HIGH-RESOLUTION DENSITY-GRADIENT ANALYSIS OF SEA URCHIN POLYSOMES

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Mature unfertilized sea urchin eggs exhibit minimal incorporation of radioactive protein precursors into 5-10% trichloroacetic acid (TCA)-precipitable material (Nakano and Monroy, 1958; Hultin, 1961; Stafford et al., 1964; MacKintosh and Bell, 1967). One might therefore expect polysomes to be scarce or non-existent in unfertilized eggs. Indeed, recent studies have failed to convincingly demonstrate (by RNase-sensitivity of 260 mμ absorbance and radioactivity) their presence in mature sea urchin eggs before fertilization (Hultin, 1964; Malkin et al., 1964; Stafford et al., 1964). The marked rise in protein synthesis which immediately follows fertilization of sea urchin eggs, however, is not mirrored by obvious changes in optical density profiles (Hultin, 1964; Malkin et al., 1964; Stafford et al., 1964; Stavy and Gross, 1967); nor is polysomal material from older embryos resolved into discrete sedimentation classes (Monroy and Tyler, 1963; Hultin, 1964; Siekevitz et al., 1966). We now report a method for ribosome-polysome isolation which is consistently effective in yielding sedimentation profiles highly resolved by 260 mμ absorbancy and radioactivity, whether at 15 minutes or 15 hours after fertilization. We also show that the isolation of intact sea urchin polysomes is sensitive to KCl concentrations.

MATERIALS AND METHODS

Embryos from Lytechinus variegatus were grown to the desired developmental stage in Millipore-filtered sea water (MSW) containing penicillin

(150 units/ml) and streptomycin (75 μg/ml). They were then washed twice in about 9 volumes (each time) of isotonic NaCl-KCl (Chambers, 1940) at 0-5°C, followed by two washes in the cold homogenization buffer indicated in the text. Packed embryos were homogenized in 1.0 to 2.0 volumes of the homogenization buffer with a motor-driven Teflon pestle and a Duall tube. Thorough homogenization of single-celled embryos generally required 5-12 strokes, while older, multicellular embryos required more than 12.

Embryos to be used for amino acid incorporation studies were washed one or two times in MSW by gentle centrifugation, suspended in 100 ml of MSW and incubated at 25-26°C for 3.5 minutes in the presence of 0.2 μc of uniformly labeled L-phenylalanine-^{14}C (sp. act. 333 mc/mmole, New England Nuclear Corp.) per ml of the incubation medium. The embryo suspension was then diluted 1:1 with 0-5°C isotonic NaCl-KCl, and further washed and homogenized as described above.

Crude homogenates were centrifuged at 15,000x*g* for 15 minutes at 4°C. The supernatants (S-15) were collected and adjusted so that a fixed number of OD_{260} units could be layered onto the gradients. The ionic composition of the sucrose solution of the gradient matched that of the layered sample unless otherwise indicated.

Absorbance at 260 mμ was continuously read from the top to the bottom of the gradient through a 1-cm flow-cell spacer on a Gilford multiple sample absorbance recorder. The flow-cell spacer used was custom-designed to eliminate the absorbance artifacts in the optical path (Schlieren patterns) which occurred when passing our extended sucrose density gradients through the flow-cell spacer supplied by Gilford. Results are corrected for blank absorbancies.

Drop fractions were collected into tubes containing a final concentration of 5% TCA and 90 μg/ml of carrier bovine serum albumin. The fractions were heated at 90-95°C for 15 minutes, chilled, collected onto Whatman GF/C glass filter pads and washed with six 4-ml aliquots of 5% TCA and six 4-ml aliquots of ethanol-ether (3:1). The pads were dried, placed in vials containing 10 ml

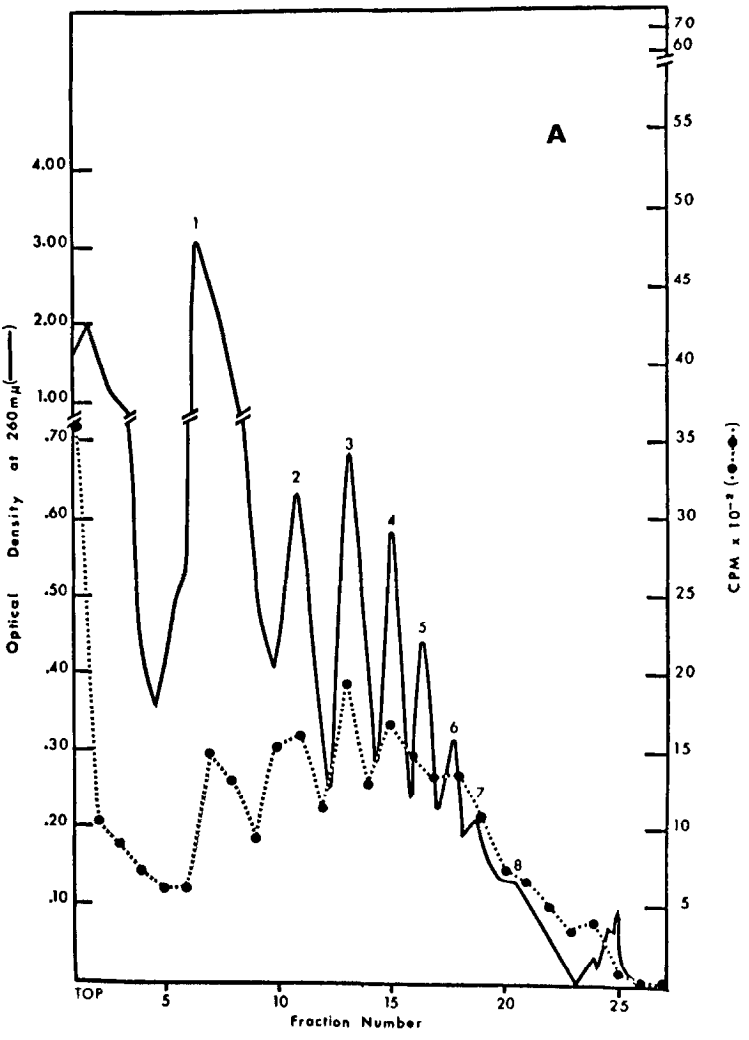
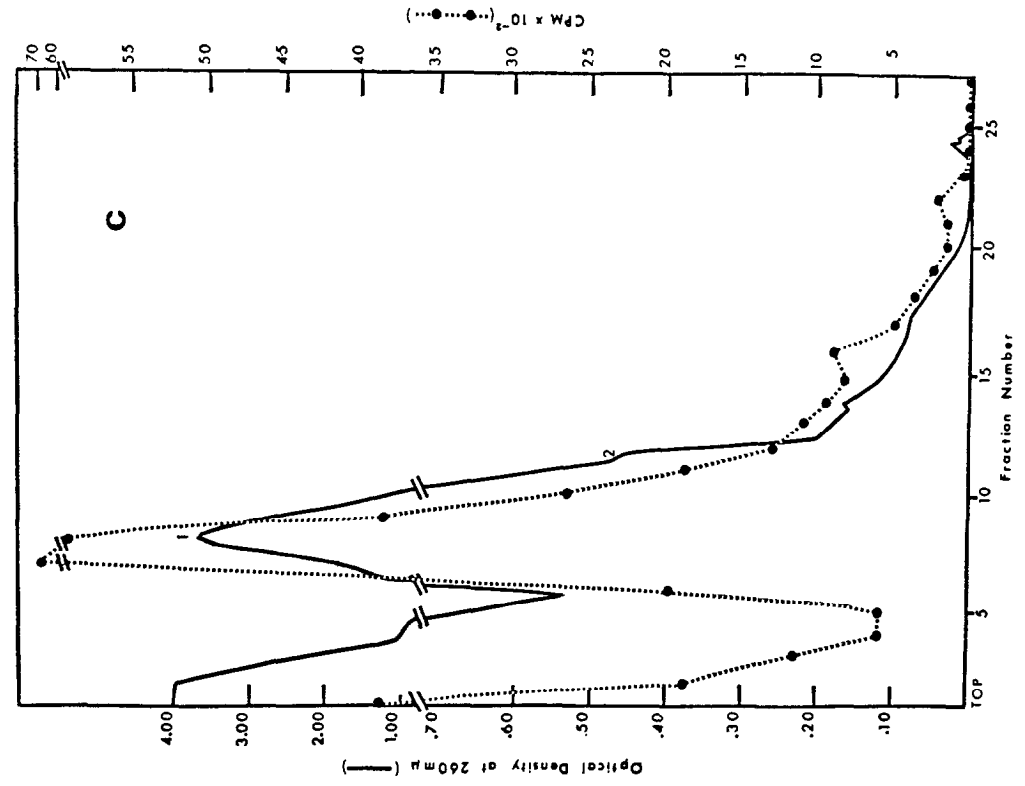
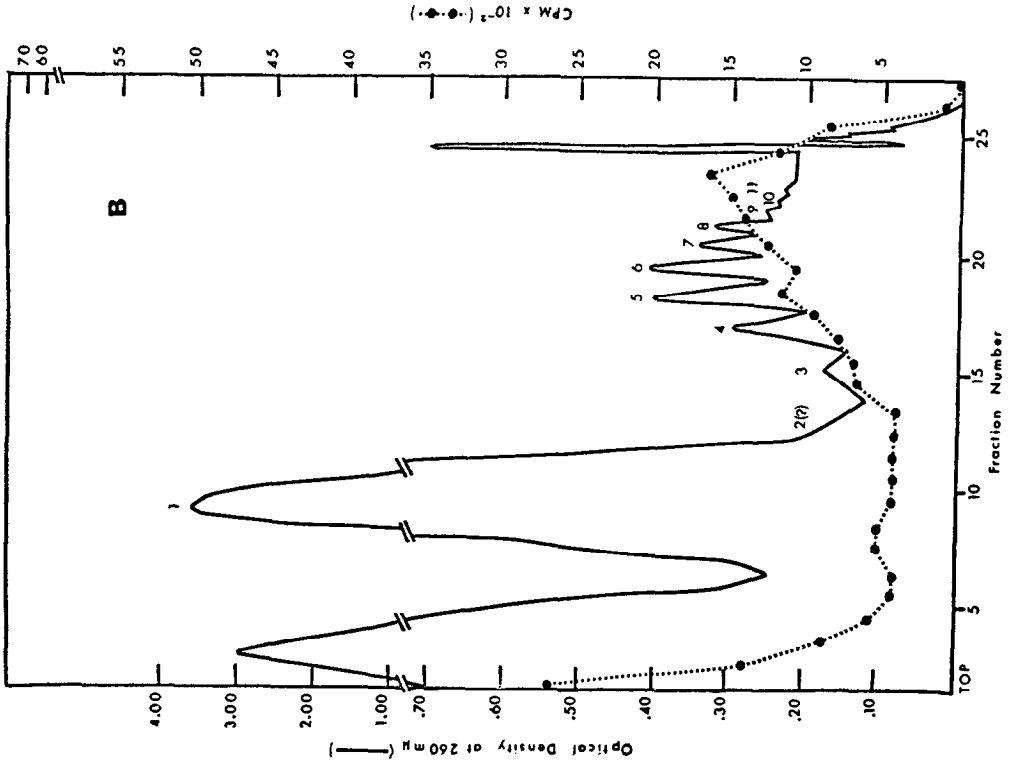


Figure 1. Sucrose density-gradient centrifugation of 15,000xg supernatants extracted from 10-hr-old sea urchin blastulae. Embryos were labeled with L-phenylalanine-^{14}C for 3.5 min, washed and then homogenized in RSB (A) or in buffer B (B,C) as described earlier. The sample in (C) is identical to (B) except that it has been treated with 5 μg/ml (final concentration) of RNase for 30 min at 0-5° C. About 28 OD_{260} units of S-15 were layered onto a 28-ml 50-17% (w/v) buffer B linear sucrose density gradient in a Beckman SW 25.1 rotor tube. The gradient was centrifuged at 24,000 rpm for 3.75 hr at 4° C in a Beckman Model L-2 preparative ultracentrifuge. The gradients were continuously read at 260 mμ and collected as described in the Methods section. Absorption in fractions 24-25 marks interface of the displacing sucrose solution at the bottom of the gradient. Numbers assigned to peaks are arbitrary points of reference.


C
CPM x 10⁻³ (•···•)
Optical Density at 260mμ (——)
Fraction Number
TOP
1
2


B
CPM x 10⁻³ (•-•-•)
Optical Density at 260mμ (——)
Fraction Number
TOP
1
2(?)
3
4
5
6
7
8
9
10
11

of scintillation fluid (5 g PPO and 0.3 g dimethyl-POPOP per liter of toluene, Packard Instruments Co., Inc.) and counted at an average efficiency of 74%.

RESULTS AND DISCUSSION

Use of two different homogenization media in this laboratory revealed that two types of optical density profiles are obtainable from the same culture of sea urchin embryos. The type I pattern (Fig. 1A) is characterized by predominantly slower-sedimenting polysomal peaks and an apparent reduction in the faster-sedimenting peaks relative to the size of the ribosomal peak (peak 1)

Table 1

Homogenization Buffer[1] with Resulting Sedimentation Pattern[2]

Molarity KCl	Molarity $MgCl_2$	Pattern type
0.01	0.0015 (RSB)	I
0.01	0.01	I
0.24	0.01 (Buffer A)	II
0.24	0.0015	II
0.05	0.01	II
0.10	0.01	II
0.43	0.018 (Buffer B)	II

[1]All buffers contained 0.01 M Tris-HCl, pH 7.8 at 25° C.
[2]Pattern type I and II refers to Fig. 1A and 1B, respectively.

The type II pattern (Fig. 1B) is characterized by a reduction in the slower-sedimenting polysomal peaks and an apparent increase in the faster-sedimenting peaks relative to the size of peak 1.

The type I pattern results when blastulae or gastrulae are homogenized in the low ionic strength RSB buffer (0.01 M Tris-HCl, pH 7.8; 0.01 M KCl; 0.0015 M $MgCl_2$) (Penman et al., 1963) whereas the type II pattern is obtained when embryos are homogenized in buffer A (0.01 M Tris-HCl, pH 7.8; 0.24 M KCl; 0.01 M $MgCl_2$) (Stafford et al., 1964). The increased KCl concentration is indispensible for type II isolations (Table 1).

The apparent increase in smaller polysomes coupled with depletion of the heavier UV-absorbing materials (type I pattern) suggests polysomal breakdown during or after homogenization. Results from ^{14}C-phenylalanine incorporation

studies on blastula (Fig. 1A) and gastrula embryos are consistent with this possibility. Whenever type I patterns are obtained the radioactivity is highest in the region of peak 1 and the slower-sedimenting polysomal peaks, and lowest in the area of the faster-sedimenting polysomal peaks. The converse holds true for type II patterns. We emphasize, however, that we have not eliminated the possibility of preferential extraction or loss of classes of ribosomes at some step in the protocol. The amount of ribosome-polysome material recovered in RSB or low KCl (0.01 M) homogenates is in fact considerably less than that obtained from comparable homogenizations with buffer A.

In the studies illustrated in Figs. 1B and 1C, buffer B (see Table 1) was used for the homogenization medium. Buffer B is near-isotonic and minimizes lysis during the handling of cells prior to homogenization. The $KCl/MgCl_2$ ratio was maintained as in buffer A. Results with homogenates from either buffer A or B are essentially the same.

To demonstrate that the polysomes isolated in buffer B are genuine and not aggregates of ribosomes bound by DNA, or smaller polysomes attached to membrane fragments, 15,000xg supernatants from blastulae and gastrulae were treated with 10 μg/ml of deoxyribonuclease (Worthington, electrophoretically purified, certified RNase-free) or with 0.5% (final concentration) sodium deoxycholate (Fisher Scientific Co.) for 30 minutes at 4°C. Neither treatment produced significant change in optical density profiles.

Non-mRNA-dependent ribosomal aggregation has been attributed to high magnesium concentrations (Hamilton and Peterman, 1959; Silman et al., 1965; Breillat and Dickman, 1966). We believe this possibility is unlikely in our studies for four reasons: (1) The heavy, radioactive, UV-absorbing material observed on sucrose gradients is sensitive to mild RNase treatments (Fig. 1C). (2) Although unfertilized eggs contain an abundance of ribosomes (Nemer, 1962; Wilt and Hultin, 1962; Monroy and Tyler, 1963; Hultin, 1964; Stafford et al., 1964; Malkin et al., 1964), never by sucrose density-gradient techniques have we detected UV-absorbing polysomes in a buffer B preparation from unfertil-

ized eggs. In contrast, polysomes are readily detected at 15 minutes after fertilization (Iverson and Cohen, in preparation). (3) It is possible to obtain the type II sedimentation pattern from gastrula embryos at a concentration of 0.0015 M $MgCl_2$. (4) When the ionic composition of 15,000x*g* supernatants from gastrulae homogenized in RSB is changed prior to layering on the sucrose density gradient so that the KCl and $MgCl_2$ concentrations match those of buffer B, the sedimentation pattern (type I) is unchanged.

The polysome pattern designation is at present more qualitative than quantitative. Experiments are in progress to characterize quantitatively both the recovered ribosome-polysome classes and the ribosomal subunits under conditions of varying KCl and $MgCl_2$ concentrations, during and subsequent to initial isolations.

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