUMBER

FIGURE 3-Electrophoregrams of RNA from normal and lobeless Ilyanassa embryos. Typical percentage distributions of RNA extracted at $4^{o}C$, pH 8.3 and analyzed on "small-pore" gels (see appendix B). The "small-pore" distributions were chosen for this visual comparison for their greater resolution and sharper appearance.

All embryos were incubated for 24 hr in ^{3}H-uridine at the concentrations: (a-e) 300 embryos, 250 µC/ml; (d-f) 300 embryos, 125 µC/ml; (g) 300 embryos; (h) 243 embryos; (i) 442 embryos; (j) 250 embryos; (k) 303 embryos; (l) 296 embryos; (g-l) 250 µC/ml.


NORMAL EMBRYOS
LOBELESS EMBRYOS
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
(I)
(J)
(K)
(L)
I
II
III
IV
V
VI
PERCENT OF TOTAL DPM
DAYS OF DEVELOPMENT
RELATIVE MOBILITY
0
0.4
0.8
1.2
1.6
2.0
5
10

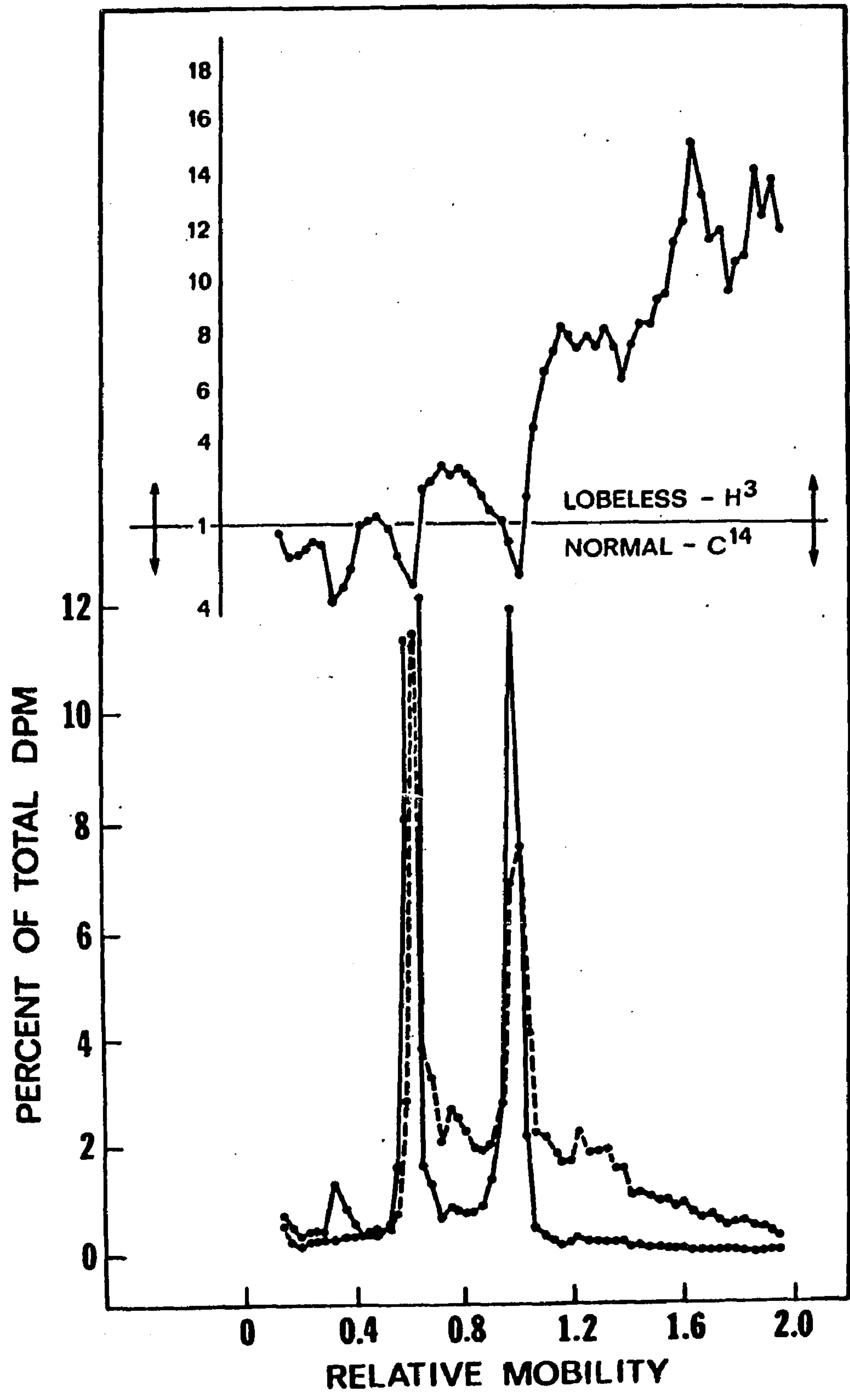
either an increased rate of synthesis of that class of RNAs or from a decreased rate in other classes; i.e., only proportionate differences in the representation of nascent RNAs were revealed. The RNA distributions showed no striking changes either during development or in a lobeless-normal comparison; however, several trends were apparent. Nascent RNAs from normal embryos exhibited considerable size-heterogeneity (dispersion) during early development (Fig. 3 a-c) which decreased with development as synthesis of ribosomal RNA assumed an ever-larger proportion of the overall distribution (d-f). This shift from heterogeneous RNA to rRNA during development paralleled the increasing prominence of the ribosomal RNA precursor (at relative mobility (RM) = 0.3). There was, concomitant with the shift towards rRNA synthesis, a change in the relative proportions of the ribosomal RNA moieties (a-f). Between stages 5 and 6 (e,f), the 26S rRNA became larger in proportion to 18S rRNA (the 18S rRNA was smaller in (d) for the peak radioactivity was spread among two gel slices). The RNAs intermediate in size between the rRNAs and those just smaller than 18S RNA remained an almost constant fraction of the whole.

Lobeless embryos presented a slightly different pattern of nascent RNAs during embryogenesis (Fig. 3g-1). The primary difference lay in the non-ribosomal, heterogeneous RNAs which remained a larger part of the total nascent RNA later into development and were still a greater share by stage-6 (g,1) than in normal embryos. The rRNA precursor did not assume the magnitude found in the normal distributions.

Experiments were designed to exactly compare RNA transcription in lobeless and normal embryos by radioactively labeling one group with ^{14}C-uridine, the other with ^{3}H-uridine, and combining the two groups of embryos for RNA extraction and electrophoresis. After normalizing the carbon radioactivity by a factor (total tritium DPM in gel/total carbon DPM), the carbon:tritium ratio (or the reciprocal) for each gel slice was plotted against the relative mobility with the rationale (Terman, 1970) that a ratio of unity across the gel would imply no difference between the two RNA populations and any significant deviations from unity would disclose proportionate differences in identifiable (by molecular weight) classes of RNAs. Thus, it was inferred from Fig. 4 that lobeless embryos synthesized considerably more low molecular weight RNA (<18S) and less ribosomal RNA than normal embryos (or, in the proportionate sense, normal embryos synthesized relatively more ribosomal RNA and less low molecular weight RNA than lobeless embryos). This result was, however, misleading for a control experiment (carbon- and tritium-labeled normal embryos assayed together) presented the identical pattern. Fig. 5 shows that the modified electrophoretic distribution of nascent RNAs from carbon-labeled embryos was a consequence of the high exogenous uridine concentration (200 μg/ml) required for sufficient precursor incorporation. This effect was observed when unlabeled uridine was added to the 24 hr incubation of embryos with ^{3}H-uridine. As in Fig. 4, with a high exogenous uridine concentration the ribosomal precursor and ribosomal RNAs formed a considerably

FIGURE 4-Electrophoregram of Ilyanassa RNA. Percentage distributions of RNAs co-extracted from stage-4 normal and lobeless embryos and electrophoresed together. After normalization to the same total DPM, the carbon:tritium ratio (or the reciprocal) in each slice is plotted in the upper portion of the figure.

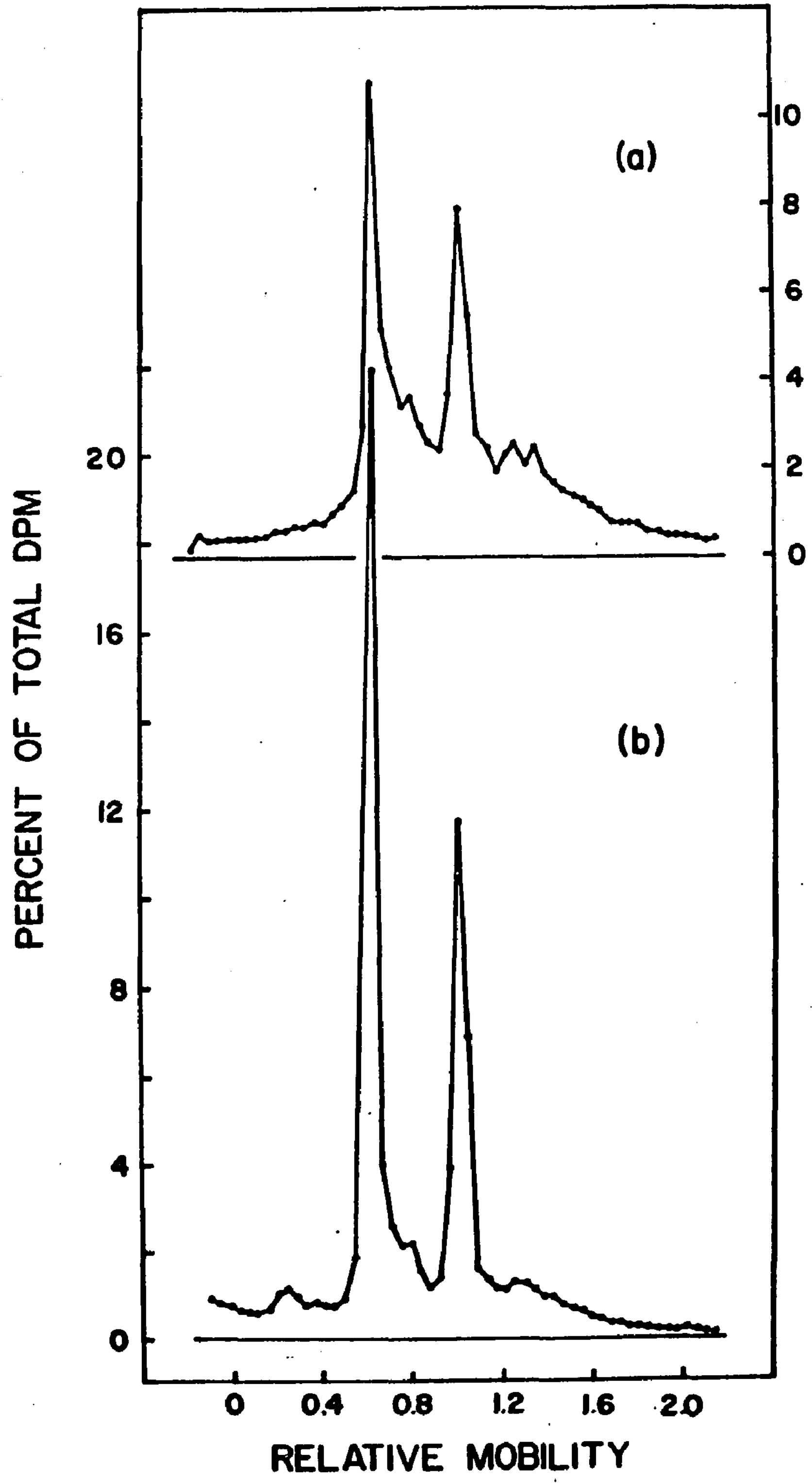
(____) 550 normal embryos incubated 24 hr in 25 μc/ml ^{14}C-uridine; (----) 303 lobeless embryos incubated 24 hr in 250 μC/ml ^{3}H-uridine. Total radioactivity in all slices: carbon, 39,840 DPM; tritium, 6,256,615 DPM.



18
16
14
12
10
8
6
4
1
4
LOBELESS - H3
NORMAL - C14
PERCENT OF TOTAL DPM
12
10
8
6
4
2
0
0
0.4
0.8
1.2
1.6
2.0
RELATIVE MOBILITY

FIGURE 5-Electrophoregrams of Ilyanassa RNA. Effect of high uridine concentration on the distribution of nascent RNAs.

230 stage-4 embryos were incubated 24 hr in ^{3}H-uridine at 250 µC/ml. (a) control embryos, exogenous uridine concentration 2.4 µg/ml (9.8 x 10^{-3} µM/ml), 7,742,820 DPM total in all gel slices; (b) as above with 200 µg/ml (0.9 µM/ml) of unlabeled uridine (equivalent to 50 µC/ml of ^{14}C-uridine at a specific activity of 55.6 µC/uM) added during the incubation period, 131,096 DPM total.



PERCENT OF TOTAL DPM
RELATIVE MOBILITY
(a)
(b)
0
4
8
12
16
20
0
2
4
6
8
10
0
0.4
0.8
1.2
1.6
2.0

greater fraction of the newly synthesized RNAs.

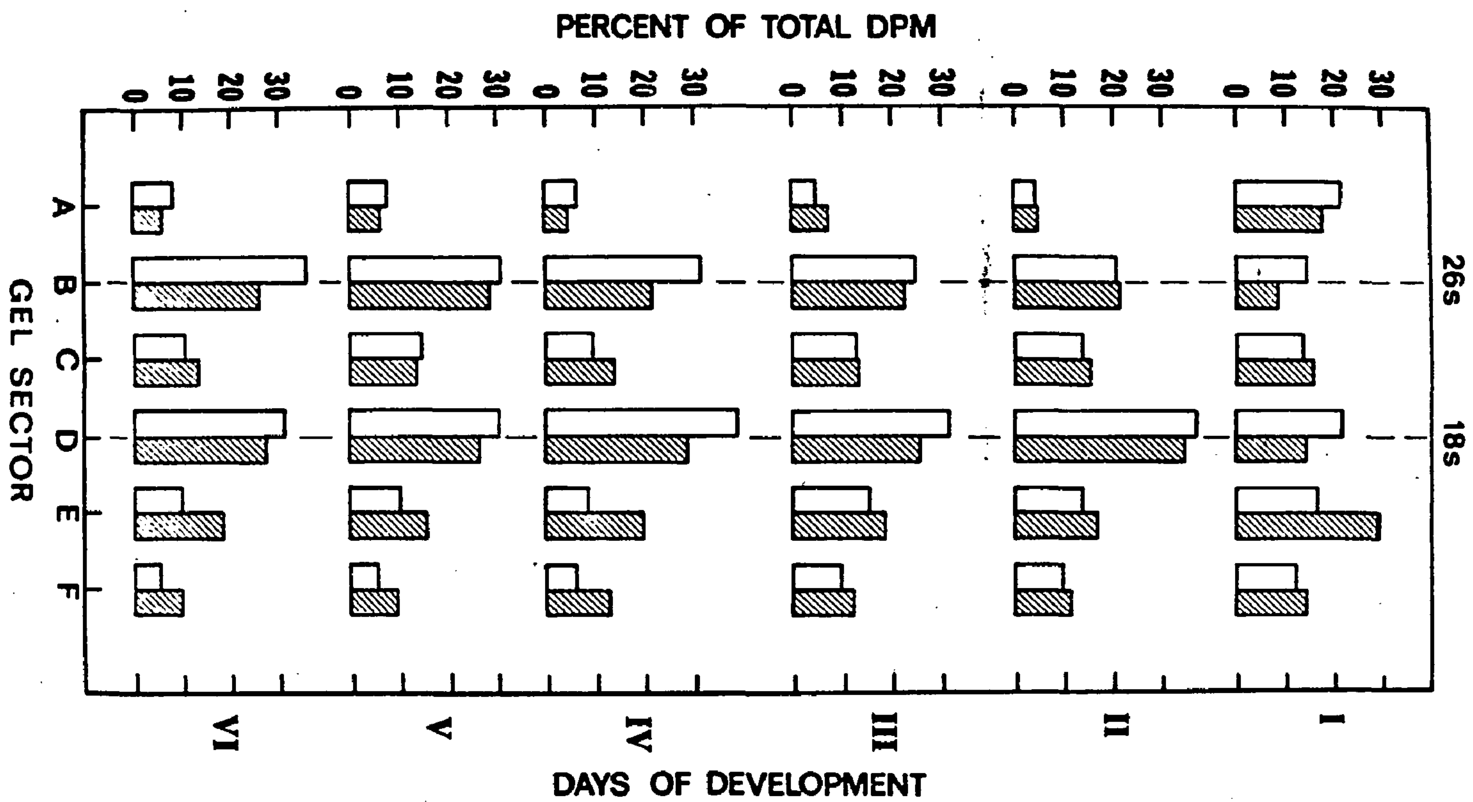
The impossibility of performing the dual-label analysis prevented an exact comparison of normal and lobeless embryo RNA transcription. The data from Fig. 3 and a duplicate set of profiles (all embryos radioactively labeled with tritium) were compressed to compensate from some of the inherent difficulties in comparing electrophoretic profiles. Only RNAs in the molecular weight ranges common to all gels were considered and slicing discrepancies were minimized by summing the gel-slice radioactivities in six (later, seven) visually distinct profile regions, or sectors (see appendix B). Corresponding sectors from all electrophoretic profiles contain RNA molecules in the same molecular weight range and the six sector sums, or areas, of each gel represent the distribution of nascent RNAs and these can be contrasted with each other.

In Fig. 6 are summarized the normal and lobeless embryo sector areas. This is the most readily interpretable form of the results because sources of error in the biology and methodology of the problem were balanced our or at least reduced: conversion og gel slice radioactivities to percentage distributions compensated for variations in precursor concentration, pool size, specific activity of the nascent RNA and recovery; condensing the slice DPM into sectors decreased gel slicing errors. The same general trends of the nascent RNA distributions (Fig. 3) are apparent even though the data were compressed. The lobeless and normal distributions are quite similar in sectors A and C. As noticed in Fig. 3, the lobeless embryos lag the normals in ribosomal

FIGURE 6-Sector areas of nascent RNA distributions from normal and lobeless embryos. The sectors were defined as: A) RNAs larger than 26S RNA, relative mobility (RM) < 0.5, molecular weight (MW) $> 1.5 \times 10^6$; B) 26S RNA, RM 0.5-0.7, MW $1.2\text{-}1.5 \times 10^6$; C) size intermediate between the 26S and 18S peaks, RM 0.7-0.9, MW $0.8\text{-}1.2 \times 10^6$; D) 18S RNA, RM 0.9-1.1, MW $0.6\text{-}0.9 \times 10^6$; E) smaller than 18S RNA, RM 1.1-1.4, MW $0.4\text{-}0.6 \times 10^6$; F) low MW RNA, RM > 1.4, MW $< 0.4 \times 10^6$. Sector A was, for some tests, subdivided into high molecular weight RNA (HMW) and ribosomal precursor RNA (PREC). The radioactivities in each of the six gel sectors were summed and expressed as a percentage of the total in the six sectors (see appendix B); each area is the average of two replicates.

Open bars, normal embryos; shaded bars, lobeless embryos (data in Table B2).

Stage I-normal embryos, "small-pore" and "large-pore" replicates (SP,LP); lobeless embryos, (SP,LP); II) normal, (SP,LP); lobeless, (SP,SP); III) normal, (SP,LP); lobeless, (SP,LP); IV) normal, (SP,LP); lobeless, (SP,LP); V) normal, (SP,SP); lobeless, (SP,LP); VI) normal, (SP,SP); lobeless, (SP,LP). See appendix B for definitions.



PERCENT OF TOTAL DPM
0
10
20
30
GEL SECTOR
A
B
C
D
E
F
26s
18s
VI
V
IV
III
II
I
DAYS OF DEVELOPMENT

RNA synthesis and show a greater proportion of their nascent RNA synthesis in sectors E and F, especially during the first, fourth, fifth and sixth days of development.

Are the normal-lobeless differences noted in Figs. 3 and 6 significant? These distributions of nascent RNAs can be compared statistically by a three-factor analysis of variance (anova) on stage of development x normal-lobeless x profile and the degree of confidence in the answer can be determined. Details of the method, a complete set of sector areas, data transformations and anova summaries are presented in appendix B. Each gel profile is considered as a subject with the sector areas representing repeated measurements on the proportion of the subject's nascent RNAs falling into the six or seven sectors. The factors are: A, stage of development; B, normal-lobeless; C, sector areas (profile). The anova summary (see Winer, 1962, p. 340 ff.) is broken down into two categories that analyze the variance between subjects and within subjects. The errors (between) and (within) form the denominators for their respective F-ratios which indicate the probability that differences calculated in main effects or interactions could have arisen by chance. The profile (factor C), or gel RNA distribution, is the criterion of measurement and the F-ratio of its interaction estimates the probability that profiles are significantly different, for example, between stages, considering all profiles together (A x C interaction), or between normal and lobeless embryos, considering all stages together (B x C interaction); the latter is most pertinent to this work.

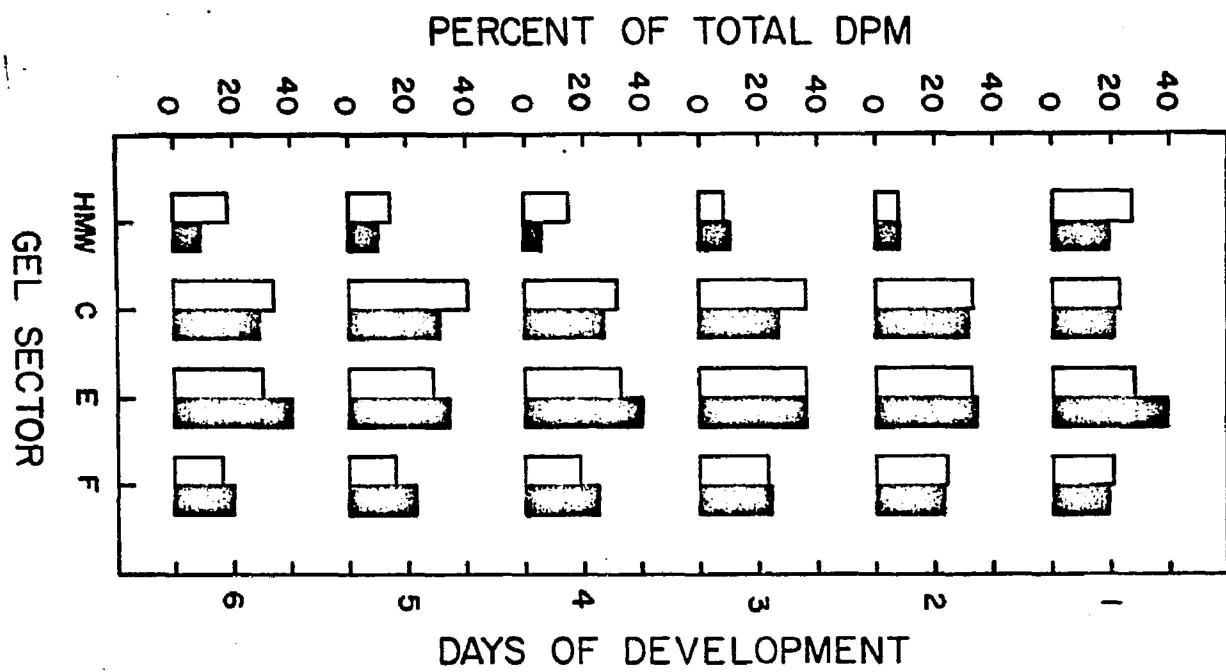
Thus, for the entire body of data (Tables B2, B4), nascent RNA distributions in normal embryos were significantly different ($P < .05$) from lobeless embryo nascent RNA distributions (B x C interaction). Also, the distributions (normal and lobeless together) varied significantly ($P < .01$) during development (A x C interaction) as was obvious from Figs. 3 and 6.

To estimate the time when these alterations occurred, gastrulation was chosen as a developmental landmark and the analysis was partitioned into data for days 1 and 2 (Table B5) and days 3-6 (Table B6); newly transcribed RNA was significantly different ($P < .05$) in normal and lobeless embryos after gastrulation but not before (B x C interaction). Pooling the lobeless and normal profiles, transcriptive patterns varied significantly with time prior to gastrulation ($P < .05$) but not afterward (A x C interaction).

Polar lobe substances thus affect the synthesis of RNA in the post-gastrula _Ilyanassa_ embryo. Since the lobe's morphological effect is organ-specific, these lobe-controlled transcripts are likely to exist amongst the DNA-like, or non-ribosomal, RNAs. To test this premise, the non-ribosomal RNAs (data from Fig. 6; sectors HMW, C, E and F) were reanalyzed independently. The sector area percentage distributions (Fig. 7) again showed that lobeless embryos contained proportionately more low molecular weight RNA in sectors E and F than normal embryos during the period of organogenesis (days 4-6). Furthermore, it was now more obvious that lobeless embryos contained proportionately less high molecular

FIGURE 7-Sector areas of nascent, non-ribosomal RNA distributions from normal and lobeless *Ilyanassa* embryos. The data from Fig. 6 (Table B2) were re-computed (Table B7) for the non-ribosomal sectors HMW, C, E and F.

Open bars, normal embryos; shaded bars, lobeless embryos.


PERCENT OF TOTAL DPM
0 20 40 0 20 40 0 20 40 0 20 40 0 20 40 0 20 40
GEL SECTOR
HMW
C
E
F
6 5 4 3 2 1
DAYS OF DEVELOPMENT

weight RNA during the same period. Lobeless embryos also consistently had slightly less RNA in sector C. The stage-1 pattern showed possible lobeless-normal differences but the stage-2 patterns were nearly identical. The sector area percentage distributions are listed in Table B7 and the log transforms are in Table B9. The anova summary (Table B8) sustains the supposition that embryonic dRNAs were significantly different ($P<.05$) in normal and lobeless embryos during embryogeny (B x C interaction). With regard to timing, the normal-lobeless, non-ribosomal transcriptive difference was again significant ($P<.05$) during organogenesis (Table B10b) but not prior to gastrulation (Table B10a).

Re-computation of the ribosomal RNA distributions (Tables B11-B14) verifies that normal-lobeless transcriptive differences lie in the non-ribosomal, dRNAs; the B x C interaction was not significant (Table B12). These data do show that the proportions of ribosomal RNA precursor and processed (26S and 18S) ribosomal RNAs changed considerably during development (A x C interaction) and that these changes occurred mostly during the first two days (Table B14a, b).

The results have pointed to the polar lobe's effect on new RNAs in the later stages of embryogenesis yet extensive determination for lobe-dependent structures occurs during the first 24 hr of development (Clement, 1952, 1962, 1971; Collier, 1966). Thus a more intensive analysis of transcription during the first day was undertaken by preparing five replicate groups of normal and lobeless embryos. Also, phenol-chloroform (1:1) was substituted for phenol to more completely extract nuclear

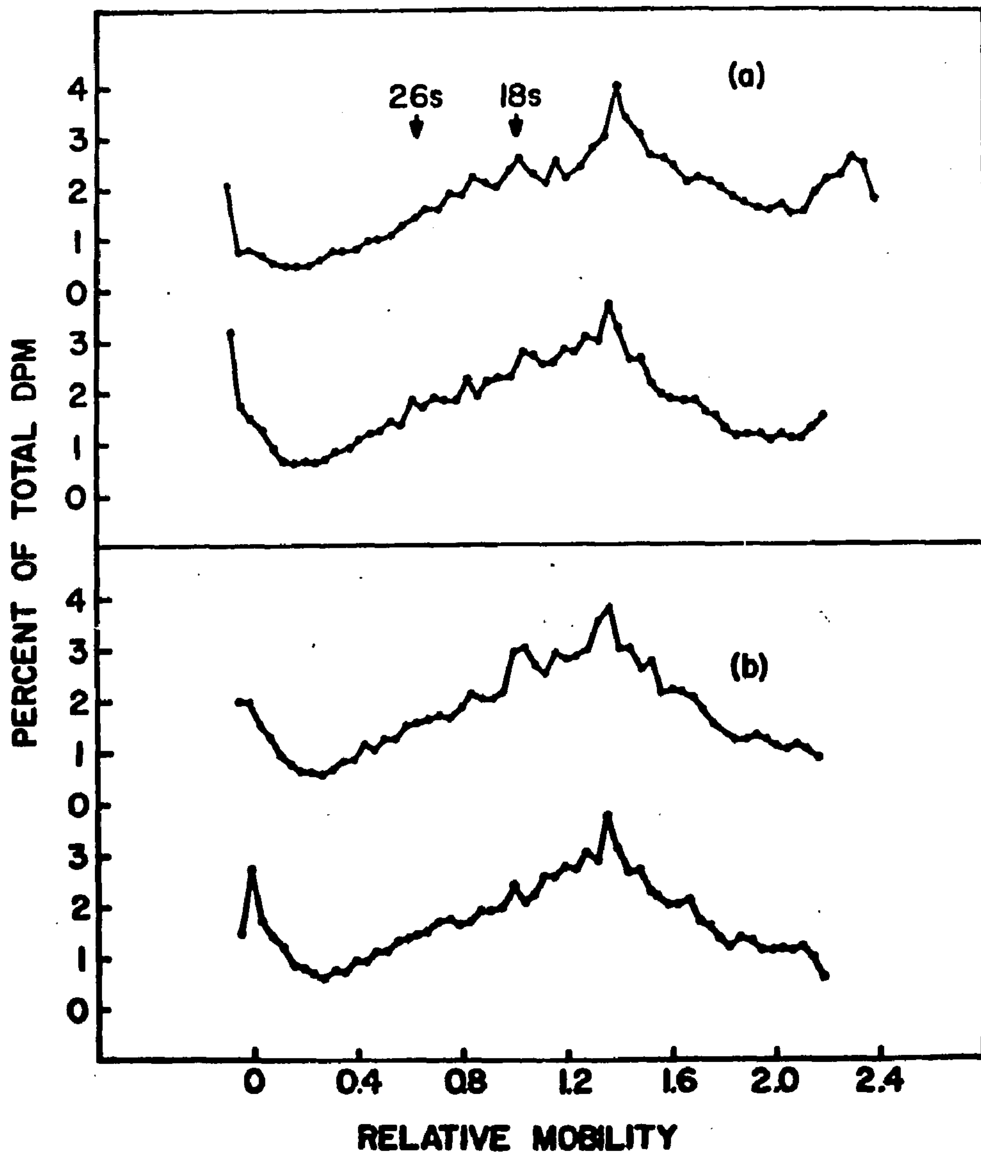
RNA (Brawerman et al., 1972; Perry et al., 1972); the extraction efficiency was 84 % (radioactivity distributed over the electrophoretic gel/acid precipitable radioactivity remaining in the extraction residue + gel radioactivity). The data are listed in Table B15 (a, percentage distributions and experimental details; b, log transforms of sector DPM; c, anova summary). Typical electrophoretic distributions of nascent RNAs from one-day embryos are displayed in Fig. 8 which reveals their considerable heterodispersity. The normal and lobeless profiles were undistinguishable from each other. A graph of the sector area percentage distributions (Fig. 9) shows the increased representation of low molecular weight RNA (sectors E and F) released by the chloroform procedure. Most important was the similarity of the lobeless and normal embryo nascent RNA distributions. A two-factor anova (lobeless-normal x profile) supports the visual interpretation that there was no significant difference (A x B interaction) between normal and lobeless profiles of nascent RNAs from the major period of embryonic determination. This result upholds the inferences, drawn from Tables B5 and B10a, that while the polar lobe of Ilyanassa influences RNA transcription, this influence is not detectible during the period of determination.

DISCUSSION

A survey of transcription in the course of Ilyanassa embryogeny was made by examining the patterns of RNAs synthesized during consecutive 24 hr periods through morphogenesis. In the cleavage period, nascent RNAs were mostly heterodisperse

FIGURE 8-Electrophoregrams of Ilyanassa RNA. Representative distributions of nascent RNAs extracted with phenol-chloroform (1:1) from stage-1 (a) normal and (b) lobeless embryos incubated in 250 µC/ml ^{3}H-uridine for 24 hr.

(a) upper curve, N3 from Table B15a; lower curve, N4. (b) upper curve, L3; lower curve, L4.

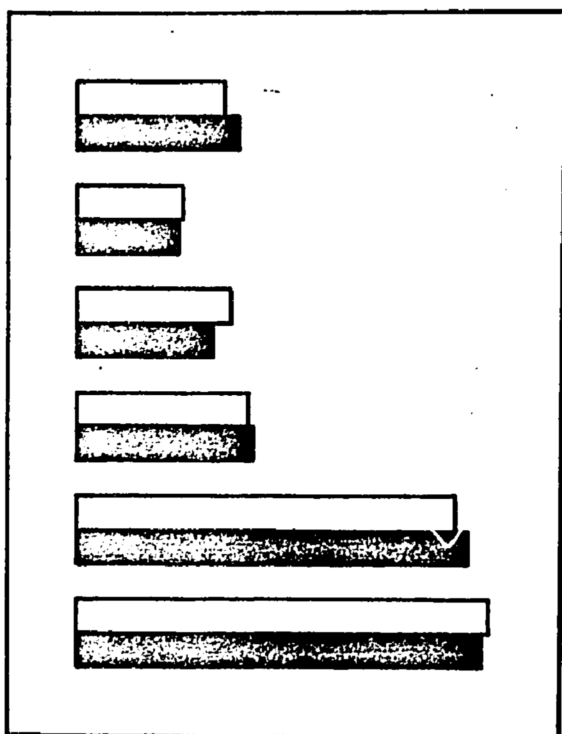


(a)
26s
18s
(b)
PERCENT OF TOTAL DPM
4
3
2
1
0
0
0.4
0.8
1.2
1.6
2.0
2.4
RELATIVE MOBILITY

FIGURE 9-Sector areas of nascent RNA distributions from stage-1 normal and lobeless *Ilyanassa* embryos. Compare Fig. 6.

Data are averages of five replicates listed in Table B15a.

Open bars, normal embryos; shaded bars, lobeless embryos.



PERCENT OF TOTAL DPM
0
10
20
30
A
B
C
D
E
F
GEL SECTOR

in size and as embryos grew older, ribosomal RNA comprised an ever-larger fraction of the embryonic gene products. Lobeless embryo nascent RNAs initially resembled those of normal embryos but later, substantial heterogeneity persisted and transcription of ribosomal RNA did not achieve the magnitude found in normal embryos. A statistical examination of the data corroborated these inferences by establishing within acceptable confidence limits that: 1) distributions of nascent RNAs in lobeless embryos were dissimilar to those in normal embryos; 2) these differences occurred not in the course of cleavage and determination but after gastrulation; 3) they originated only in the non-ribosomal RNAs.

The normal transcription pattern in Ilyanassa is similar to that of another mollusc (Kidder, 1972) and some "regulative" embryos, e.g., the sea urchin (Slater and Spiegelman, 1970; see Kidder, 1972). In Ilyanassa, the normal-lobeless changes in nascent RNAs are mirrored by changes in nascent proteins which also occurred in organogenesis so that the lobeless embryo "appears to retain continuously the younger condition" (Teitelman, 1973).

The disparity between normal and lobeless embryo RNAs was genuine and not an injury artifact. The lobe's effect is organ-specific and cell division proceeds so that lobeless embryos contain no fewer cells than normal ones through at least the fourth day of development (Davidson et al., 1965; Collier, unpublished observations). Moreover the general loss of cytoplasm is not accountable for Wilson (1904a) demonstrated that the removal of a comparable amount of non-lobe cytoplasm

from the Dentalium egg created a dwarf, but otherwise normal, larva. Similarly, removal of the 4D macromere (Clement, 1956, 1962) resulted in a normal Ilyanassa embryo.

It was desirable to assay all RNA transcripts during Ilyanassa embryogenesis so six long (24 hr), consecutive periods of incubation in radioactive precursor were used to completely bracket early development. Long pulses ensured adequate incorporation and biased the results toward the stable messages assumed to be important in organogenesis (Collier, 1966; Sussman and Sussman, 1965; see also Davidson, 1968). However, this approach had some minor drawbacks: there was not a complete representation of short-lived transcripts although a reasonable sample can be expected from those synthesized near the end of each labeling period; ribosomal RNA became heavily labeled.

The RNA extraction procedures were scrutinized to find a suitable compromise between yield and breakdown. Increasing the extraction pH (Hadjavassiliou and Braverman, 1965) or temperature (Scherrer and Darnell, 1962; Collier and Yuyama, 1969) increased the recovery of RNA so these conditions were tested sequentially; while the elevated pH enhanced the recovery, raising the temperature caused no appreciable gain over the high pH, low-temperature procedure and exacerbated the problem of breakdown. About 70 % of the acid-precipitable radioactivity was recovered which compared favorably with the low-temperature yield (75-80 %) reported for the worm Urechis (Davis and Wilt, 1972). More importantly, these authors stated

that, on the basis of sucrose gradient analyses, no major classes of RNA had been lost. The addition of chloroform (Brawerman et al., 1972; Perry et al., 1972) during extraction of the stage-1 embryos increased the yield to 84 %.

The decrease of 18S RNA in proportion to 26S RNA during development paralleled the lessening proportion of heterodisperse, non-ribosomal RNA to total RNA. These events may be correlated for dRNA is known to aggregate with ribosomal RNA (Collier, 1965a, 1971; Collier and Yuyama, 1969); this association depends upon RNA concentration and temperature and does not involve covalent binding (Wagner et al., 1967). While aggregation might be a non-specific artifact (Gillam et al., 1967), it might alternatively represent an in vivo mechanism for the passage of transcripts from the nucleus to cytoplasm (Hadjiolov, 1967). The extraction pH used here was probably not high enough to prevent association (Hayes et al., 1966), whatever the basis, so that the likelihood of some aggregation, together with the changing proportions of 18S and 26S RNA in development, support the conclusion, from the nascent RNA distributions, that the fraction of non-ribosomal, heterodisperse RNA decreases during development. This interpretation also argues against the presence of considerable, degraded 26S RNA in the electrophoresis profiles.

The small peaks on either side of the 18S peak (22-23S and 14-16S) raise the specter of bacterial contamination. This was quite unlikely (Daigneault et al., 1970; Kidder, 1972a) because embryos were incubated in the presence of antibiotics continuously after their removal from capsules

prior to first cleavage and the concentration used here (100 μg/ml each, penicillin and streptomycin) proved maximally effective for incubation of another mollusc, the coot clam (Kidder, 1972). These peaks are a common feature of RNA electrophoresis profiles (cf. MacGregor and Mahler, 1969; Fraser and Loening, 1973). Chamberlain and Metz (1972) speculated that the larger ribosomal subunit was the source of 23S RNA in the sea urchin and, in the case of mammalian RNAs, this was verified on the basis of methyl content, base composition and hybridization competition (Nair and Knight, 1971). But this is probably not the case for Ilyanassa because increasing disruption of rRNA secondary structure revealed a 23S minor component in rat liver 28S rRNA but not in Ilyanassa 26S rRNA (see appendix A). Instead, these minor peaks are thought to have originated in mitochondrial RNA for 11-16S RNAs were found enriched in mitochondrial fractions of sea urchins (Chamberlain and Metz, 1972; Dubois et al., 1971) as was 23S RNA (Dubois et al., 1971). Finally, Swanson and Dawid (1970) measured the electrophoretic S-values of amphibian mitochondrial ribosomal RNA to be 13S and 21S which supports the assumption that these lesser components of Ilyanassa nascent RNA distributions represent mitochondrial RNA. Their contribution to this analysis was not significant as they were detectable only as small bumps over a larger baseline of heterodisperse RNAs.

In contrast to the normal pattern, lobeless embryos contained proportionately more low molecular weight, nascent RNA which, if considered as an accumulation of messages in the

absence of translation (Peterkofsky and Tomkins, 1968; Reif-Lehrer and Amos, 1968), raises the possibility that the polar lobe contents exert an effect upon translation of new and/or old transcripts as well as, or instead of, transcription. Pre-formed, or "masked" messages are present in the unfertilized egg of many, if not all, organisms (see reviews by Spirin, 1966; Tyler, 1967; Davidson, 1968) and both transcription control and translation control function during sea urchin development (Ellis, 1966; Terman, 1970). In Ilyanassa, patterns of protein synthesis were modified in lobeless embryos during organogenesis (Teitelman, 1973), the same period when nascent RNA distributions were affected. This suggests that substances contained in the polar lobe and selectively distributed in the course of determination might control translation during organogenesis.

The polar lobe has been thought of as a storage container (Waddington, 1956) and distribution mechanism (Clement, 1962) for substances that affect morphogenesis. The regional localization of new transcripts (Flickinger et al., 1966; Flickinger and Daniel, 1972; Bachvarova and Davidson, 1966; Markman, 1961) and proteins (Denis, 1961; Donohoo and Kafatos, 1973) illustrates the function but not the identity of these substances. Chemical analyses have disclosed a chemical concentration of phosphorous compounds (Collier, 1960b) and nucleotides (Berg and Kato, 1959) in the lobe. The electron microscope has revealed no unusual structures in the polar lobe (Crowell, 1964) although a concentration of particles resembling ribosomes embedded in a matrix was found there

(Pucci-Minafra _et al_., 1969). These were often associated with the multimembranous vesicles postulated (Crowell, 1964) to contain the chemicals concentrated in the lobe and were distributed to the micromeres during cleavage. Polar lobe morphogenetic factors could, if they are RNAs, originate from lampbrush chromosomes present in the oocyte (Davidson and Mirsky, 1965) for these are known to transcribe during _Xenopus_ oogenesis (Davidson _et al_., 1966; Crippa _et al_., 1967). The lobe contains RNA in proportion to its hyaline protoplasm (Collier, 1960a). The factors might be proteins synthesized in the lobe itself (Clement and Tyler, 1967). Nascent lobe proteins, to the extent that a single experiment can be believed, did not differ from those made in normal, four-day embryos (Teitelman, 1973). Removal of the visible components from the vegetal region of the egg by centrifugation (Morgan, 1933, 1935; Clement, 1968) did not prevent polar lobe formation or normal development so that the morphogenetic factors are invisible and/or located within or near the vegetal cortex of the egg which forms the lobe cortex. The egg cortex has been shown to contain important morphogenetic factors in _Xenopus_ (Curtis, 1960) and the squid (Arnold, 1968) and, in _Limnaea_ (Raven, 1963, 1967), sub-cortical patterns were observed to distribute to the early blastomeres. In _Ilyanassa_ (Dan and Dan, 1942), the animal pole was observed to shrink while the vegetal pole stretched in the course of the second maturation division which illustrates a possible mechanism for transmitting cortex material during cleavage. A precise distribution, rather than the mere presence, of morphogenetic

factors is required as shown by experiments applying pressure during Ilyanassa cleavage (Styron, 1967); when polar lobe formation was inhibited and equal cleavage occurred (i.e., equal distribution of lobe contents to both of the first blastomeres), each extruded a lobe and the ensuing cleavage gave rise to poorer development than occurred in lobeless embryos.

That cytoplasmic substances can enter nuclei and affect transcription has been supported by studies of nucleocytoplasmic interaction (Merriam, 1969; Gurdon and Brown, 1965; Gurdon and Woodland, 1969; Yamana and Shiokawa, 1966; Harris, 1967; Zetterberg, 1966a, b; Arms, 1968) and there is evidence for these substances among at least three classes of molecules: 1) simple ions (Beritashvili et al., 1969; Kroeger and Lezzi, 1966); 2) proteins (Riggs and Bourgeois, 1968; Riggs et al., 1968; Ptashne, 1967; Lee and Dahmus, 1973), including histones (Allfrey et al., 1963; see reviews by Bonner et al., 1968; Bonner and Ts'o, 1964) and non-histones (Georgiev et al., 1966; Paul and Gilmour, 1968; Teng et al., 1971); 3) chromosomal RNA (Bonner and Widholm, 1967; Mayfield and Bonner, 1971; Bekhor et al., 1969; Huang and Huang, 1969). It is also possible that RNA and protein act together as a complex (Benjamin et al., 1966).

How these controllers might affect transcription/translation is of interest. As measured by the priming ability of isolated chromatin (Paul and Gilmour, 1968; Georgiev et al., 1966; see Bonner et al., 1968) and RNA/DNA hybridization (Davidson et al., 1966; Crippa et al., 1967; Denis, 1966;

Shearer and McCarthy, 1967), only 2-10 % of the genome is active at any given time. Since loss of active cytoplasm, i.e. the polar lobe in Ilyanassa, causes a loss of function, is is reasonable to consider genomic control mainly from the standpoint of activation. This does not exclude repression-control, however, as Scarano et al. (1964) have illustrated such a case in the sea urchin.

In Ilyanassa, then, removal of the polar lobe at the first cleavage resulted in a failure of the nascent RNA pattern to evolve, with development, to one characteristic of morphogenesis. That period in lobeless embryos was distinguished by more newly synthesized RNA in the low molecular weight range or, in the proportionate sense, less high molecular weight RNA. Repeated measurements failed to show any differences in transcription during the first 24 hours. Evidence that cleavage and gastrulation can proceed without concurrent transcription (Collier, 1966; Feigenbaum and Goldberg, 1965) (although organogenesis was later blocked) supports the evidence presented here that determination of lobe-dependent structures is not a transcriptional event. The elucidation of a timetable of organ-related transcription occurring 1-2 days prior to organ differentiation (Collier, 1966) provides additional information for an interpretation of the present results: since lobeless embryos do not achieve the normal nascent RNA pattern during organogenesis, determination of lobe-dependent structures can be thought of in terms of segregation of preexistent morphogenetic factors that control transcription/translation after gastrulation. The increased

number of transcripts, presumably messages, in the low molecular weight region of the gel profiles of lobeless embryos could reflect an accumulation of untranslated messages. Thus morphogenetic factors might control the translation of organ-specific proteins and, lacking these factors, messages would accumulate. Alternatively, the lobeless nascent RNA pattern could represent a decreased number of high molecular weight transcripts. This suggests, simply, that morphogenetic factors localized in the lobe activate organ-specific transcription; no factors, no organs. It is not possible at this time to state unequivocally which aspect of the lobe's effect, transcription of translation, predominates, if indeed one does predominate. Rather, this work presents substantial evidence concerning the biochemical level at which morphogenetic factors contained in the Ilyanassa polar lobe operate. Further investigations should be directed towards discriminating between these two closely linked and interdependent mechanisms.

APPENDIX A

Ribosomal RNA

When _Ilyanassa_ tissues were homogenized at $4^{o}C$ and then treated with phenol for 2.5 minutes at either 55°, 50° or $45^{o}C$ the recovery of RNA was complete but sedimentation of the RNA through a sucrose gradient disclosed only a single 18S (the S-values serve as convenient appellations and do not imply accurate measurement of absolute sedimentation rates) component (Fig. A1a-c). After homogenization at $4^{o}C$ and phenolization at $40^{o}C$, or both operations at $25^{o}C$, the RNA sedimented in a sucrose gradient (Fig. A1d, e) as an 18S and 26S RNA but the ratio of 18S to 26S was smaller than anticipated from the size (see below for molecular weight measurements on RNA) of these classes of RNA (Amaldi and Attardi, 1968; Click and Tint, 1967). RNA extracted at $4^{o}C$ and phenolized at the same temperature sedimented as 18S and 26S RNA with a ratio of 1.0 to 1.9 (Fig. A1f); however, if this RNA was extracted in the absence of magnesium ions and sedimented through sucrose containing ethylenediaminetetraacetate (EDTA), an atypical 18S to 26S ratio of 1.0 to 0.9 was observed (Fig. A1g).

When ribosomes were isolated and deproteinized by incubation with 1 % SDS and then sedimented through a sucrose gradient, 18S and 26S RNA in a ratio of 1.0:2.3 was obtained (Fig. A2). This ratio exceeded the molecular weight ratio (see below) of the 18S and 26S RNAs and might have resulted from aggregation of the RNA in the presence of magnesium.

FIGURE A1-Sucrose gradient profiles of _Ilyanassa_ RNA. _Ilyanassa_ tissue was homogenized at 4^oC (a-d, f, g) or 25^oC (e) and incubated 2½ min at (a) 55°; (b) 50°; (c) 45°; (d) 40°; (e) 25°; (f, g) 4^oC. RNA was extracted with phenol-SDS-TNS and sedimented through sucrose in a Spinco SW 25.1 rotor in the presence of (a-f) 10^{-2} M magnesium or (g) 10^{-3} M EDTA. The dashed line idealizes the low molecular weight region of the gradient, here omitted for the sake of clarity.

ABSORBANCY 254mu

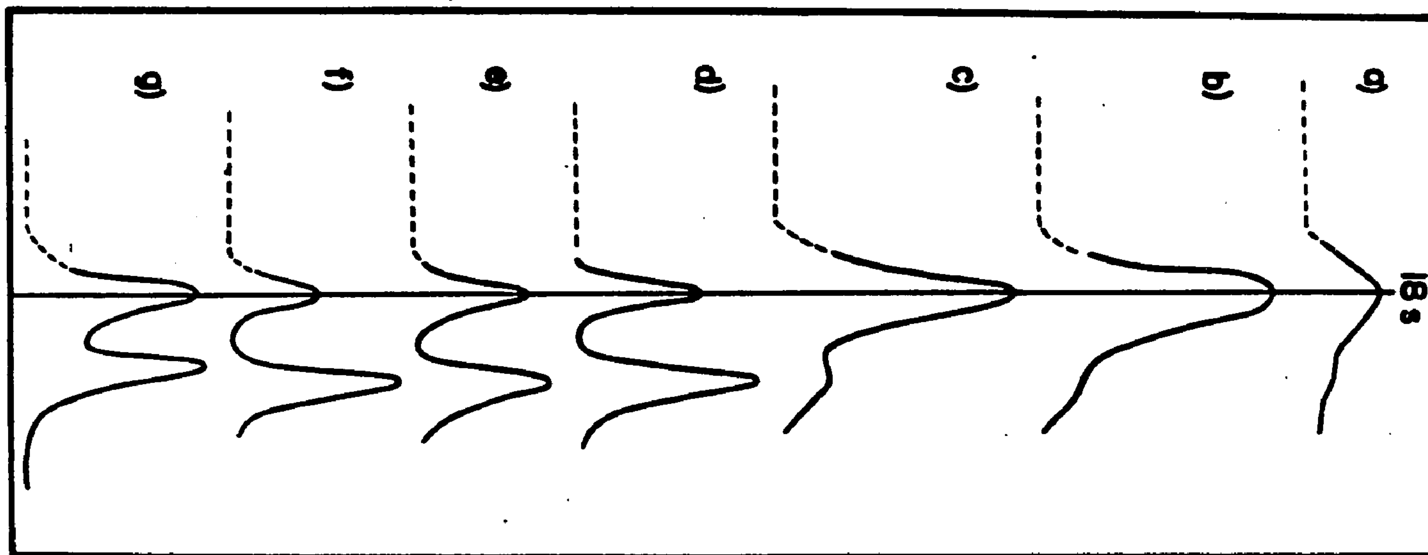
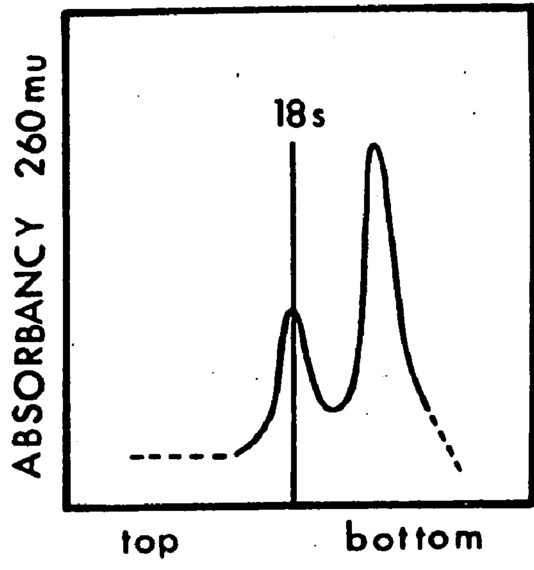


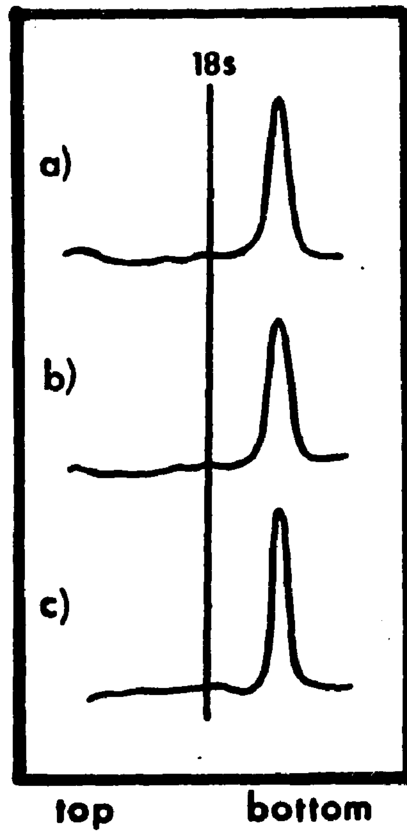
FIGURE A2-Sucrose gradient profile of RNA extracted from *Ilyanassa* ribosomes. *Ilyanassa* digestive gland tissue was broken in a Dounce homogenizer; the 12,000 x g supernatant was layered over 0.5 M sucrose and centrifuged for 2 hr at 105,000 x g in a Spinco 30 rotor. The ribosomal pellet was incubated 45 min at 25°C in 1 % SDS and the RNA was sedimented through sucrose in a Spinco SW 25.1 rotor.


ABSORBANCY 260mu
18s
top
bottom

Since 26S RNA may be more labile to ribonuclease (RNase) than the 18S RNA (Fenwick, 1968), the assay procedure was tested for sensitivity to exogenous nuclease activity by adding pancreatic RNase to the sucrose gradient. As expected, in the presence of SDS, 26S RNA was stable in RNase concentrations up to 5 µg/ml (Fig. A3). Without SDS present, 26S RNA degraded in 0.001 µg/ml RNase. To assay for endogenous nuclease activity during extractions, 26S ^3H-RNA was prepared from embryos and sedimented through a sucrose gradient; only 0.1 % of this RNA sedimented at 18S after incubation at 40°C while 32 % sedimented at 18S after incubation at 55°C. When this ^3H-RNA was added to digestive gland tissues and homogenized and phenolized at 4°C, there was no detectable change in the sedimentation of the labeled 26S molecule. It is therefore unlikely that the unusual sensitivity of the 26S RNA was caused by nuclease activity during extraction or in subsequent handling.

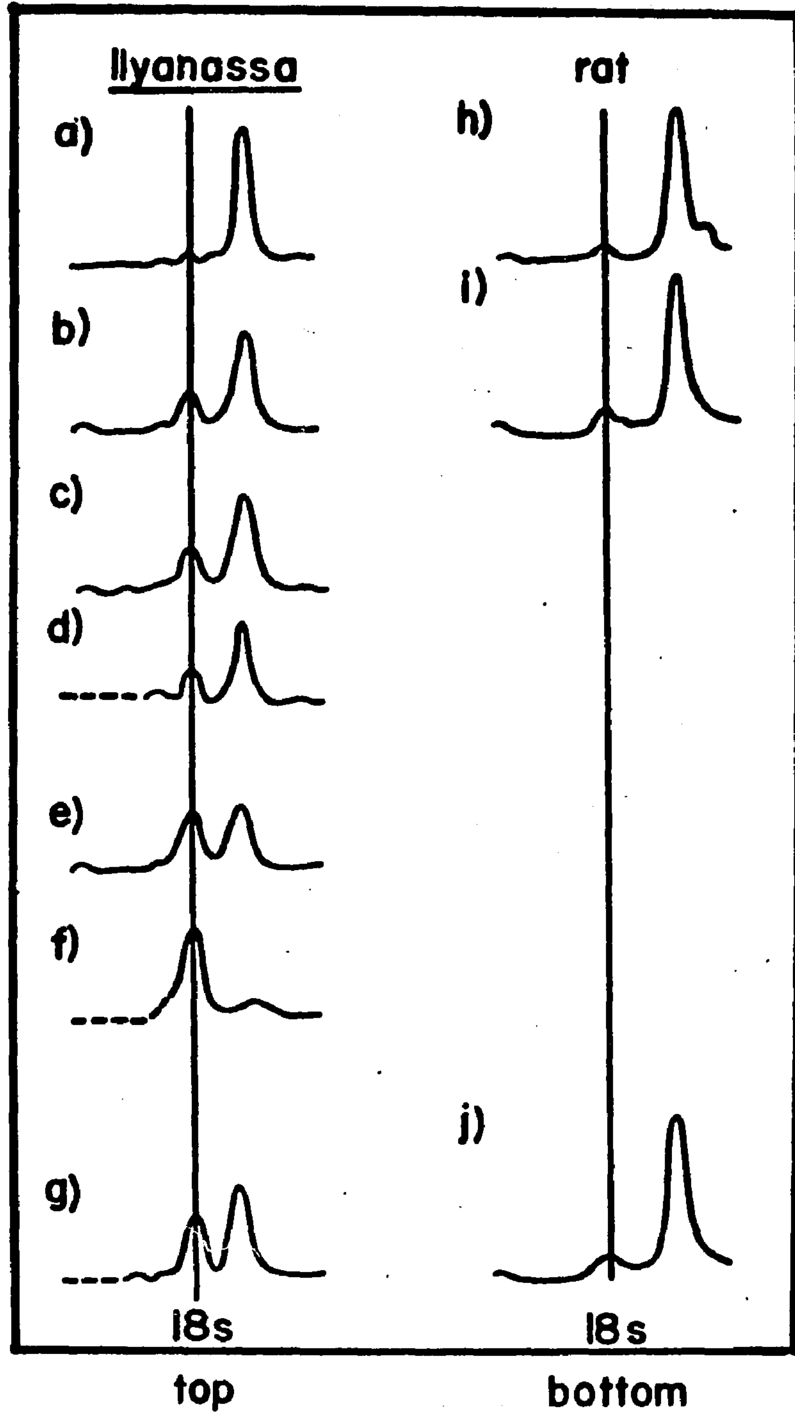
The 26S rRNA was sensitive to the detergents SDS and TNS (Fig. A4). When 26S RNA was heated for 2½ min at 55°C in 0.1 M sodium acetate containing SDS and/or TNS, and sedimented once again through sucrose, the purified 26S RNA then sedimented as an 18S and a 26S component. When the SDS concentration was increased (Fig. A4b, c, e) there was a proportional increase in the size of the 18S RNA fraction. In the presence of 1 % SDS and 1 % TNS, which were the concentrations used previously (Fig. A1), almost all of the 26S RNA sedimented as an 18S fraction (Fig. A4f).

FIGURE A3-Sucrose gradient profiles of Ilyanassa 26S RNA; treatment with ribonuclease. Ilyanassa digestive gland 26S RNA was collected from sucrose gradients and precipitated with ethanol. It was redissolved in buffer and sedimented through sucrose in a Spinco SW 41 rotor (a). The buffer and the sucrose gradients contained (b) 1 µg/ml pancreatic ribonuclease or (c) 5 µg/ml ribonuclease.



18s
a)
b)
c)
ABSORBANCY 260mu
top
bottom

FIGURE A4-Sucrose gradient profiles of Ilyanassa digestive gland 26S RNA and rat liver 28S RNA; treatment with detergents and urea. Ilyanassa and rat liver RNAs were extracted and purified on sucrose gradients. Ilyanassa 26S RNA (a) was incubated 2½ min at 55°C in gradient buffer containing (b) 0.5 % SDS; (c) 1 % SDS; (d) 1 % TNS; (e) 2 % SDS; (f) 1 % SDS and 1 % TNS; (g) 10 min incubation at 25°C in 6 M urea. Rat liver 28S rRNA (h) was incubated 2½ min at 55°C in 0.5 % SDS (i) or 10 min at 25°C in 6 M urea (j).



Ilyanassa
rat
a)
b)
c)
d)
e)
f)
g)
h)
i)
j)
ABSORBANCY 254 mu
18s
18s
top
bottom

Ten minutes incubation in 6 M urea (Fig. A4g) at 25°C also changed the sedimentation properties of 26S rRNA so that nearly half of it sedimented as an 18S component.

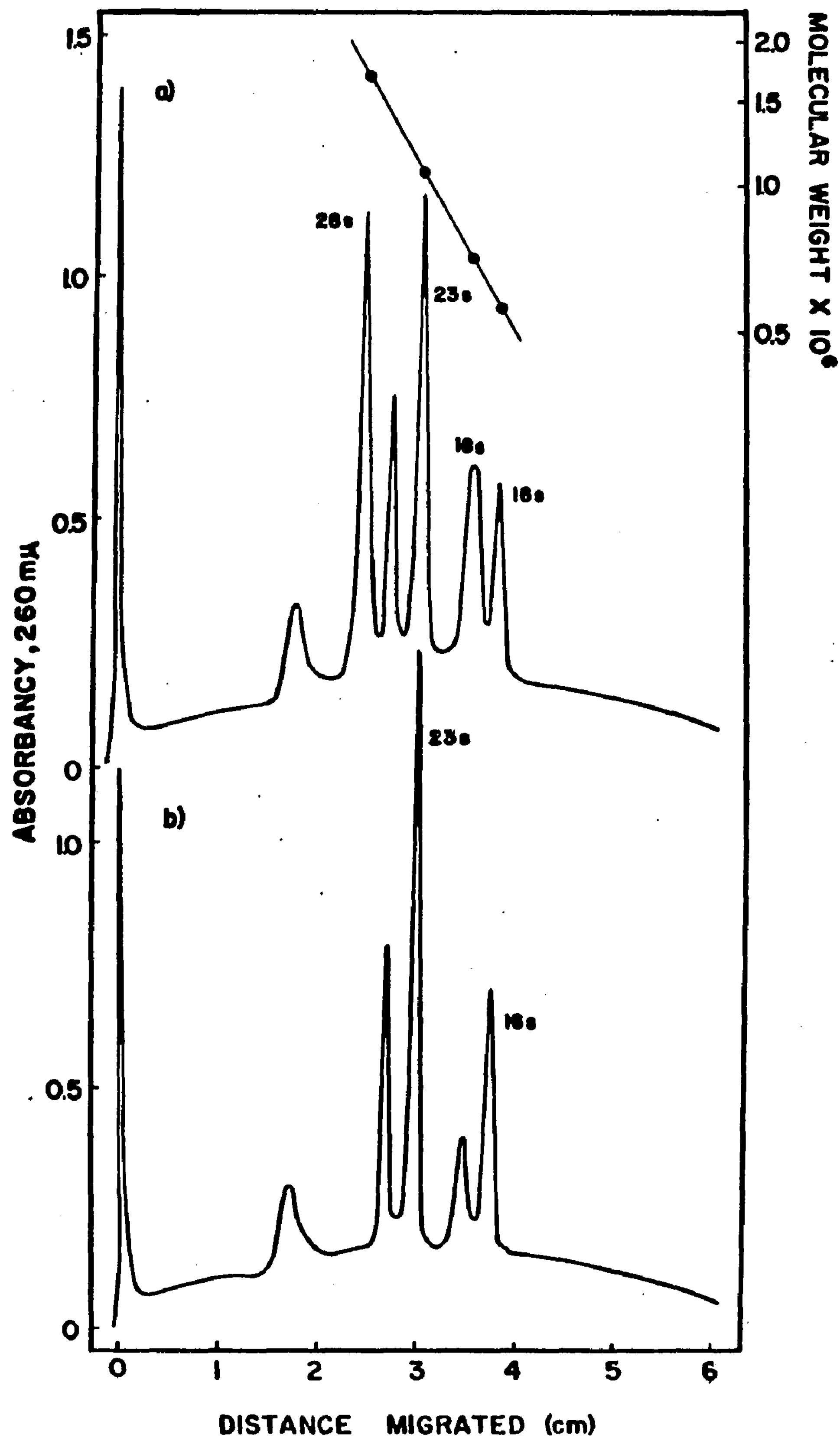
When rat liver 28S rRNA was heated in 0.5 % SDS or treated with 6 M urea its sedimentation properties were unchanged (Fig. A4h-j).

These observations showed that the sedimentation properties of _Ilyanassa_ 26S rRNA were altered after heating 45° to 55°C in the presence of detergents and after treatment with 6 M urea and that this effect was not caused by RNase and that these conditions did not produce a similar change in the sedimentation properties of rat liver RNA.

To resolve the nature of this change in the 26S RNA, the molecular weight and hyperchromicity of the heated rRNAs were determined.

In Fig. A5a are the electrophoretic profiles of _Ilyanassa_ rRNA run with rat liver and _E. coli_ rRNA as standards. The upper portion of this figure shows the linearity of the mobility with the logarithms of the molecular weights of the standards (average coefficient of determination for 15 gels = 0.992; i.e., 99.2 % of the variation in molecular weight was accounted for by this relationship. This resulted only when both magnesium and EDTA were absent from the RNA preparations and the electrophoresis system; the _Ilyanassa_ 26S RNA was stable under these conditions. The molecular weights of the standards were (Loening, 1968): rat liver 28S, 1.75×10^6; 18S, 0.70×10^6; _E. coli_ 23S, 1.07×10^6; 16S, 0.56×10^6.

FIGURE A5-Electrophoretic profile of Ilyanassa RNA. (a) Ilyanassa RNA was electrophoresed together with rat liver 28S and 18S RNA and E. coli 23S and 16S RNA. The straight line above the curve demonstrates the linearity of the distance migrated (peak position) to the logarithm of the molecular weight. (b) The Ilyanassa 18S RNA was revealed when electrophoresed with E. coli RNA.



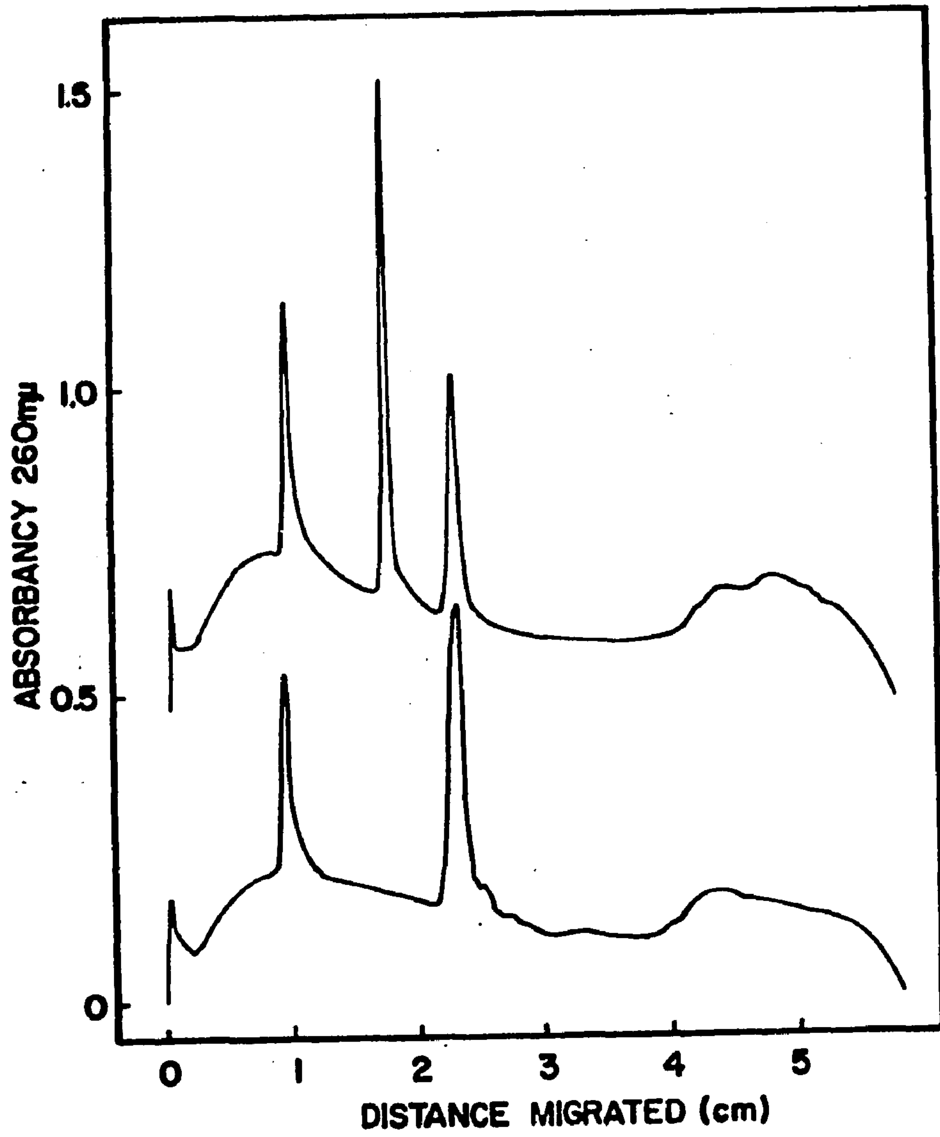
a)
b)
28s
23s
16s
18s
23s
16s
ABSORBANCY, 260 mμ
MOLECULAR WEIGHT X 10^6
DISTANCE MIGRATED (cm)

From Fig. A5a the molecular weight of the large rRNA of Ilyanassa was calculated to be $1.37 \pm 0.004 \times 10^6$ (mean $\pm$ S.E.). The smaller rRNA could not be distinguished from rat liver 18S rRNA. However, the shape of this peak suggested that it might contain two components. In Fig. A5b Ilyanassa rRNA was electrophoresed with E. coli rRNA as a standard; the smaller Ilyanassa rRNA was found to have a molecular weight of 0.71 $\pm$ 0.002×10^6, quite close to the value of 0.70×10^6 for rat liver 18S rRNA with which its migration was coincident.

Fig. A6a is an electrophoretic profile of RNA extracted from the digestive gland with cold phenol; from left to right the major elements are: 1) a DNase-labile component, 2) the large ribosomal RNA (1.37×10^6 daltons) and 3) the small rRNA component (0.71×10^6 daltons). The profile shown in Fig. A6b resulted from heating the material of Fig. A6a for 5 min at 60°C in electrophoresis buffer. The bulk of the RNA now migrated as an 18S (or slightly smaller) molecule; thus, the 26S RNA behaved similarly both on acrylamide gels and sucrose gradients.

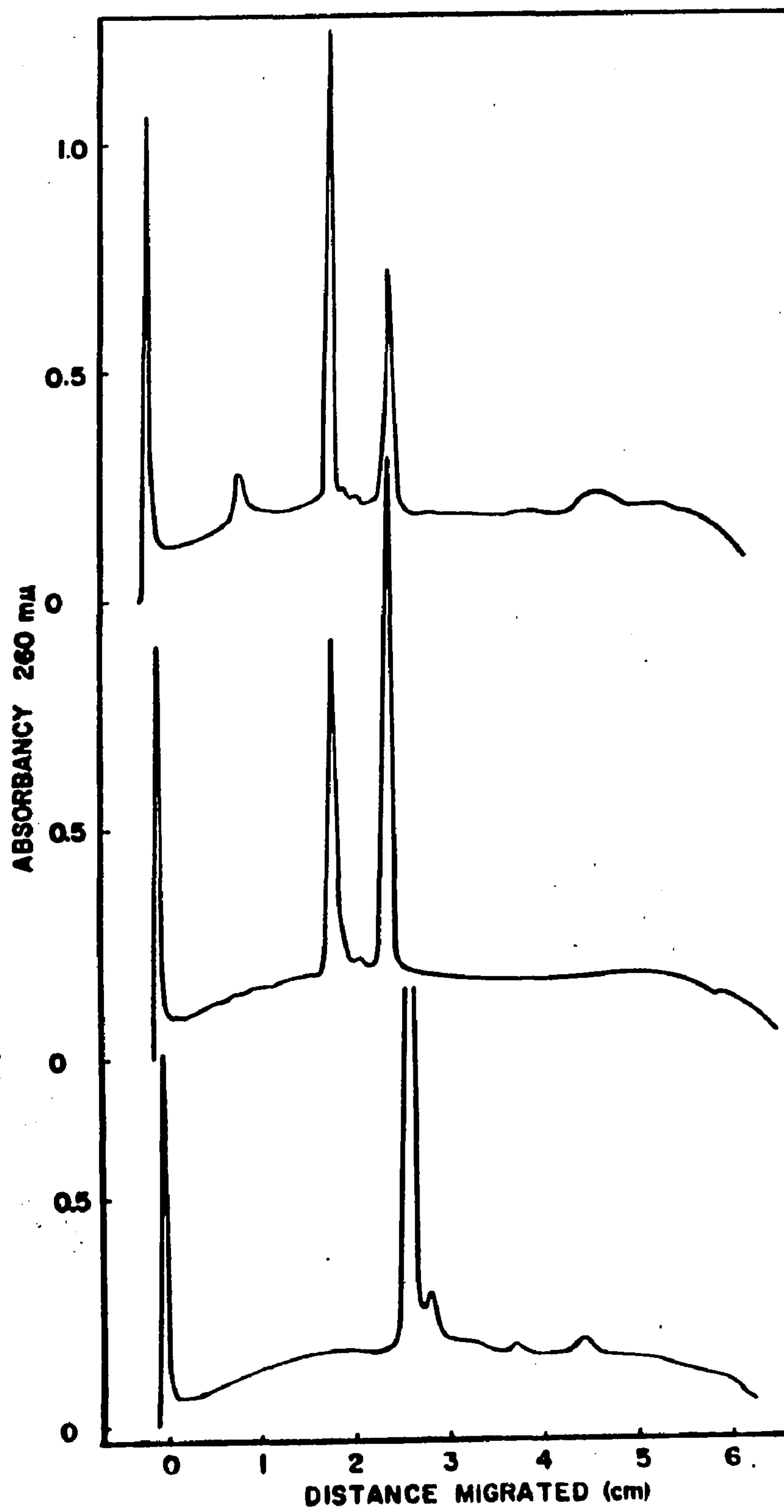
When Ilyanassa RNA was both extracted and electrophoresed in the absence of magnesium, a typical electrophoretic profile resulted (Figs. A6a, A7a). However, the 26S RNA collected from a magnesium-free sucrose gradient of this preparation showed considerable conversion to 18S material (Fig. A7b). When heated to 60°C for 5 min and quickly cooled in an ice bath, the 18S and 26S components of 26S RNA (Fig. A7b) gave rise to the profile shown in Fig. A7c. There were four clearly distinguishable peaks, from left to right, with molecular weights of

FIGURE A6-Electrophoretic profiles of Ilyanassa RNA. (a) Control. (b) Preparation similar to (a) but heated in electrophoresis buffer for 5 min at 60°C and quickly cooled prior to electrophoresis.



ABSORBANCY 260mμ
1.5
1.0
0.5
0
0
1
2
3
4
5
DISTANCE MIGRATED (cm)

FIGURE A7-Electrophoretic profiles of Ilyanassa RNA. Ilyanassa digestive gland RNA was extracted with phenol-SDS without magnesium and, after ethanol precipitation, sedimented through sucrose that also lacked magnesium; 26S RNA was collected from the gradient, ethanol-precipitated, redissolved in electrophoresis buffer, heated to 60°C for 5 min and quickly cooled. (a) Control electrophoregram of Ilyanassa RNA after phenol extraction and ethanol precipitation (cf. Fig. A6); (b) 26S RNA after collection from the sucrose gradient and ethanol precipitation; (c) the RNA from (b) after heating and cooling.



1.0
0.5
0
0.5
0
0.5
0
ABSORBANCY 260 mμ
0
1
2
3
4
5
6
DISTANCE MIGRATED (cm)

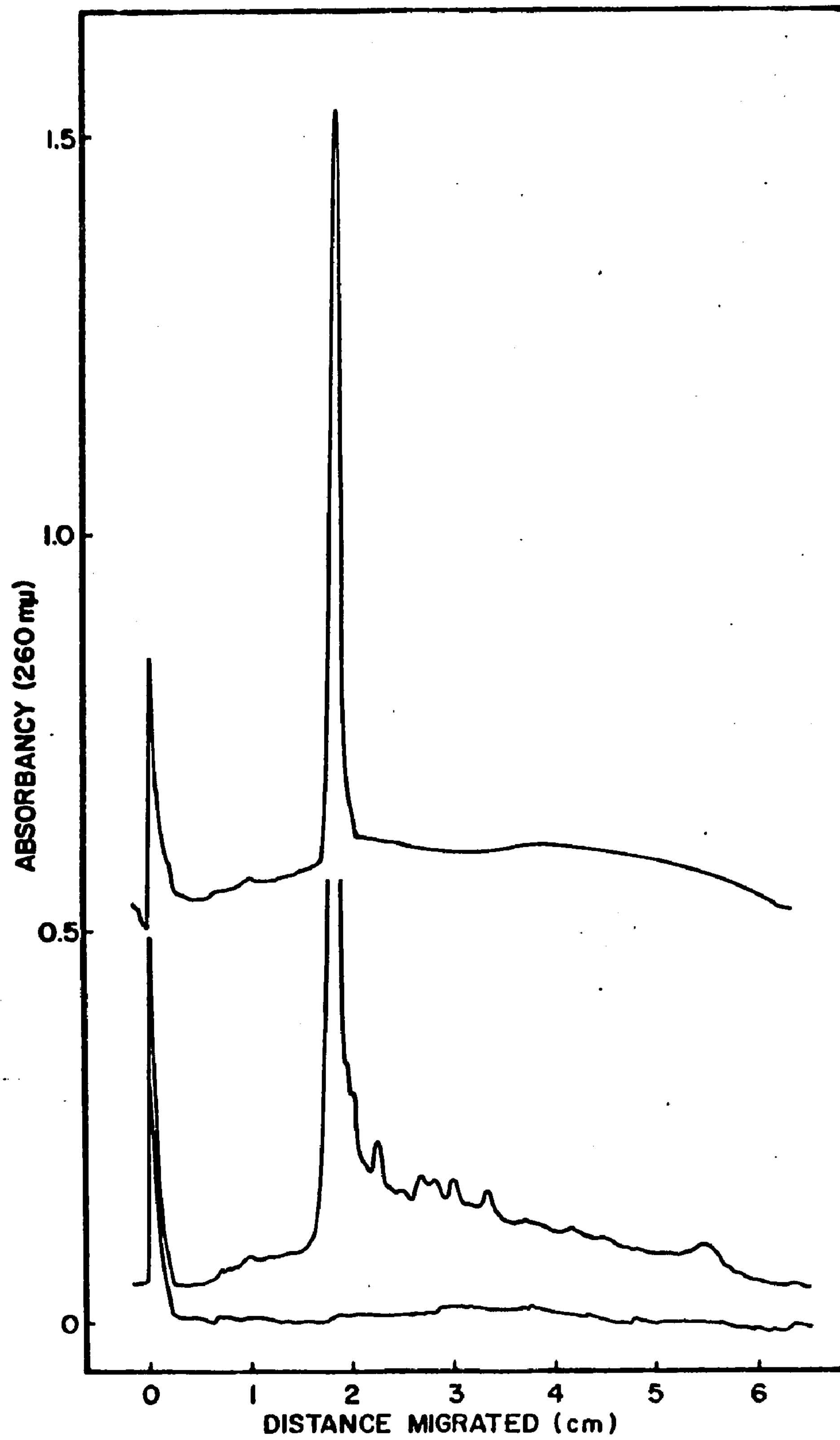
0.63 ± 0.005, 0.5 ± 0.005, 0.15 ± 0.002 and 0.041 ± 0.001 x 10^6 daltons, respectively. The molar quantities of these RNAs were determined from their amounts (by measuring the area under the absorbancy curve in several different runs) and from their molecular weights relative to the molecular weight of their source. Since the 26S RNA remainder and its product after magnesium deprivation (Fig. A7b) measured 1.31 ± 0.006 and 0.66 ± 0.001 x 10^6 daltons, the source was assumed to be a molecule of 1.32 x 10^6 daltons. Thus the molar ratios, from left to right in Fig. A7c, were 1.89, 0.08, 0.17 and 1.50, respectively. The 0.63 and 0.041 x 10^6 dalton pieces (approximately 18S and 7S, respectively), on a molar basis, constituted the major portion of the breakdown products that resulted from heating the large rRNA molecule. The molar ratios suggest that upon heating the 26S molecule gave rise to two 18S pieces and one to two 7S pieces. No difference in the electrophoretic profiles (Fig. A7c) or molar ratios was apparent whether the preparation (Fig. A7b) was heated to 45°, 60°, 75°, 90° or 100°C.

When purified rat liver 28S rRNA was heated and electrophoresed (Fig. A8), a similar pattern of fragments resulted which differed from the *Ilyanassa* pattern in the larger number of pieces and in the failure of the molecule to break down to 18S RNA. The molar ratio of the 7S fraction to 28S RNA averaged 2.1:1 for three measurements; another preparation four months old (stored under ethanol at -40°C) averaged 3.5:1.

In Fig. A9 are shown the thermal melting profiles of 26S

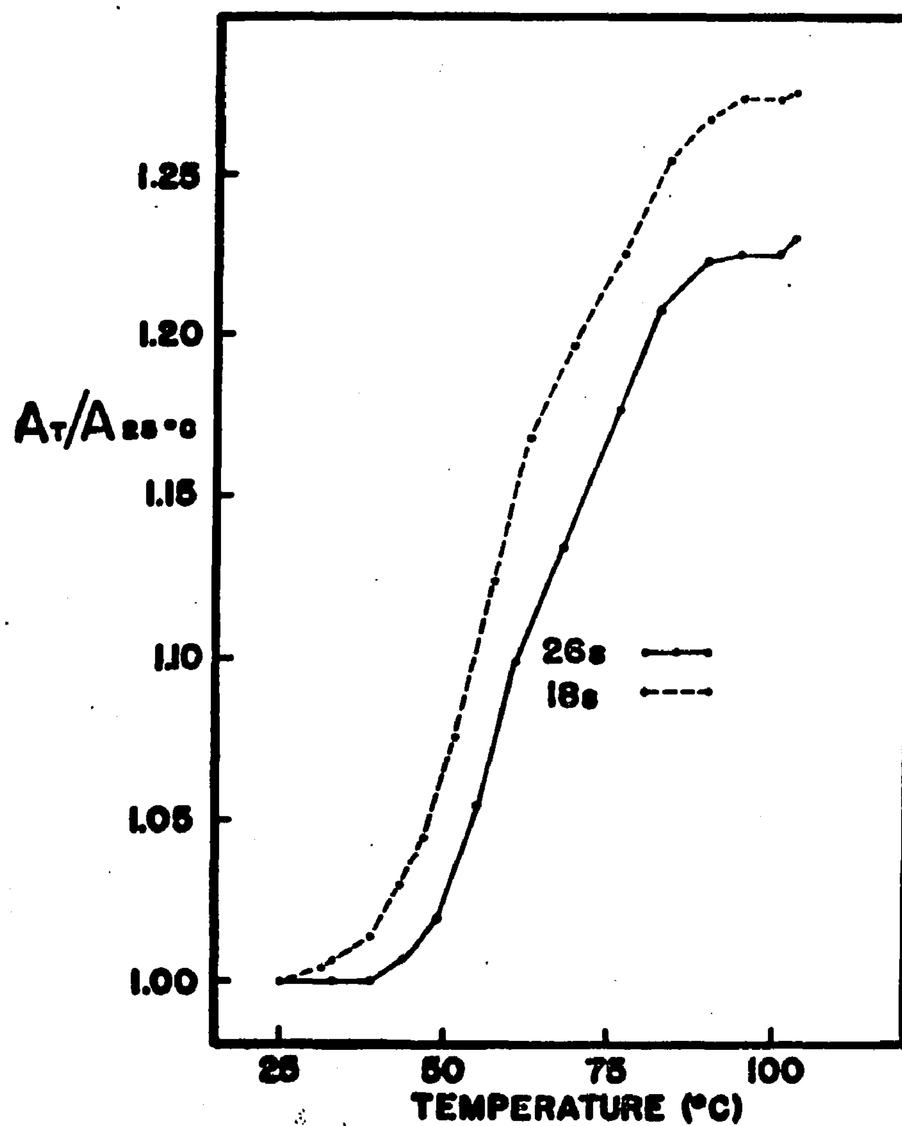
FIGURE A8-Electrophoretic profiles of rat liver RNA. The RNA was extracted and processed as in the legend to Fig. A7. (a) Electrophoregram of 28S RNA collected from the sucrose gradient; (b) preparation similar to (a) after heating 10 min at 60°C and cooling.

The lowest (absorbancy = 0) trace was made at 310 mμ to measure absorbancy caused by imperfections in the quartz electrophoresis and scanning tubes (see materials and methods).



1.5
1.0
0.5
0
ABSORBANCY (260 mμ)
0
1
2
3
4
5
6
DISTANCE MIGRATED (cm)

FIGURE A9-Melting profiles of _Ilyanassa_ ribosomal RNAs. _Ilyanassa_ 26S and 18S ribosomal RNAs were purified by sedimentation through sucrose containing magnesium; after ethanol precipitation the RNAs were redissolved in 0.15 M sodium acetate, pH 5; the absorbancy was measured at different temperatures (A_T) and expressed as a ratio (A_T/A_{25^oC}) to the absorbance at 25°C.


1.25
1.20
1.15
1.10
1.05
1.00
$A_T/A_{25°C}$
26s
18s
25
50
75
100
TEMPERATURE (°C)

and 18S Ilyanassa RNA prepared in 10^{-2} M magnesium; they could not be separated in pure form in the absence of magnesium (Fig. A7b). The 18S RNA showed a hyperchromicity of 1.27 and had a melting point (Tm) of 59°C. The Tm of 26S RNA was 64°C and its hyperchromicity was 1.23.

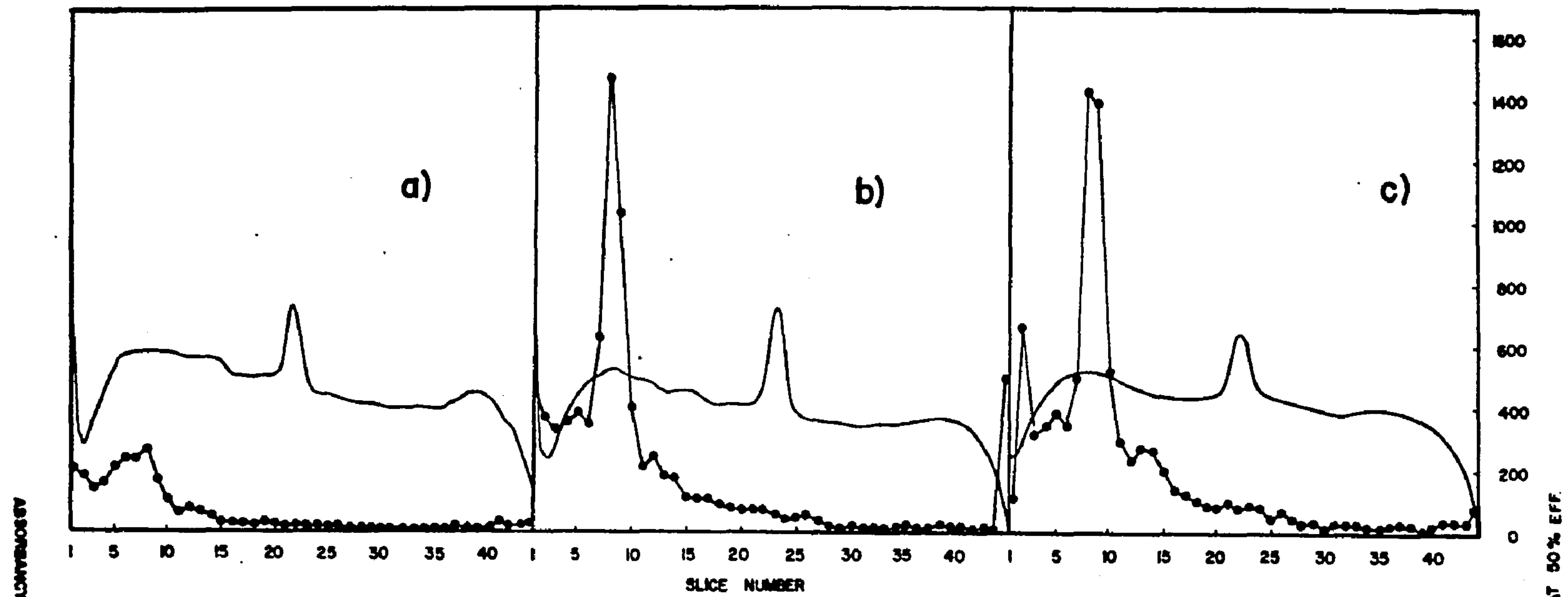
Ribosomal RNA Precursor

The question arises as to whether Ilyanassa 26S rRNA is transcribed with its "lesion" or the latter is introduced into the molecule during its processing from the ribosomal precursor. It was therefore necessary to examine the ribosomal precursor molecule. A series of short, radioactive pulses of increasing duration were employed with the rationale that, if a large molecule were first labeled after which the rRNAs became labeled, a precursor-product relationship could be inferred.

In the shortest (10 min) pulse time (Fig. A10a), three radioactive, high molecular weight RNA components were present with peaks in slices 6, 8 and 12. During a longer labeling period (15 min, Fig. A10b) the middle component (slice 8) became the predominant nascent RNA species. This condition persisted during the first 20 min of RNA synthesis (Fig. A10c); by 30 min (Fig. A10d) fully processed rRNA appeared coincident with the rRNA absorbance peaks at slices 17 and 24. As pulse time further increased (Fig. A10e, f, A11) the size of the predominant high molecular weight RNA peak. e.g., Fig. A10b, slice 8, decreased in proportion to increasing amounts of radioactivity appearing in rRNA. These observations are consistent with a precursor-product relationship between the

FIGURE A10-Electrophoregrams of Ilyanassa RNA. For each determination, 300 3-day old Ilyanassa embryos were radioactively labeled with ^{3}H-uridine and the RNA was extracted as described in Materials and Methods. In (a-c), the homogenate was incubated 2½ min at 55°C to quantitatively extract all the RNA. All other operations and the entire extractions (d-f) were performed at 4°C. Pulse times and precursor concentrations were: (a) 10 min, 250 µC/ml; (b) 15 min, 250 µC/ml; (c) 20 min, 250 µC/ml; (d) 30 min, 100 µC/ml; (e) 60 min, 100 µC/ml; (f) 120 min, 100 µC/ml.

In (d-f), the predominant absorbance peaks, e.g. slices 20 and 25 in (d), belong to E. coli ribosomal RNA added as a molecular weight marker. Migration is from left to right.


a)
b)
c)
SLICE NUMBER
ABSORBANCY 260mμ
NET CPM AT 50% EFF.

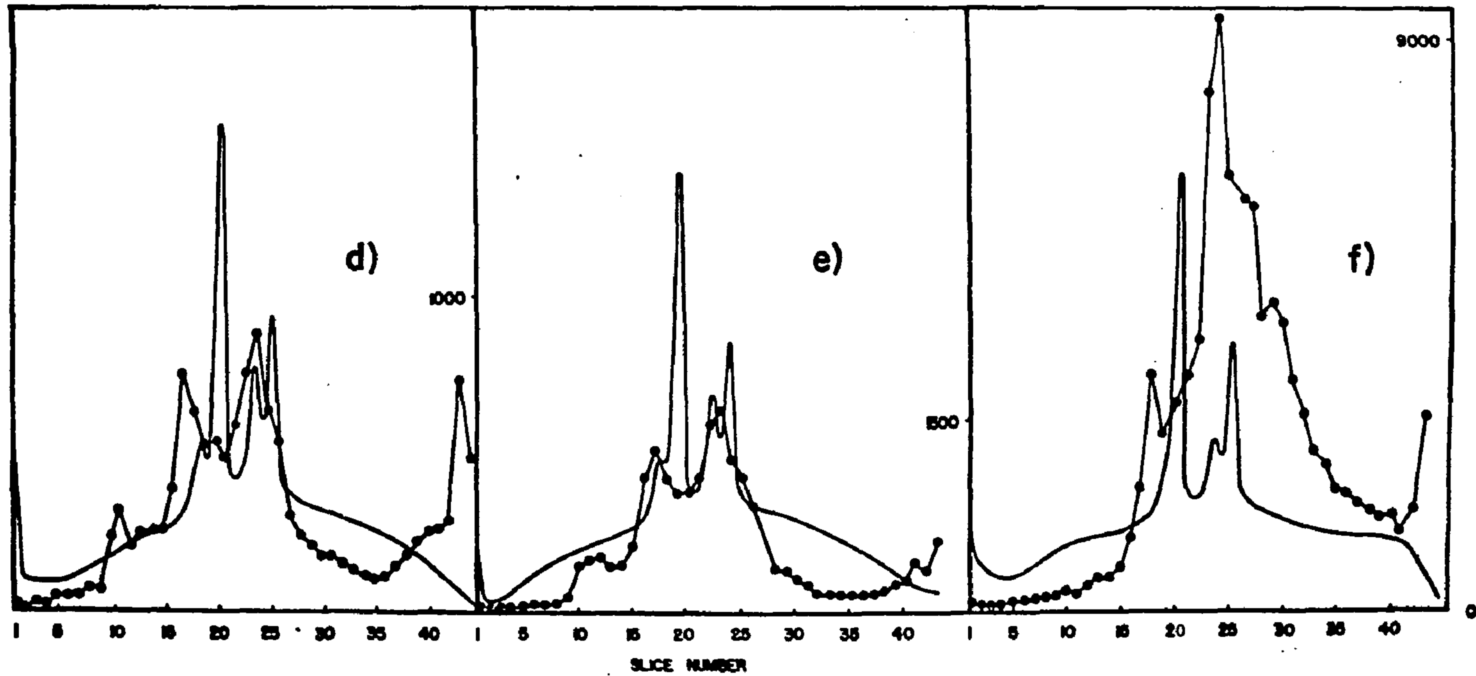
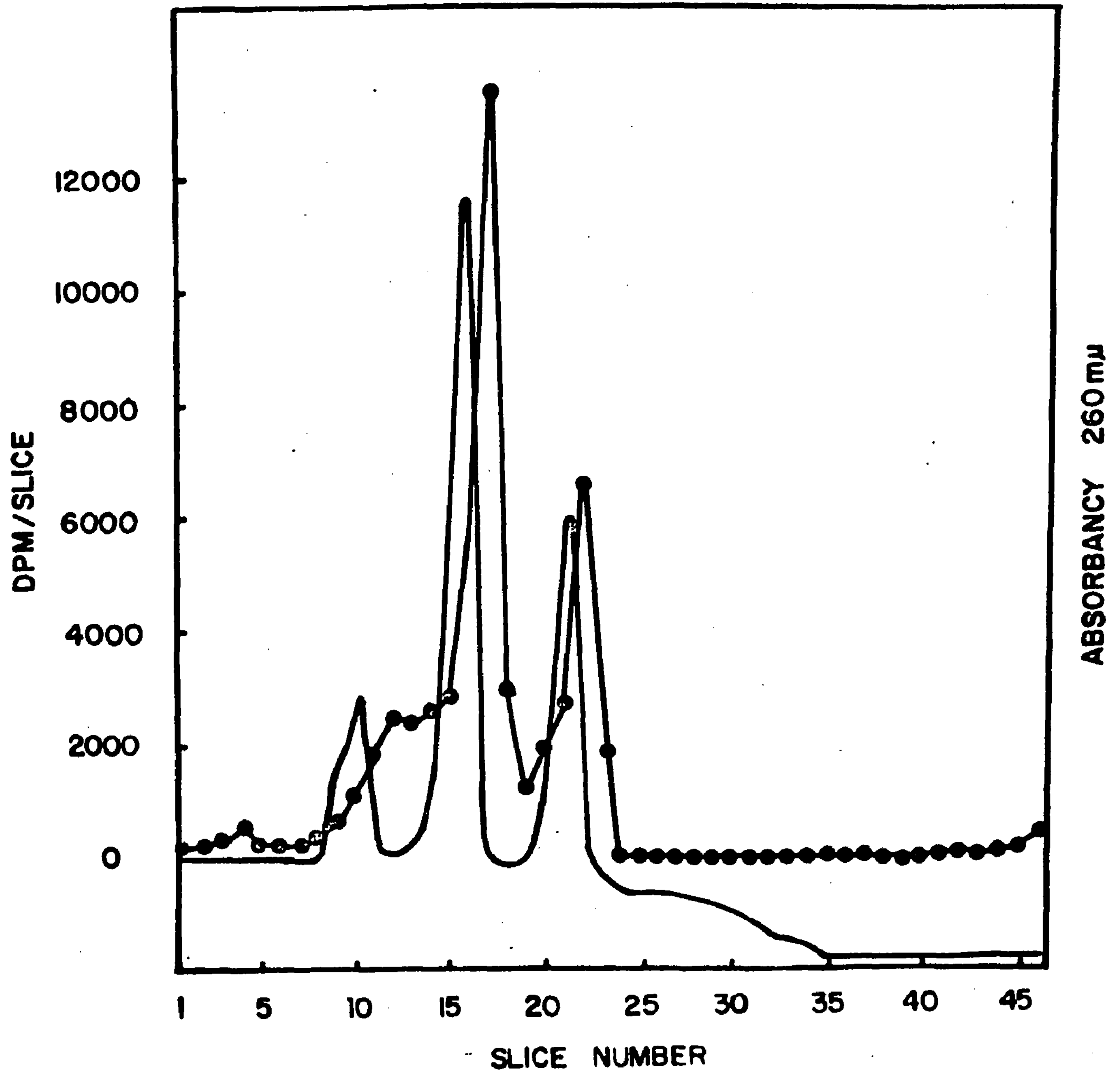

d)
e)
f)
SLICE NUMBER

FIGURE A11-Electrophoregram of Ilyanassa RNA. Three hundred 3-5-day old Ilyanassa embryos were radioactively labeled with ^{3}H-uridine for 48 hr at 10 μC/ml. RNA was extracted at 4°C as described in Materials and Methods and was not treated with deoxyribonuclease prior to electrophoresis.

Migration is from left to right.


12000
10000
8000
6000
4000
2000
0
DPM/SLICE
ABSORBANCY 260 mμ
1
5
10
15
20
25
30
35
40
45
SLICE NUMBER

rapidly-labeled, high molecular weight RNA peak and ribosomal RNA.

In Fig. A10d-f, if the molecular weights of the rRNAs were 1.37 and 0.7 x 10^6, then the precursor peak measured 2.4-2.5 x 10^6 D. Using this value in Fig. A10a-c, along with 0.7 x 10^6 for the 18S rRNA absorbance peak, the sizes of the shoulders on the precursor peak averaged 3.2 and 1.8 x 10^6 D.

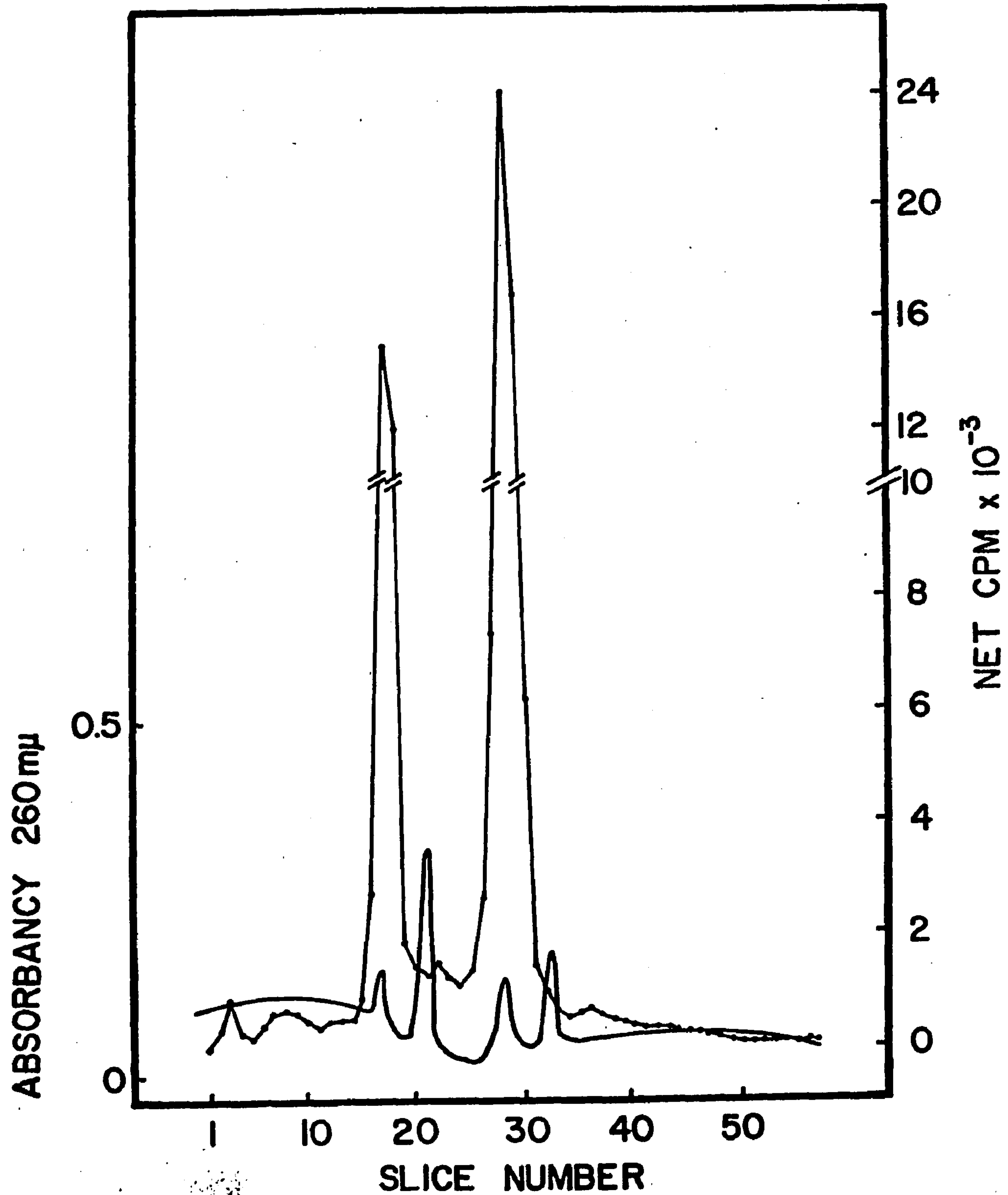
The precursor peak and rRNAs were methylated in the same sequence as their synthesis when embryos were incubated with ^{3}H-methyl-L-methionine. After 48 hr (Fig. A12), the RNAs in slices 8 (2.4 x 10^6 D), 12 (1.8 x 10^6 D), 17 (1.37 x 10^6 D) and 28 (0.7 x 10^6 D) were also labeled. During 30, 60 and 120 min pulses, the 2.4 x 10^6 D RNA first became labeled and then diminished in proportion to increasing radioactivity in rRNA (there was insufficient incorporation during shorter pulse times).

Since some of these preparations were treated with hot phenol during extraction (Fig. A10a-c), breakdown components of the labile 26S rRNA appeared in some of the profiles. The minor, 0.5 x 10^6 D component was apparent in slice 27 (Fig. A10f) and only the 18S rRNA was observed in the absorbancy profile (Fig. A10a-c). Similarly, much 26S RNA was converted to 18S rRNA (Fig. A10d-f) presumably from mechanical fragility or localized heating during the rRNA extraction. Compare the intact 26S rRNA in Fig. A11 where gentler RNA extraction procedures were employed.

When RNA from embryos labeled for periods shorter than 30 min was extracted with hot phenol, no radioactivity was

FIGURE A12-Electrophoregram of Ilyanassa RNA. Three hundred 3-day old Ilyanassa embryos were radioactively labeled with L-methionine-methyl-H^3 for 48 hr at 30 μC/ml. RNA was extracted at 4^oC as described in Materials and Methods. The absorbance peaks at slices 21 and 33 belong to E. coli ribosomal RNA added as a molecular weight marker.

Migration is from left to right.



24
20
16
12
10
8
6
4
2
0
NET CPM x 10^{-3}
0.5
0
ABSORBANCY 260 mμ
1
10
20
30
40
50
SLICE NUMBER

detected coincident with the 18S rRNA absorbance peak (e.g., Fig. A10b, slice 23) where breakdown products of the 26S rRNA would appear. That is, the ribosomal precursor molecule (Fig. A10b, slices 7-10) was not heat-labile.

Samples that were not treated with DNase prior to electrophoresis (Fig. A11) often showed an additional absorbance component that migrated more slowly than did 26S rRNA; compare the DNase-labile component in Figs. A6, A8.

Characterization of Ilyanassa rRNA

Sucrose gradient analysis of Ilyanassa ribosomal RNA disclosed that the larger component, nominally 26S, was degraded to an 18S fragment by heat, treatment with urea or Mg^{2+} deprivation. A detailed electrophoretic study of the rRNA brought to light several smaller degradation fragments and established the molecular weight of the larger rRNA component as 1.37×10^6 and that of the smaller component as 0.71×10^6. A similar lability of the larger ribosomal RNA has been reported in protozoa (Loening, 1968; Stevens and Pachler, 1972), arthropods (Applebaum et al., 1966; Hastings and Kirby, 1966; Hayashi et al., 1966; Greenberg, 1969; Fristrom et al., 1970), molluscs (Kidder, 1972a) and amphibia (Brown and Littna, 1964); rat liver nuclear 28S RNA is also heat-labile (Venkov and Hadjiolov, 1967). Although the larger subunit is commonly the more labile one (Huppert and Pelmont, 1962; Midgley, 1965), perhaps because of its greater ribonuclease sensitivity (Fenwick, 1968), the smaller, maternal ribosomal subunit is labile in an echinoderm (Nemer and Infante, 1967). These authors interpreted the decreasing proportion of labile RNA

during development as a replacement of maternal ribosomes. A similar situation might occur in Ilyanassa for a decreasing lability with developmental stage was noted (R. Koser, unpublished observations). In Ilyanassa, the size of the smaller rRNA is similar to that of other eukaryotes and the larger rRNA is slightly smaller than in other invertebrates (Loening, 1968; Attardi and Amaldi, 1970).

Because the fragmentation of the 26S rRNA occurred under conditions insufficient to rupture the phosphodiester bond, this RNA probably contains "hidden breaks" (Gould, 1967) which were revealed by alterations of the secondary structure of the RNA. That these breaks did not occur from exogenous ribonuclease activity in extraction or subsequent handling of the RNA was shown by the failure of the 26S RNA to degrade when ribonuclease was added to sucrose gradients and by the stability of radioactive 26S RNA added during the homogenization of Ilyanassa tissue.

There remains the possibility, which could not be eliminated, that an endogenous nuclease (a ribosomal or cellular component released during extraction) produced a lesion in the 26S RNA in specific regions (Applebaum et al., 1966) so that breakdown products of distinct size resulted when the secondary structure was disrupted. The loci acted upon may be helical regions or other portions of the molecule sufficiently complementary to be stabilized by base-pairing; in this case the breaks would not be evident unless the RNA were heated, i.e., the breaks would be hidden.

Ilyanassa 26S RNA fragmented during electrophoresis in low-salt buffers unless stabilized by addition of magnesium. Conversely, it was stable during electrophoresis in higher salt concentration (e.g., 0.04 M Tris, 0.02 M sodium acetate) even at 0.001 M EDTA. Clearly, the integrity of Ilyanassa 26S rRNA is dependent upon its secondary structure. This is supported by the fact that the temperature required to break the molecule (40-45°C) coincided with the onset of its melting as determined from melting profiles. These showed a broad melting range which, together with the increasing extent of breakdown commensurate with extraction or treatment temperature detected in the sucrose gradient profiles, indicates heterogeneity among Ilyanassa ribosomal RNAs; heterogeneity of ribosomal RNAs has been demonstrated on the basis of partial sequencing in rat (Higashi et al., 1972) and mouse (Hashimoto and Muramatsu, 1973) tissues.

As the molecular weight of the larger rRNA increases with evolution (Loening, 1968), so also does the number of hydrogen bonded fragments in that molecule: there are none in E. coli (Stanley and Bock, 1965; Pene et al., 1968), four in Ilyanassa, five to six in the sea urchin (Sy and McCarty, 1970) and about seven in the rat. These may represent the increments by which the larger ribosomal RNA grew in size during the course of evolution. Furthermore, the scission of this molecule, rather than being an artifact of preparation or analysis, is likely a natural consequence of its processing from the ribosomal precursor as is the detachment of 7S RNA from the larger

subunit during processing from the 32S RNA intermediate in HeLa cells (Pene et al., 1968).

The studies of pulse-labeled Ilyanassa RNAs suggest that a rapidly-labeled, 2.4×10^6 D RNA species is the precursor to rRNA. The identification was made on the basis of kinetic data and is supported by the parallel methylation of the pertinent RNA species (Perry et al., 1970). The molecular weight measurements are only approximate for the radioactivity profiles did not perfectly match with the absorbance profiles. Moreover, the introduction of magnesium for DNase treatment could alter the relationship between mobility and molecular weight (Loening, 1969). The presence of even trace amounts of magnesium tended to lower the apparent molecular weight of rRNAs in electrophoresis (R. Koser, unpublished observations); thus the actual size for the Ilyanassa rRNA precursor is probably somewhat larger than 2.5×10^6D.

The results are consistent with the scheme discussed by Perry et al. (1970) in which eukaryotes from yeast to reptiles had a ribosomal precursor molecule of $2.6\text{-}2.7 \times 10^6$ molecular weight that formed 18S rRNA directly and the larger rRNA through a slightly larger intermediate. Only 20-30 % of the precursor was lost in the process. This pattern has been described in plants (Loening et al., 1969), yeast (Taber and Vincent, 1969), arthropods (Greenberg, 1969; Ringborg et al., 1970) and amphibia (Landesman and Gross, 1969; Loening et al., 1969). Contrary to this general trend, however, it was not possible to detect nascent 18S rRNA prior to the appearance of 26S RNA and the intermediate of 1.8×10^6 D was detected in

even the shortest pulse times. In Ilyanassa, then, the 18S and 26S rRNAs appear simultaneously. It is possible that 1.8×10^6 D RNA is not an intermediate for the processing of 26S RNA. In any event, the two species are processed at the same rate in Ilyanassa.

The results clearly show that the ribosomal precursor molecule is not heat-labile for, when RNA from embryos labeled for periods shorter than 30 min was extracted with hot phenol, no radioactivity was detected coincident with the 18S rRNA absorbance peak where breakdown products of the 26S rRNA would appear. Again, breaks in the larger ribosomal subunit probably result from the in vivo processing from the precursor molecule rather than from the analytical procedures employed here.

In higher organisms, a 4×10^6 D precursor was only 50 % conserved in processing to rRNA via a 2.1×10^6 dalton intermediate (Perry et al., 1970; Weinberg et al., 1967). The Ilyanassa $2.4\text{-}2.5 \times 10^6$ D precursor and 1.8×10^6 D intermediate are consistent with the snail's phylogenetic position.

APPENDIX B

Data Reduction

Many of the laborious and/or repititious calculations were performed by a PDP-8 time-sharing computer under TSS/8.22B (Digital Equipment Corporation) using programs written in Basic.

Acrylamide gel radioactivity profiles were superimposed over the optical density profiles or were converted to percentage distributions and plotted against the relative mobility (RM) of each slice (compared to the 18S rRNA peak). Inasmuch as gel concentrations were not perfectly reproducible between different batches of gels, the RMs of the ribosomal RNAs also varied between runs. Therefore all plots were expanded or contracted so that when the RM of 18S rRNA was 1.00, that of 26S rRNA was 0.62. The observed relative mobility of each slice was multiplied by a factor:

(1.00-0.62)/(1.00-(slice # 26S peak/slice #18S peak)), so that 0.38 relative mobility units separated the rRNA peaks; the curves were then shifted to the right or left by adding a constant: (1.00 - factor), to align the 18S rRNA at RM=1.00 and the 26S rRNA at 0.62. This resulted in a "relative RM," hereinafter referred to as RM, which, although zero or negative for the high molecular weight RNAs, was uniform and comparable for all gels.

Molecular weights (Table B1) were calculated assuming a linear relationship (with negative slope) between relative mobility (or distance migrated) and the logarithm of the molecular weight (Loening, 1969; Bishop et al., 1968). This

interdependence was observed (see Fig. A5, appendix A) with both acrylamide gels and composite, acrylamide-agarose gels even when the upper surface of the gel was concave or stratified (from the separate gelations of agarose and acrylamide), provided neither EDTA nor magnesium were present during the RNA extraction and electrophoresis.

TABLE B1

Molecular weight x 10^{-3} vs. relative mobility (RM). The RM (sum of left column and uppermost row) ranged from -0.6 to 2.9. Molecular weights were calculated from 26S, 18S and 4S standards.

RM	0	.1	.2	.3	.4	.5	.6	.7	.8	.9
-0	4013	4772	5674	6747	8023	9540	11345			
+0	4013	3375	2838	2387	2007	1688	1420	1194	1004	844
1	710	599	506	427	361	305	257	217	183	155
2	131	110	93	79	66	56	47	40	34	29

Radioactivity profiles from embryos at different stages of development or from normal and lobeless embryos could not be exactly compared for two reasons: 1) Two slightly different gel formulations were routinely employed. "Large-pore" gels contained, after electrophoresis until 18S rRNA **had migrated half the gel length**, RNA molecules ranging in size from 4 x 10^4 daltons (D) to 9.5 x 10^6D. When more acrylamide was added to decrease the pore size ("small-pore"), greater resolution resulted (sharper count profiles) but the molecular weight range had decreased to 13.1 x 10^4 D to 3.3-3.4 x 10^6D. 2) Corresponding slices from different gels do not contain identical sets of RNA molecules.

A method was adopted that provided a measure of profile similarity and indicated the reliability of this measure.

After all radioactivity profiles had been plotted on the same relative mobility scale as percentage distributions, six (later seven) obvious regions, or sectors, were selected and the radioactivity in each was summed, thus minimizing slicing imperfections; these sums corresponded to the areas within the sectors. Since RNA migrates according to its molecular weight, corresponding sectors from all profiles therefore contained RNA molecules in the same molecular weight range. Sectors were determined by visual inspection and RM as: A>26S rRNA, B=26S RNA, 26S RNA>C>18S RNA, D=18S RNA, 18S RNA>E>F. The area in each sector was expressed as a percentage of the total so that, taken together, the six sectors represented the size-distribution of RNA molecules synthesized during any stage of development and these distributions could be compared to one another.

Statistical Procedures

An anova (analysis of variance) (Sokal and Rohlf, 1969; Winer, 1962) was used for this comparison. In simplest terms, if the variation between two or more experimental groups, e.g. normal and lobeless embryos, exceeds the variation among replicates within the groups (expressed as the ratio of variances, or F-ratio) by an amount greater than that expected to have arisen by chance, the groups are considered significantly different. Here, the distributions of nascent RNAs (sector areas) are considered as repeated measures on the same group of subjects. For example, the distribution of nascent RNA from 250 stage-5 normal embryos was measured according to six criteria, viz., the fraction of the total

falling into molecular weight ranges (sectors) A, B, C, D, E and F. The statistician (R. Sokal, personal communication) does not regard the values for sectors as independent, randomly chosen variates as they are physically linked in the electrophoretic gel, i.e., replicate gels are not replicates in the statistical sense. Thus the anova was inititally constructed with four factors without replicates; gel replication comprised the fourth factor (stage x normal-lobeless x profile x replication). The four-way anova was then reconstituted into a three-way anova by adding the between-replicates variances into the error terms and the resulting computational expressions are those presented by Winer (1962, p. 342) for a three-factor anova with repeated measures on one factor (developmental stage x normal-lobeless x profile). The sector areas, expressed as percentages, are listed in Table B2 (sector A was subdivided to high molecular weight RNA (HMW) and the ribosomal RNA precursor (PREC)). Since radioactive decay exhibits a Poisson distribution (Tuttle, 1964) in which the variance is proportional to the mean, all sector areas were first transformed to (logarithm of sector DPM)/(logarithm of total DPM on gel) (R. Sokal, personal communication) to meet the requirement in anova, which is based upon the normal distribution, that variances by reasonably homogeneous.

The repeated measures anova is actually a univariate approximation to the considerably more complicated multivariate analysis which would be required for an exact test. Thus the significance of the F-ratios was judged by the conservative test of Greenhouse and Geisser (1959) in which, by re-

ducing the degrees of freedom to the minimum, the calculated F-ratio of the univariate test more closely approximates the F-distribution.

There were two replicates for each group; in some cases, both were on "small-pore" gels (SP,SP) while, in other cases there was one "small-pore" and one "large-pore" replicate (SP,LP). Figure B1 (left panel) demonstrates that, within the molecular weight (relative mobility) range common to both, large pore and small pore gels develop identical RNA distributions. The truncated 18S rRNA peak (RM=1) of the lower (SP) profile illustrates a major problem in slicing gels; had the gel slicer been differently oriented, the radioactivity in these two slices could have appeared in a single slice showing twice the amplitude. The right panel illustrates the reproducibility of RNA distributions on gels of similar formulation.

FIGURE B1-Reproducibility of electrophoregrams. Each profile represents the analysis of RNA extracted from 300 embryos incubated with ^{3}H-uridine at 125 μC/ml for 24 hours. (a) RNA from stage-4 embryos analyzed on "large-pore" (____) and "small-pore" (----) gels. The baseline of the lower curve is displaced by two percentage units. (b) Two different stage-6 RNA preparations, electrophoresed on "small-pore" gels in separate runs, are superimposed.

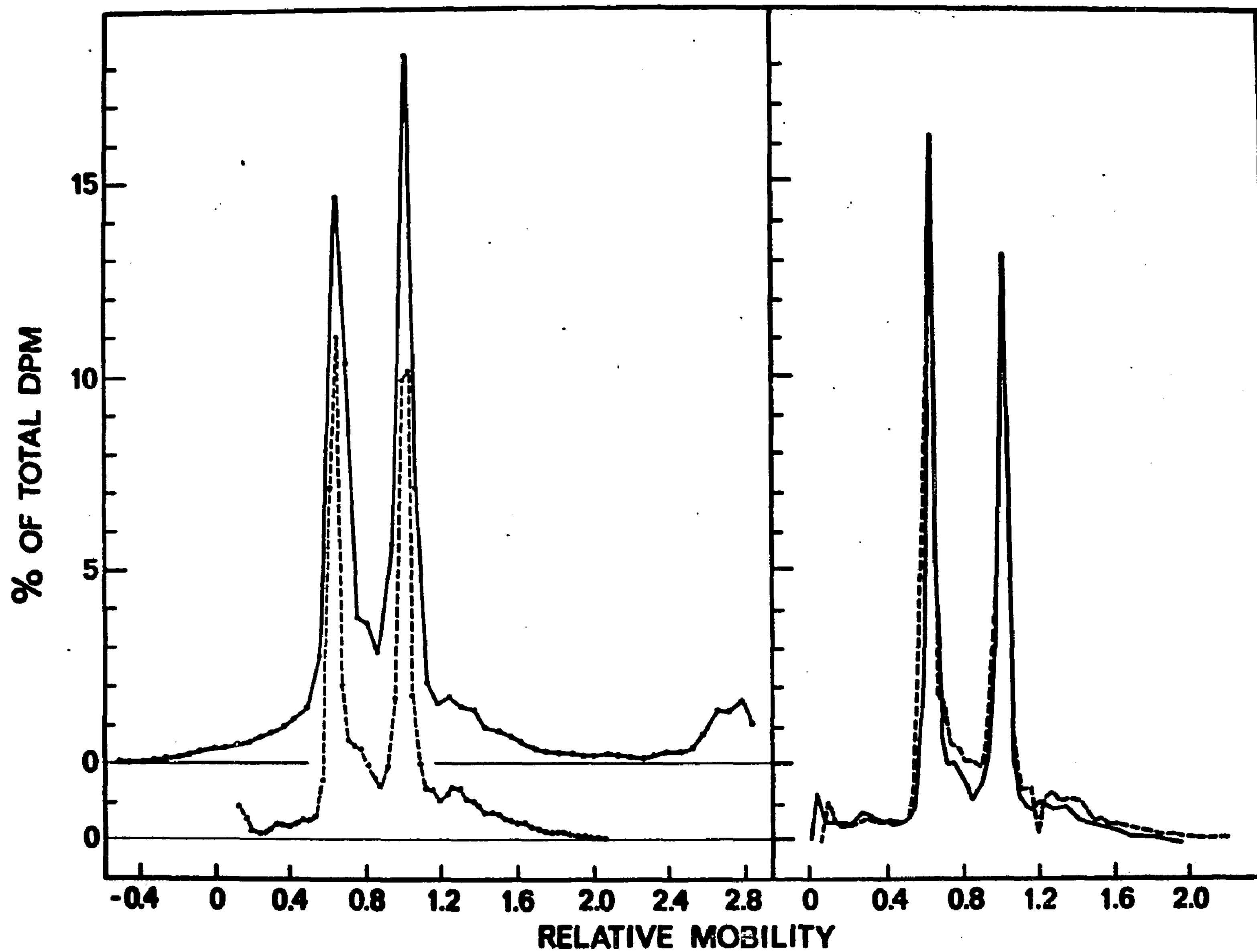

% OF TOTAL DPM
15
10
5
0
0
-0.4
0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
0
0.4
0.8
1.2
1.6
2.0
RELATIVE MOBILITY

TABLE B2

Sector areas (in percent) of normal (N) and lobeless (L) embryo nascent RNA distributions.

STAGE		SECTOR HMW	PREC	B	C	D	E	F
1	N	17.13	4.74	14.78	9.90	22.61	17.28	13.57
		15.06	5.79	13.56	17.74	21.06	15.73	11.06
	L	8.05	3.20	8.70	13.82	12.30	37.06	16.86
		19.93	4.28	8.53	17.30	16.25	21.95	11.76
2	N	3.32	1.57	23.63	12.50	36.84	13.03	9.11
		3.45	1.05	17.78	15.35	36.98	14.52	10.87
	L	5.60	0.92	22.70	15.01	30.41	15.73	9.62
		2.82	0.44	20.15	15.86	29.03	18.23	13.46
3	N	3.55	1.63	27.24	13.02	31.08	14.83	8.66
		3.38	0.98	22.69	12.66	32.46	16.34	11.49
	L	2.90	1.25	15.43	13.95	26.40	24.98	15.09
		7.06	2.98	29.59	12.99	24.99	12.71	9.69
4	N	4.59	1.87	33.97	7.82	37.61	8.72	5.41
		4.99	1.52	28.34	12.29	33.61	12.40	6.85
	L	3.13	1.44	21.99	10.59	32.65	18.74	11.47
		3.49	1.42	20.52	16.55	23.91	20.97	13.14
5	N	5.00	2.54	31.36	16.56	28.00	10.74	5.80
		5.36	2.92	30.65	13.09	32.45	9.85	5.67
	L	3.23	2.01	23.86	11.94	29.91	16.77	12.28
		5.61	2.09	33.00	15.04	23.56	13.45	7.24
6	N	6.65	3.16	33.89	10.04	33.42	8.33	4.51
		4.81	1.68	37.39	10.95	29.03	10.41	5.73
	L	3.56	1.57	22.45	11.86	29.70	21.17	9.69
		4.97	2.27	29.40	14.10	25.53	15.13	8.60

TABLE B3
Transformations of gel sector DPM.

STAGE		SECTOR HMW	PREC	B	C	D	E	F
1	N	.8503	.7414	.8378	.8038	.8739	.8511	.8306
		.8439	.7651	.8352	.8574	.8716	.8475	.8185
	L	.7843	.7052	.7909	.8306	.8206	.9150	.8476
		.8604	.7271	.7869	.8481	.8427	.8687	.8147
2	N	.7780	.7290	.9059	.8644	.9349	.8671	.8438
		.7818	.7044	.8880	.8785	.9355	.8749	.8561
	L	.7961	.6682	.8951	.8658	.9158	.8692	.8344
		.7402	.6044	.8834	.8659	.9099	.8761	.8539
3	N	.7872	.7375	.9171	.8701	.9256	.8784	.8441
		.7844	.7055	.9056	.8684	.9283	.8846	.8622
	L	.7497	.6901	.8679	.8608	.9059	.9019	.8663
		.8270	.7706	.9205	.8668	.9095	.8653	.8476
4	N	.8035	.7461	.9311	.8374	.9376	.8444	.8139
		.8094	.7339	.9198	.8667	.9307	.8673	.8295
	L	.7736	.7227	.9010	.8533	.9269	.8906	.8585
		.7855	.7282	.8988	.8850	.9085	.9001	.8703
5	N	.8135	.7714	.9278	.8881	.9208	.8611	.8228
		.8174	.7795	.9262	.8731	.9298	.8554	.8209
	L	.7823	.7522	.9091	.8652	.9234	.8867	.8669
		.8269	.7677	.9334	.8862	.9132	.8795	.8423
6	N	.8328	.7869	.9333	.8582	.9324	.8467	.8089
		.8100	.7441	.9384	.8615	.9226	.8583	.8210
	L	.7975	.7479	.9093	.8705	.9263	.9057	.8583
		.8172	.7694	.9254	.8807	.9168	.8849	.8506

TABLE B4

Anova summary of all profiles.

Source of Variation	Sum of Squares	Deg. of Freedom	Con-serv. Deg.	Mean Square	F
Between Subjects	0.03315163	23			
A (Stage)	0.02485943	5		4.971886E-3	14.81
B (N-L)	6.618500E-4	1		6.618500E-4	1.97
A x B	3.602982E-3	5		7.205963E-4	2.15
Error Between	4.027367E-3	12		3.356139E-4	
Within Subjects	0.6490059	144			
C (Profile)	0.5371189	6	1	0.08951982	278.40
A x C	0.06514645	30	5	2.171548E-3	6.75**
B x C	0.01509666	6	1	2.516111E-3	7.83*
A x B x C	8.492470E-3	30	5	2.830823E-4	0.88
Error Within	0.0231514	72	12	3.215472E-4	

*P <.05 **P <.01

TABLE B5

Anova summary on profiles prior to gastrulation.

Source of Variation	Sum of Squares	Deg. of Freedom	Con-serv. Deg.	Mean Square	F
Between Subjects	7.172585E-3	7			
A (Stage)	2.186298E-3	1		2.186298E-3	6.91
B (N-L)	3.609180E-3	1		3.609180E-3	11.40
A x B	1.106262E-4	1		1.106262E-4	0.35
Error Between	1.266479E-3	4		3.166199E-4	
Within Subjects	0.2379832	48			
C (Profile)	0.1780877	6	1	0.2968129	69.27
A x C	0.03821898	6	1	6.369829E-3	14.87*
B x C	8.487701E-3	6	1	1.414617E-3	3.30ns
A x B X C	2.903938E-3	6	1	4.839897E-4	1.13
Error Within	0.01028395	24	4	4.284978E-4	

TABLE B6

Anova summary on profiles after gastrulation.

Source of Variation	Sum of Squares	Deg. of Freedom	Con-serv. Deg.	Mean Square	F
Between Subjects	5.893707E-3	15			
A (Stage)	2.588272E-3	3		8.627574E-4	2.50
B (N-L)	1.211166E-4	1		1.211166E-4	0.35
A x B	4.224777E-4	3		1.408259E-4	0.41
Error Between	2.761841E-3	8		3.452301E-4	
Within Subjects	0.4110212	96			
C (Profile)	0.3777418	6	1	0.6295697	234.93
A x C	8.213997E-3	18	3	4.563332E-4	1.70ns
B x C	9.030342E-3	6	1	1.505057E-3	5.62*
A x B x C	3.170967E-3	18	3	1.761648E-4	0.66
Error Within	0.01286316	48	8	2.679825E-4	

ns not significant *P<.05

TABLE B7
Percentage distribution of non-ribosomal nascent RNA.

STAGE		SECTOR HMW	C	E	F
1	N	29.59	17.10	29.86	23.44
		25.27	29.76	26.40	18.57
	L	10.63	18.23	48.89	22.25
		28.10	24.39	30.94	16.57
2	N	8.75	32.93	34.32	23.99
		7.81	34.73	32.86	24.60
	L	12.19	32.66	34.22	20.93
		5.60	31.49	36.19	26.72
3	N	8.85	32.50	37.02	21.62
		7.71	28.86	37.23	26.20
	L	5.09	24.51	43.89	26.50
		16.63	30.60	29.94	22.83
4	N	17.31	29.47	32.85	20.37
		13.66	33.64	33.95	18.74
	L	7.12	24.11	42.66	26.10
		6.44	30.56	38.73	24.27
5	N	13.11	43.46	28.19	15.23
		15.78	38.53	29.00	16.69
	L	7.31	27.00	37.91	27.78
		13.57	36.38	32.54	17.51
6	N	22.51	34.00	28.20	15.28
		15.08	34.31	32.64	17.97
	L	7.70	25.62	45.74	20.95
		11.62	32.94	35.35	20.10

TABLE B8
Anova summary on profiles of non-ribosomal RNA

Source of Variation	Sum of Squares	Deg. of Freedom	Con-serv. Deg.	Mean Square	F
Between Subjects	0.01249313	23			
A (Stage)	9.593964E-3	5		1.918793E-3	12.63
B (N-L)	7.038116E-4	1		7.038116E-4	4.63
A x B	3.719330E-4	5		7.438660E-5	0.49
Error Between	1.823425E-3	12		1.519521E-4	
Within Subjects	0.1225739	72			
C (Profile)	0.08058453	3	1	0.02686151	62.54
A x C	0.01684666	15	5	1.123110E-3	2.61ns
B x C	6.135941E-3	3	1	2.045314E-3	4.76*
A x B x C	3.542900E-3	15	5	2.361933E-4	0.55
Error Within	0.01546288	36	12	4.295243E-4	

ns not significant *$P < .05$

TABLE B9

Log transforms of non-ribosomal sector DPM.

STAGE		HMW	C	E	F
1	N	.8917	.8429	.8925	.8710
		.8815	.8956	.8853	.8550
	L	.8034	.8507	.9372	.8681
		.8867	.8741	.8953	.8396
2	N	.8304	.9227	.9255	.9007
		.8255	.9276	.9238	.9040
	L	.8424	.9162	.9197	.8829
		.7790	.9114	.9221	.8988
3	N	.8360	.9239	.9328	.8964
		.8278	.9165	.9336	.9099
	L	.7808	.8965	.9394	.9022
		.8760	.9181	.9166	.8979
4	N	.8777	.9148	.9226	.8891
		.8648	.9260	.9266	.8862
	L	.8176	.9018	.9412	.9073
		.8175	.9211	.9369	.9058
5	N	.8655	.9448	.9162	.8754
		.8765	.9362	.9172	.8802
	L	.8250	.9124	.9351	.9143
		.8733	.9359	.9288	.8895
6	N	.9005	.9280	.9156	.8747
		.8724	.9279	.9245	.8843
	L	.8367	.9132	.9501	.9004
		.8617	.9287	.9332	.8969

TABLE B10

Anova summaries of nascent RNA distributions of non-ribosomal RNAs a) prior to gastrulation (stages 1 and 2) and b) after gastrulation (stages 3-6).

Source of Variation	Sum of Squares	Deg. of Freedom	Con-serv. Deg.	Mean Square	F
a) Between Subjects	3.373623E-3	7			
A (Stage)	2.147198E-3	1		2.147198E-3	16.49
B (N-L)	6.830692E-4	1		6.830692E-4	5.25
A x B	2.264977E-5	1		2.264977E-5	0.17
Error Between	5.207062E-4	4		1.301765E-4	
Within Subjects	0.04344106	24			
C (Profile)	0.02102113	3	1	7.007043E-3	10.15
A x C	0.01166153	3	1	3.887177E-3	5.63
B x C	1.699448E-3	3	1	5.664825E-4	0.82ns
A x B x C	7.719994E-4	3	1	2.573331E-4	0.37
Error Within	8.286715E-3	12	4	6.905595E-4	
b) Between Subjects	2.490044E-3	15			
A (Stage)	8.158684E-4	3		2.719561E-4	1.67
B (N-L)	1.983643E-4	1		1.983643E-4	1.22
A x B	1.726151E-4	3		5.753835E-5	0.35
Error Between	1.303196E-3	8		1.628995E-4	
Within Subjects	0.07913351	48			
C (Profile)	0.06075001	3	1	0.02025	67.70
A x C	3.998756E-3	9	3	4.443063E-4	1.49
B x C	5.098820E-3	3	1	1.699607E-3	5.68*
A x B x C	2.106667E-3	9	3	2.340741E-4	0.78
Error Within	7.178783E-3	24	8	2.991160E-4	

ns not significant *P < .05

TABLE B11
Percentage distribution of ribosomal RNA

STAGE		SECTOR PREC	B	D
1	N	11.25	35.08	53.67
		14.33	33.55	52.13
	L	13.21	35.95	50.84
		14.71	29.35	55.93
2	N	2.53	38.09	59.39
		1.88	31.85	66.27
	L	1.70	42.02	56.28
		0.88	40.61	58.51
3	N	2.71	45.44	51.85
		1.75	40.43	57.83
	L	2.90	35.82	61.28
		5.17	51.40	43.43
4	N	2.55	46.25	51.21
		2.40	44.65	52.95
	L	2.56	39.22	58.22
		3.10	44.76	52.14
5	N	4.10	50.66	45.24
		4.42	46.43	49.15
	L	3.61	42.77	53.62
		3.57	56.26	50.17
6	N	4.48	48.10	47.42
		2.46	54.90	42.64
	L	2.93	41.79	55.28
		3.97	51.40	44.63

TABLE B12
Anova summary of nascent ribosomal RNA distributions.

Source of Variation	Sum of Squares	Deg. of Freedom	Con-serv. Deg.	Mean Square	F
Between Subjects	0.0132823	23			
A (Stage)	8.351803E-3	5		1.670361E-3	7.87
B (N-L)	6.251335E-4	1		6.251335E-4	2.94
A x B	1.757145E-3	5		3.514290E-4	1.65
Error Between	2.548218E-3	12		2.123515E-4	
Within Subjects	0.5462465	48			
C (Profile)	0.5066786	2	1	0.2533393	1077.58
A x C	0.02971554	10	5	2.971554E-3	12.64***
B x C	2.641678E-4	2	1	1.320839E-4	0.56ns
A x B x C	3.944874E-3	10	5	3.944874E-4	1.68
Error Within	5.642414E-3	24	12	2.351006E-4	

ns not significant ***P<.001

TABLE B13

Log transforms of DPM in ribosomal RNA sectors

STAGE		SECTOR PREC	B	D
1	N	.8000	.9041	.9430
		.8269	.9027	.9419
	L	.8027	.9003	.9340
		.8142	.8812	.9437
2	N	.7525	.9350	.9649
		.7321	.9229	.9723
	L	.6986	.9359	.9575
		.6369	.9309	.9589
3	N	.7624	.9480	.9567
		.7324	.9401	.9638
	L	.7338	.9228	.9632
		.7994	.9549	.9435
4	N	.7611	.9498	.9564
		.7557	.9472	.9584
	L	.7511	.9364	.9633
		.7664	.9459	.9562
5	N	.6851	.8554	.8481
		.8002	.9508	.9545
	L	.7812	.9440	.9589
		.7931	.9643	.9434
6	N	.8042	.9539	.9529
		.7624	.9615	.9453
	L	.7772	.9449	.9626
		.7965	.9580	.9491

TABLE B14

Anova summaries of ribosomal RNA distributions (a) before and (b) after gastrulation.

Source of Variation	Sum of Squares	Deg. of Freedom	Con-serv. Deg.	Mean Square	F
a) Between Subjects	4.839420E-3	7			
A (Stage)	1.609325E-3	1		1.609325E-3	7.04
B (N-L)	1.729250E-3	1		1.729250E-3	7.56
A x B	5.860329E-4	1		5.860329E-4	2.56
Error Between	9.148121E-4	4		2.287030E-4	
Within Subjects	0.1989605	16			
C (Profile)	0.1692336	2	1	0.08461678	344.40
A x C	0.02419496	2	1	0.01209748	49.24**
B x C	1.564026E-3	2	1	7.820129E-4	3.18ns
A x B x C	2.002001E-3	2	1	1.001000E-3	4.07
Error Within	1.965523E-3	8	4	2.456903E-4	
b) Between Subjects	3.511429E-3	15			
A (Stage)	1.811981E-3	3		6.039937E-4	2.96
B (N-L)	9.536743E-7	1		9.536743E-7	0.004
A x B	6.484985E-5	3		2.161662E-5	0.11
Error Between	1.633644E-3	8		2.042055E-4	
Within Subjects	0.3472872	32			
C (Profile)	0.3401785	2	1	0.1700892	740.05
A x C	2.786160E-3	6	3	4.643599E-4	2.02ns
B x C	1.215935E-4	2	1	6.079674E-5	0.26ns
A x B x C	5.230904E-4	6	3	8.718173E-5	0.38
Error Within	3.677368E-3	16	8	2.298355E-4	

ns not significant **$p < .01$

TABLE B15

RNA transcription during the first 24 hours. Five groups of normal (N1-N5) and lobeless (L1-L5) embryos at early cleavage (1-4 cells) were incubated in 250 uC/ml ^{3}H-uridine for 24 hr. After RNA extraction and electrophoresis, the gels were sliced and the radioactivities (total DPM on each gel and DPM/embryo listed below) were plotted as distributions (Figs. 8,9) and subjected to a sector analysis.

a)

# EMBRYOS		TOT. DPM	DPM/EMB.	SECTOR AREA A	B	C	D	E	F
N1	308	49851	162	11.64	7.08	13.15	13.94	25.52	28.67
N2	168	50827	303	11.45	8.59	10.62	11.46	21.58	36.30
N3	295	58205	197	9.49	6.36	11.22	15.91	23.98	33.03
N4	252	53607	213	13.41	7.62	11.21	13.93	28.64	25.19
N5	295	52535	178	7.68	9.32	10.36	7.91	38.18	26.55
L1	330	121781	369	11.79	5.15	13.13	15.52	27.41	27.00
L2	181	59667	330	10.26	6.37	8.27	10.46	25.27	39.37
L3	298	69821	234	12.84	8.48	8.49	14.01	29.41	26.77
L4	218	49121	225	14.29	7.43	8.44	13.17	29.66	27.00
L5	244	75516	309	9.76	9.82	11.84	11.49	30.02	27.06

b)

	TRANSFORMATIONS OF SECTOR DPM A	B	C	D	E	F
N	.7998	.7534	.8111	.8165	.8728	.8837
	.7973	.7704	.7903	.7973	.8566	.9052
	.7825	.7456	.7980	.8303	.8682	.8977
	.8141	.7618	.7975	.8177	.8843	.8724
	.7618	.7797	.7895	.7644	.9106	.8769
L	.8157	.7443	.8250	.8394	.8884	.8871
	.7905	.7467	.7707	.7923	.8735	.9143
	.8149	.7775	.7776	.8228	.8896	.8812
	.8181	.7569	.7688	.8104	.8863	.8776
	.7918	.7923	.8091	.8064	.8923	.8830

c)

Source of Variation	Sum of Squares	Deg. of Freedom	Conserv. Deg.	Mean Square	F
Between Subjects	2.080000E-3	9			
A (N-L)	3.100000E-4	1		3.100000E-4	1.42
Error Between	1.770000E-3	8		2.222222E-4	
Within Subjects	0.1437	50			
B (Profile)	0.12964	5	1	0.02593	77.88
A x B	7.400000E-4	5	1	1.480000E-4	0.44ns
Error Within	0.01332	40	8	3.330000E-4	

ns not significant

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