RADIOACTIVE LABELING OF RNAs OF SEA URCHIN EGGS DURING OOGENESIS [1]

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In studies with sea urchins it is often of considerable value for the investigator to have available unfertilized eggs in which one or more specific substances have been radioactively labeled. The low metabolic activity of the unfertilized egg and its relative impermeability to many substances of biological interest hamper attempts at direct labeling of the shed eggs. For instance, uptake of phosphate (Whiteley, 1949; Litchfield and Whiteley, 1959; Chambers and Whiteley, 1966; Whiteley and Chambers, 1966) and nucleosides (Piatigorsky and Whiteley, 1965; Mitchison and Cummins, 1966; Siekevitz, Maggio and Catalano, 1966) is very greatly suppressed and that of amino acids (Mitchison and Cummins, 1966; Tyler, Piatigorsky and Ozaki, 1966) and potassium (Tyler and Monroy, 1959) is considerably reduced in unfertilized eggs, as is also the utilization of amino acids until after fertilization (see Monroy, 1965; Tyler and Tyler, 1966b, for reviews).

It has been shown (Tyler, 1949; Tyler and Tyler, 1964a) that sea urchins, after having been induced to spawn by potassium chloride-injection, can produce additional batches of ripe eggs in the laboratory. The yield after ten days to two weeks may often approach the quantity originally obtained. It is, then, possible to label eggs during oogenesis, as has been done in various other animals particularly among the mammals, birds, amphibians, and insects (see Discussion for references), but with the specific advantages that sea urchin eggs provide.

This method was first applied in experiments (Tyler and Hathaway, 1958) to label, with S^{35}, the gelatinous coat (fertilizin) of the egg. Another *in vivo* procedure without preliminary shedding of the eggs consisted of a four-hour incubation of the injected female for labeling of protein (Nakano and Monroy, 1957, 1958; Immers, 1959, 1961; Erb and Maurer, 1962) and of polysaccharide (Immers, 1961). In more recent studies, Gross, Malkin and Hubbard (1965) have again used the longer labeling periods and report effective *in vivo* labeling of RNA with H^3-uridine or P^{32}-phosphate in a period of one week. Also attempts have been made by Holland and Giese (1965) to label the DNA of unfertilized eggs by long periods of maintenance of the injected animals. Only the small oocytes were found to be radioactive in those experiments; but later experiments by Pikó, Tyler and Vinograd (1967) have shown that DNA of ripe eggs can be labeled by the long-term incubation of injected animals in the laboratory.

The present report is for the purpose of demonstrating the effectiveness of the labeling that can be accomplished in sea urchins by the long-term procedure, and

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of providing some of the parameters for incorporation of C^{14}- and H^3-uridine into the RNA of the ripe unfertilized egg. The results of one experiment of this type have been reported elsewhere (Tyler and Tyler, 1966a, p. 644).

Materials and Methods

1) *Injection and care of the animals*

Lytechinus pictus and *Strongylocentrotus purpuratus* females were induced to spawn most, or all, of their mature eggs by injecting about 0.5 ml. of 0.55 *M* potassium chloride into their perivisceral cavity. The sea urchins were kept continually moist with artificial sea water in order to prevent injury by dehydration of the external epidermis or the gills (Tyler and Tyler, 1966a). This was accomplished either by immersing the sea urchin in sea water every few minutes or by allowing the animal to shed in a moist chamber. Injections were done with a small hypodermic needle (30-gauge, $\frac{3}{8}$-inch) carefully inserted through the peristome.

One or two days after being shed, the sea urchins were injected with 0.4 to 0.6 ml. of C^{14}- or H^3-uridine in artificial sea water, at concentrations specified in the individual experiments. The equipment for maintaining the animals was that described by Tyler and Tyler (1966a). The sea urchins were placed, in pairs, in covered, transparent plastic boxes (16 cm. × 35 cm.) containing about 1500 ml. of artificial sea water. They were fed eel grass (*Zostera*). Gentle rocking was provided to allow for aeration and circulation of the water, and constant illumination provided so as to supply additional oxygen by the photosynthetic activity of the eel grass. The constant illumination is thought also to retain the animals in gamete-ripening condition. The temperature in the room was kept below the critical limits for the species but high enough to permit reasonably rapid maturation of the gamets; namely 20° C. for *Lytechinus pictus* and 15° to 17° C. for *Strongylocentrotus purpuratus*. *Lytechinus pictus* proved to be the hardier of the two species. With *Strongylocentrotus purpuratus* there were fewer long-term survivors and thus only a few experiments are reported for this sea urchin. The water was changed approximately every two weeks and new eel grass was added at that time. Algae would grow along the sides of the boxes and this was allowed to remain.

As has been noted elsewhere (Tyler and Tyler, 1966a) sea urchins often die even under, presumably, optimal laboratory conditions present at marine stations. Probably due to the precautions taken in the initial handling of the sea urchins, only few animals died in the present experiments. Sea urchins that survived the first week in the plastic boxes seldom died thereafter even after incubations that lasted longer than one year. It appears, then, that the sea urchins can readily adapt to these conditions if they are not damaged during or after their collection.

2) *Assay of the labeled material in the shed eggs*

At various times after the labeling injection, eggs were obtained from the sea urchins by potassium chloride-induced spawning. The suspensions were screened for oocytes; those containing more than 1% were discarded. This occurred in very few cases.

The assay of radioactivity was performed by the filter-paper procedure of Mans and Novelli (1960) as described previously (Tyler, 1966), the measurements being

made in a Packard Tricarb Scintillation Counter with efficiencies of 50% for C^{14} and 2 to 4% for H^3. For this purpose the eggs were washed thoroughly, the gelatinous coat removed by brief exposure to pH 5-sea water, the suspension adjusted to 10.0 ml. and 6 aliquots placed on strips of filter paper and allowed to dry. Two additional aliquots were added to an equal volume of 0.6 *M* potassium hydroxide, incubated at 37° C. for 18 hours and then dried on filter paper strips. Two of the first six filter papers were assayed directly for radioactivity. Four strips, including the two containing the eggs subjected to alkaline hydrolysis, were processed [ice-cold 5% trichloroacetic acid (TCA)] for incorporation of C^{14}- or H^3-uridine into nucleic acid. Finally, the remaining two filter papers were processed [hot (ca. 90° C.) 5% TCA] for incorporation of label into protein, as described elsewhere (Tyler, 1966).

3) *Extraction of RNA*

RNA was extracted from the unfertilized eggs labeled during oogenesis by a procedure quite similar to that utilized by Gross, Malkin and Hubbard (1965). RNA-labeled unfertilized eggs were homogenized in 0.01 *M* sodium acetate, pH 5.0, followed by low-speed centrifugation as given with the results. The supernatant fraction and washed pellet were made to 2% with respect to sodium dodecyl sulfate, to 0.5% with respect to naphthalene disulfonic acid and to 0.3% with respect to purified bentonite. An equal volume of phenol (Mallinckrodt Chemical Co.), supplemented with 0.1% 8-hydroxyquinoline and saturated with 0.01 *M* sodium acetate buffer (pH 5.0), was added to the fractions of the homogenate. The mixture was mechanically shaken at 4° C. for 20 minutes, centrifuged and the phenol phase removed. The phenol extraction was repeated three times, the aqueous phase set aside and the interfacial gel re-extracted with a 0.01 *M* solution of Tris-hydrochloric acid at pH 7.4. The resulting aqueous phase was added to the original one and the RNA was precipitated with 66% (v/v) ethanol and 0.1 *M* sodium chloride overnight at −20° C. The precipitate was washed in absolute alcohol, dried from ether and redissolved in 0.01 *M* potassium acetate, pH 5.2, containing 10^{-3} *M* magnesium chloride and 15 μg./ml. of DNase (Worthington, electrophoretically pure). The solution was incubated at 4° C. for 30 minutes followed by numerous extractions with buffer-saturated phenol at 4° C. until an interface was no longer visible. The aqueous phase was then precipitated as above, washed with absolute alcohol, with ether, air-dried and redissolved in 0.01 *M* sodium acetate buffer, pH 5.0.

Tests with samples of the labeled material thus obtained showed that it became completely soluble in ice-cold 5% TCA after treatment with RNase (50 μg./ml., 37° C. for 30 minutes) or hydrolysis with potassium hydroxide (0.3 *M*, 37° C. for 18 hours) or hot TCA (5%, 90 to 100 ° C. for 15 minutes). The preparations gave 260mμ/280mμ absorption ratios close to 2.

The sedimentation pattern of the labeled RNA was examined by sucrose density-gradient centrifugation under the conditions specified in the section on Results. Sedimentation coefficients have been assigned as approximate values by analogy with those determined under similar conditions by Slater and Spiegelman (1966a) who employed markers of 23S and 16S RNA from *Bacillus megaterium*. The sedimentation coefficients in the present study have also been calculated by the

method of Martin and Ames (1961) and these values are in close agreement with those of Slater and Spiegelman (1966a).

Results

1) *Retention of radioactivity by Lytechinus pictus after receiving an injection of H^3-uridine*

To test the retention of radioactively labeled uridine injected into the body cavity of the adult female, the following experiment was performed. Two sea

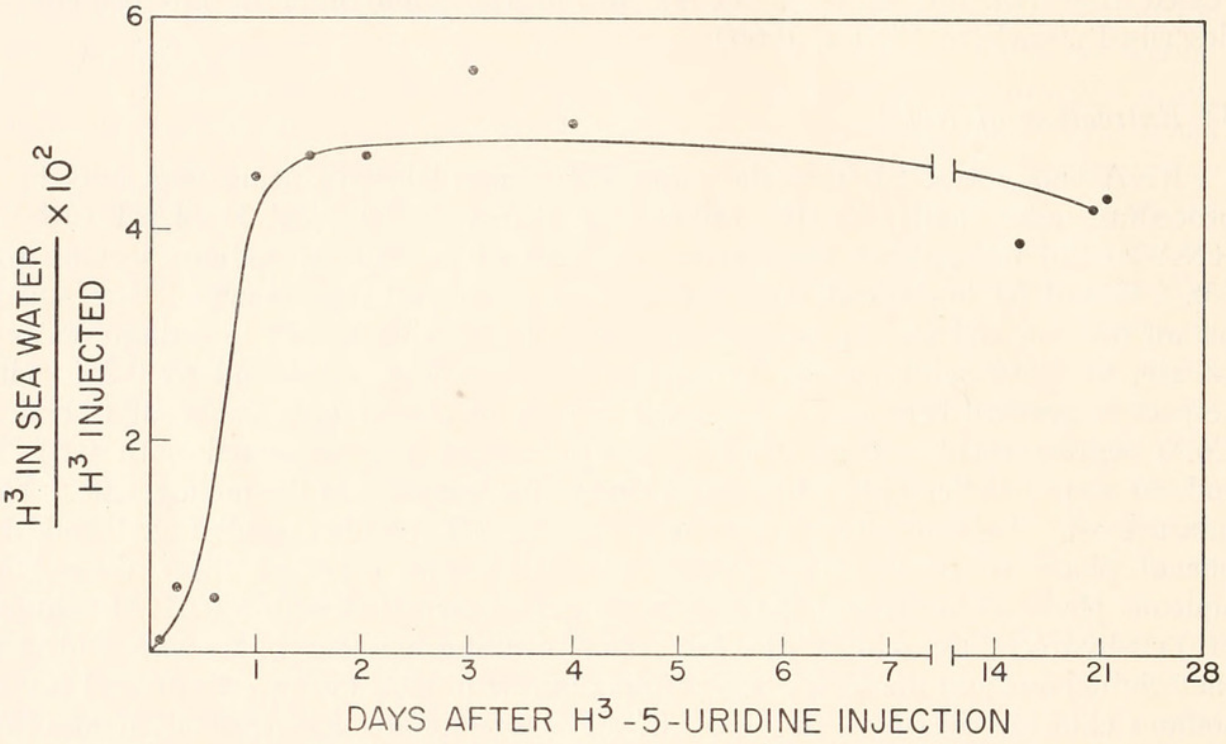

Figure 1. Retention of radioactivity by *Lytechinus pictus* after receiving an injection of H^3-5-uridine. Two females were induced to spawn by injection of potassium chloride and then injected with 125 μc. of H^3-5-uridine (sp. act. 25,000 c/M) in 0.5 ml. artificial sea water. They were maintained in the same plastic box (16 cm. $\times$ 35 cm.) containing 1500 ml. artificial sea water. Duplicate 0.5-ml. aliquots of the sea water were removed at the specified times and assayed for radioactivity.

urchins, weighing approximately 15 grams apiece, were each injected with 125 μc. of H^3-5-uridine in 0.5 ml. artificial sea water. They were placed into the same plastic box containing 1500 ml. of artificial sea water but lacking eel grass. The sea urchins were kept rocking gently, as usual, and duplicate 0.5-ml. samples of the medium were removed and assayed for radioactivity at the indicated times. The sea water was not changed for the three-week duration of the experiment. The results are illustrated in Figure 1.

For the first 12 hours less than 1% of the injected radioactivity appeared in the medium. By 24 hours, however, almost 5% could be accounted for in the sea water. This value did not increase during the next 21 days. A slight decrease was observed in the third week. It is evident then that sea urchins retain most of the nucleoside introduced into their body cavity under these conditions. It is of

interest to note at this time that Erb and Maurer (1962) injected H^3-leucine and H^3-lysine into the coelomic cavity of the sea urchin *Psammechinus miliaris* and observed that only 0.1% of the label escaped from the animal after 4 hours in 45 ml. of sea water.

2) *Uptake and incorporation of C^{14}- and H^3-uridine by maturing oocytes of Lytechinus pictus and Strongylocentrotus purpuratus*

The data obtained from the radioactivity-measurements of the shed, labeled, mature eggs of *Lytechinus pictus* and *Strongylocentrotus purpuratus* are given in Table I. The experiments are grouped according to the type of injected isotope (column 2). They are listed within each group in an order corresponding to increasing time-intervals between injection and collection of the eggs (column 4). The measurements were done in duplicate and both values are reported (columns 6, 7, 8 and 9). The data have been adjusted, for comparative purposes, to relate to 10^2 eggs. The actual number of eggs that the animal shed is given in column 5. The data in column 10 list the percentage of the injected label recovered in the shed eggs. The values in column 11 show the percentage of the total label accumulated by the eggs that is incorporated into ice-cold TCA (5%)-insoluble material. Finally, the sensitivity of the incorporated label to acid and alkaline hydrolysis provides information regarding the types of macromolecules into which the label has become incorporated (columns 12 and 13, respectively). Thus, column 12 lists values for the percentage of the macromolecular radioactivity that is nucleic acid, and column 13 lists values for the minimum percentage of the macromolecular radioactivity that is RNA. The latter are minimum values because the potassium hydroxide digestion leaves some protein (50 to 60%) which may be labeled and might account, along with DNA, for some of the radioactivity shown in column 9.

The values listed in column 10 of the table show that the recovery varied considerably in the different tests. This is not surprising since there are many variables that can affect the results of experiments of this type. For instance, among the factors that can be expected to influence the final yield of radioactivity in the shed eggs are (1) seasonal differences of the animals during the time various tests were made, (2) variations in the extent of spawning both before and after administering the radioactive material, (3) possible individual differences in the rate and number of maturing eggs, and (4) differences in the rate at which the label may be utilized by the various tissues of the animal.

In addition there are differences depending upon the particular isotope employed. This can be seen in Figure 2 where the data for per cent recovery at various times after injection are plotted.

For example, the experiments with C^{14}-2-uridine all gave relatively poor recovery. Possibly this may be due to the lower specific radioactivity of C^{14}- than of H^3-uridine, along with saturation of the oocyte's uptake-sites at relatively low concentrations of exogenous uridine. Such saturation of uptake-sites at low concentrations has been found to be true for fertilized sea urchin eggs (Piatigorsky and Whiteley, 1965). The present results with C^{14}-uridine are, then, interpretable in some manner without recourse to the improbable assumption that the C^{14}-labeled uridine is accumulated by the oocyte, and is incorporated into nucleic acid, less readily than the H^3-uridine. Likewise the differences in per cent recovery obtained

TABLE I

Uptake and incorporation of C^{14}- and H^3-uridine by maturing oocytes of the sea urchins *Lytechinus pictus* and *Strongylocentrotus purpuratus* during long-term *in vivo* labeling of spawned females. Each animal received only one injection and was maintained under the conditions specified in Materials and Methods

(1) Expt. No.*	(2) Isotope (c/M)	(3) μc. injected	(4) Days labeled	(5) No. of eggs shed	(6) (7) (8) (9) cpm per 10^2 eggs**				(10) % recovery of injected label	(11) % label in macromolecules***	(12) % label in nucleic acids relative to label in macromolecules****	(13) Minimum % label in RNA relative to label in macromolecules*****
					No TCA	Cold TCA	Hot TCA	KOH				
1	C^{14}-2-uridine (25.2)	10	3	5.0×10^4	231 256	34 33	13 14	—	1.10	14	60	—
2	C^{14}-2-uridine (25.2)	10	8	8.0×10^5	20 20	8.7 8.6	—	—	1.47	43	—	—
3	C^{14}-2-uridine (25.2)	10	9	1.5×10^5	7.9 12	1.4 1.9	—	—	0.13	17	—	—
4	C^{14}-2-uridine (25.2)	10	15	3.0×10^5	49 48	26 28	14 18	—	1.32	56	41	—
5	C^{14}-2-uridine (25.2)	10	20	8.0×10^5	12 12	3.0 3.1	0.3 0.3	—	0.84	25	90	—
6	C^{14}-2-uridine (25.2)	10	24	1.3×10^5	7.0 6.8	1.1 1.1	0.2 0.1	—	0.09	16	86	—
7	C^{14}-2-uridine (25.2)	10	28	8.2×10^4	28 30	4.2 3.7	0.3 0.4	—	0.21	14	91	—
8	H^3-6-uridine (6550)	100	7	3.8×10^4	230 225	77 76	12 10	—	2.00	34	86	—
9	H^3-6-uridine (6550)	100	9	4.6×10^4	17 13	10 8.0	2.1 2.4	—	0.16	60	75	—
10	H^3-6-uridine (6550)	100	10	8.8×10^4	439 418	268 302	25 18	—	8.57	67	92	—

* Experiments 1–22, *Lytechinus pictus*; experiments 23–28, *Strongylocentrotus purpuratus*.

** 0.005 or 0.01 of the shed eggs were assayed for each determination. The values represent the cpm's left on the filter papers after the indicated treatments as described under Materials and Methods (No TCA = total uptake; Cold TCA = protein and nucleic acid; Hot TCA = protein; KOH = DNA and some protein).

*** $\left[\frac{\text{column (7)}}{\text{column (6)}}\right] \times 100.$

**** $\left[\frac{\text{column (7)} - \text{column (8)}}{\text{column (7)}}\right] \times 100.$

***** $\left[\frac{\text{column (7)} - \text{column (9)}}{\text{column (7)}}\right] \times 100 \times \text{column (12)}.$

****** Uniformly labeled H^3-uridine (experiments 23–28).

TABLE 1—(*Continued*)

(1) Expt. No.*	(2) Isotope (c/M)	(3) μc. injected	(4) Days labeled	(5) No. of eggs shed	(6) cpm per 10^2 eggs** No TCA	(7) cpm per 10^2 eggs** Cold TCA	(8) cpm per 10^2 eggs** Hot TCA	(9) cpm per 10^2 eggs** KOH	(10) % recovery of injected label	(11) % label in macromolecules***	(12) % label in nucleic acids relative to label in macromolecules****	(13) Minimum % label in RNA relative to label in macromolecules*****
11	H^3-6-uridine (6550)	100	13	1.5×10^5	132 107	51 59	19 20	—	4.06	46	65	—
12	H^3-6-uridine (6550)	150	15	5.0×10^5	210 227	144 148	110 89	—	16.5	67	32	—
13	H^3-6-uridine (6550)	100	22	8.0×10^4	5.2 5.1	3.1 4.3	1.9 2.9	—	2.35	72	35	—
14	H^3-6-uridine (6550)	150	28	3.0×10^5	434 427	389 408	14 11	5.5 4.3	19.6	93	97	96
15	H^3-5-uridine (25,000)	150	25	7.7×10^5	193 192	143 141	9.3 9.0	4.8 6.4	22.5	74	94	90
16	H^3-5-uridine (25,000)	120	33	2.0×10^5	592 703	691 649	20 24	14 10	24.5	103	97	95
17	H^3-5-uridine (25,000)	150	34	5.3×10^5	323 279	318 387	19 21	10 10	24.4	117	94	91
18	H^3-5-uridine (20,000)	120	36	6.4×10^5	76 80	68 70	5.8 7.4	2.0 2.8	9.44	88	90	87
19	H^3-5-uridine (20,000)	120	40	2.3×10^5	741 731	575 571	36 35	13 14	32.0	78	94	92
20	H^3-5-uridine (20,000)	120	61	2.0×10^5	321 319	293 254	28 25	—	12.1	85	90	—
21	H^3-5-uridine (20,000)	125	64	6.4×10^5	192 196	188 149	13 16	9.4 9.4	23.6	87	91	86
22	H^3-5-uridine (20,000)	120	89	6.4×10^5	76 80	67 70	5.7 7.4	2.0 2.8	9.44	88	90	87
23	H^3-uridine****** (20,000)	100	13	2.0×10^5	243 230	156 154	19 20	—	10.7	66	87	—
24	H^3-uridine (20,000)	125	14	4.9×10^6	2.9 2.6	2.0 2.1	0.6 0.6	—	2.40	75	71	—
25	H^3-uridine (20,000)	125	14	1.7×10^6	4.4 3.8	2.6 3.4	1.0 0.9	—	1.28	73	68	—
26	H^3-uridine (20,000)	125	14	7.4×10^5	25 27	21 21	4.7 5.0	—	3.51	81	77	—
27	H^3-uridine (20,000)	100	16	1.5×10^6	0.6 0.6	1.0 0.3	0.1 0.1	—	0.20	108	85	—
28	H^3-uridine (20,000)	100	16	2.3×10^5	45 44	36 33	4.7 5.2	—	2.29	78	86	—

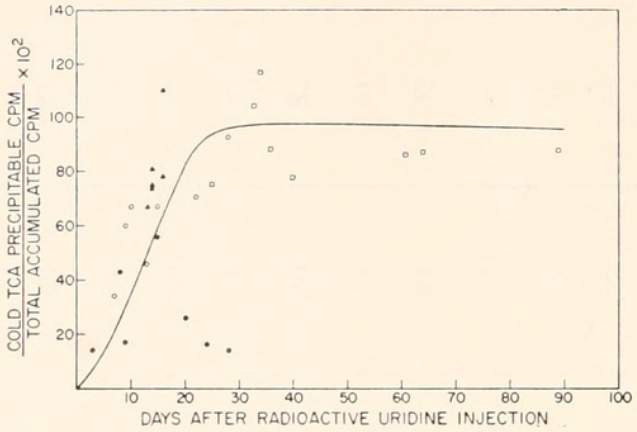

FIGURE 2. Percentage recovery of injected isotope found in mature eggs of *Strongylocentrotus purpuratus* and *Lytechinus pictus* labeled during oogenesis. Sea urchins were spawned with potassium chloride, injected with labeled uridine and incubated at 20° C. as given in Materials and Methods. At the designated time after the injection of radioactive uridine, the mature eggs were shed with potassium chloride and assayed for total radioactivity by scintillation counting. Each point on the figure specifies the percentage of radioactivity, relative to that administered to the animal, that was recovered in the shed eggs. •—*L. pictus*, C^{14}-2-uridine. ○—*L. pictus*, H^3-6-uridine. □—*L. pictus*, H^3-5-uridine. ▲—*S. purpuratus*, H^3-uniformly labeled-uridine.

with the two H^3-uridines (labeled in the 5 or the 6 positions) that were employed in the experiments with *L. pictus* may be similarly interpreted.

When expressed in terms of the amounts of uridine taken up by the oocytes in the various experiments the values for uptake of C^{14}-uridine are as high, or higher, than those obtained with H^3-uridine. For *L. pictus* values for the uptake of C^{14}-2-uridine per 10^2 eggs range from 0.25 (experiment 6) to 8.73 (experiment 1) $\mu\mu$moles while those of H^3-6-uridine are 0.01 (experiment 13) to 0.99 (experiments

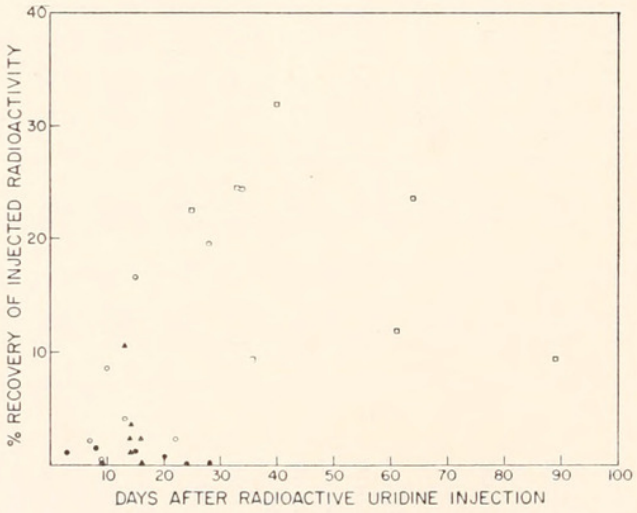

FIGURE 3. Percentage of radioactivity precipitable with ice-cold trichloroacetic acid in the shed eggs of *Strongylocentrotus purpuratus* and *Lytechinus pictus* labeled during oogenesis. The percentage of radioactivity precipitable with ice-cold 5% trichloroacetic acid, relative to the total radioactivity accumulated by the shed eggs, has been plotted from the values listed in Table I. •—*L. pictus*, C^{14}-2-uridine. ○—*L. pictus*, H^3-6-uridine. □—*L. pictus*, H^3-5-uridine. ▲—*S. purpuratus*, H^3-uniformly labeled-uridine.

10 and 14) $\mu\mu$moles and those for H^3-5-uridine are 0.05 (experiments 18 and 22) to 0.39 (experiment 16) $\mu\mu$moles.

Overall, the data for uptake as a function of time after injection show progressive increase during a period of one month. For the experiments of one month or longer there is an average recovery of some 20% of the injected material.

Values for the per cent of the total radioactivity present in the shed eggs that is incorporated into cold-acid-insoluble material are plotted in Figure 3. A plateau of about 95% is reached one month after injection.

Hence, even though these parameters are strongly influenced by factors, mentioned above, that are difficult to control, it is feasible to estimate the order of magnitude of uptake and incorporation of uridine that may be expected to appear in the eggs when sea urchins are maintained for various lengths of time under these conditions. From the present results it appears that the minimum time necessary to achieve maximum labeling of unfertilized eggs is approximately one month, if only one injection of labeled uridine is given to the animal.

The ratios of the radioactivity precipitable with cold 5% TCA, after acid and alkaline hydrolysis of the labeled eggs, to that precipitable before hydrolysis, give an index of the proportion of label that has become incorporated into total nucleic acid and RNA, respectively. Hot acid (5% TCA, 90 to 100° C. for 15 minutes) will hydrolyze nucleic acid but not protein, while dilute alkali (0.3 *M* potassium hydroxide at 37° C. for 18 hours) will degrade RNA and some protein but not DNA (Davidson, 1965). The data in column 12 of Table I show that much of the labeled uridine was incorporated into nucleic acid. The precipitable radioactivity, however, was not completely removed by the hot acid hydrolysis, indicating that some label has become incorporated into protein. The degree of specificity of incorporation of the label into nucleic acid was different when the injected uridine was labeled in different positions of the molecule. The average percentages of labeled material sensitive to acid hydrolysis, when the precursors were C^{14}-2-uridine (experiments 1–7), H^3-6-uridine (experiments 8–14), H^3-5-uridine (experiments 15–22) or uniformly labeled H^3-uridine (experiments 23–28) were 74%, 69%, 93% and 79%, respectively. Thus H^3-5-uridine gave the highest specific incorporation into nucleic acid.

As noted above, column 13 of Table I lists values for the percentage of the label of the macromolecules that is in RNA. It is evident from these figures that the radioactivity of the H^3-5-uridine is incorporated mostly (about 90%) into RNA.

3) *Sedimentation pattern of the RNA labeled during maturation of Lytechinus pictus oocytes*

The radioactive RNA of the ripe eggs that had been labeled for 33 days during oogenesis was phenol-extracted from homogenates, and fractions thereof, and analyzed by sucrose density-gradient centrifugation. Figure 4 shows the sedimentation pattern of the labeled RNA obtained from the 10,000 *g* supernatant fraction (Fig. 4A) and from the corresponding pellet (Fig. 4B).

The solid lines of the figures trace the absorbancies at 260 mμ of the successive fractions of the sucrose gradients and show the three predominant species of RNA with their characteristic peaks at approximately 28S, 18S and 4S. The dashed

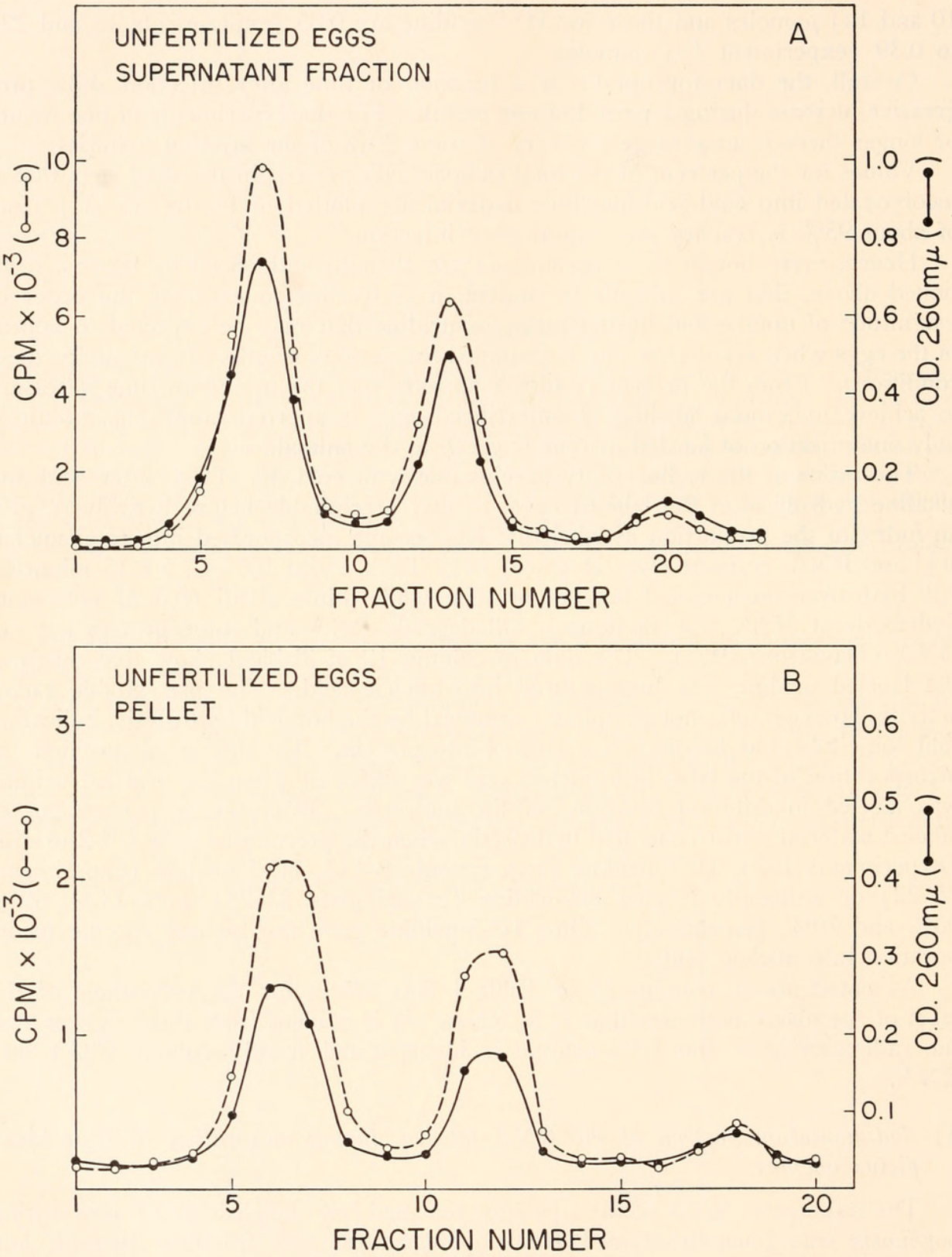

FIGURE 4. Sedimentation pattern of RNA from the supernatant fraction (A) and the mitochondrial pellet (B) of an homogenate of RNA-labeled unfertilized eggs of *Lytechinus pictus*. A spawned female was injected with 150 μc. of H^3-5-uridine (sp. act. 25,000 c/M) and shed 33 days later (experiment 16 of Table I). The labeled unfertilized eggs were washed several times by centrifugation in 0.55 *M* KCl, homogenized in 3 volumes of 0.01 *M* Na acetate buffer at pH 5.0, centrifuged at 10,000 *g* for 10 minutes and the supernatant fraction set aside at 0° C. The pellet was washed twice by centrifugation with 20 to 30 volumes of homogenization

lines give the values for the radioactivity of the same fractions and show a similar pattern. Both the supernatant fraction and the pellet contain the same types of RNA and in approximately the same relative amounts. In addition to the main components there are smaller amounts of heterogeneously sedimenting labeled RNA that appear in these profiles, as well as in others from extracts of whole homogenates. These are evident in the regions of the graphs outside the areas covered by the three main components. Presumably this represents messenger RNA which may be present also in the areas covered by the principal RNA components.

The distribution of label among the various RNAs has been estimated by a method described by Girard, Latham, Penman and Darnell (1965) which involves the assumption that the areas under the 28S and 18S regions include heterogeneously sedimenting RNA at the same level as outside these regions. Thus, the total amount of messenger RNA is obtained by a summation of the radioactivity under a baseline extending to the 4S region. The baseline is drawn at a level corresponding to the average radioactivity outside the 28S and 18S regions. Subtraction of the appropriate values from the cpm's in the 28S and 18S regions gives the amounts of label assigned to the ribosomal RNAs. This procedure gives the following percentages of label in the various types of RNA for the experiment shown in Figure 4:

28S = 49% ; 18S = 29% ; heterogeneous = 15% ; 4S = 6% (Supernatant)
28S = 42% ; 18S = 28% ; heterogeneous = 21% ; 4S = 8% (Pellet)
28S = 48% ; 18S = 29% ; heterogeneous = 16% ; 4S = 7% (Total)

In another test of this type in which the RNA was extracted from a whole homogenate of eggs, labeled with H^3-5-uridine for 64 days (experiment 21 of Table I), the distribution of radioactivity was:

28S = 48% ; 18S = 27% ; heterogeneous = 21% ; 4S = 5% (Total)

It can be concluded, then, that most of the label incorporated into RNA by the oocytes resides in the two species of ribosomal RNA. Much less radioactivity is incorporated into transfer RNA and an upper limit of 10 to 20% can be placed on the incorporation of H^3-uridine into heterogeneously sedimenting messenger RNA.

Discussion

The results of this study have shown that the RNA of ripe eggs of sea urchins can be labeled, at high specific activity, by maintaining the animals in relatively small volumes of sea water for prolonged periods after a single injection of radioactive nucleoside. About 95% of the injected isotope is retained by the sea urchin. From the measurements with animals starting one month after injection, an average of 20% with a maximum of 32% was recovered in the eggs. In most of the

buffer, all supernatant fractions combined and the pellet resuspended in about 40 volumes of buffer. The preparations were phenol-extracted at 4° C. and treated with DNase as described in Materials and Methods. Samples (0.3 ml.) of the labeled extracts were centrifuged through a linear 5 to 20% sucrose density-gradient (in 0.01 *M* Na acetate and 0.1 *M* NaCl at pH 5.0) at 37,000 rpm for 5 hours at 5 to 10° C. Three-drop (about 0.20-ml.) fractions were collected after bottom puncture of the centrifuge tube. These were diluted with an equal volume of distilled water and the 260 mμ absorption was determined in each fraction. Measurements of radioactivity were then made on the same samples by scintillation counting as given in Materials and Methods .

experiments, almost all of the label appeared in macromolecules, principally RNA. The isotope which gave the most consistently high recovery and specific incorporation into RNA was H^3-5-uridine. The shortest time for optimum RNA-labeling of unfertilized eggs was approximately one month under the present conditions.

In eggs obtained at various times less than one month after injection proportionately more of the label was found in low molecular weight (acid-soluble) materials. Evidently, then, the injected uridine can be retained by the eggs in some form in which it can be later incorporated into RNA. If the situation is analogous to that in fertilized eggs, as explored in the experiments of Piatigorsky and Whiteley (1965), it may be concluded that the uridine is stored as nucleoside triphosphates.

As noted in the introduction, *in vivo* incorporation of radioactive materials into oocytes has been accomplished in many different types of animals, among them being mammals (Sirlin and Edwards, 1959; Rudkin and Griech, 1962), chickens (Hevesy and Hahn, 1938; Chargaff, 1942; Patterson, 1961), amphibians (Ficq, 1955, 1961, 1966; Brachet and Ficq, 1956; Gall and Callan, 1962; Davidson, Allfrey and Mirsky, 1964; Brown and Littna, 1964a, 1964b; Davidson, Crippa, Kramer and Mirsky, 1966), insects (Sirlin and Jacob, 1960; Favard-Séréno and Durand, 1963a, 1963b; Bier, 1963; Zalokar, 1965) and sea urchins (Tyler and Hathaway, 1958; Gross, Malkin and Hubbard, 1965; Holland and Giese, 1965; Pikó, Tyler and Vinograd, 1967). In some organisms, notably chickens and insects (see Tyler, 1955, and Williams, 1965, for reviews), growth of the oocyte is associated with the accumulation of materials synthesized in other cells of the body. This may occur to some extent in all animals. However, at least for amphibians (Izawa, Allfrey and Mirsky, 1963), sea urchins (Piatigorsky, Ozaki and Tyler, 1967), and marine polychaete worms (Tweedell, 1966) evidence has been provided that immature oocytes isolated from the ovary are capable of intense RNA and protein synthesis. It is known also that mature sea urchin eggs while in the ovary incorporate little, if any, labeled precursors into RNA (Immers, 1961; Ficq, 1964; Gross, Malkin and Hubbard, 1965). Thus, the labeled RNA of the shed eggs in the present experiments can be assumed to have been synthesized primarily by the oocytes themselves during oogenesis.

In vivo RNA-labeling experiments (Brown and Littna, 1964b; Davidson, Allfrey and Mirsky, 1964) with the toad *Xenopus laevis* have shown that growing oocytes synthesize predominantly 28S and 18S ribosomal RNA. Much less 4S RNA was synthesized by oocytes in their tests. The most active time of RNA synthesis was found to occur during the lampbrush phase of oocyte growth (Davidson, Allfrey and Mirsky, 1964). Some non-ribosomal RNA that sediments heterogeneously in a sucrose density-gradient was also shown to be synthesized during oogenesis (Brown and Littna, 1964a; Davidson, Allfrey and Mirsky, 1964). The labeled ribosomal and heterogeneously sedimenting RNAs were conserved throughout oogenesis and during early development of the fertilized egg.

Davidson, Crippa, Kramer and Mirsky (1966) showed that RNA extracted from lampbrush stage oocytes of *Xenopus* possesses considerable capacity to stimulate the *in vitro* incorporation of labeled amino acids into protein. Hybridization studies indicated that about 1.5% of homologous DNA could be bound with RNA, labeled *in vivo,* extracted from lampbrush phase oocytes. Furthermore, since un-

labeled RNA from later stage, mature oocytes competed with the labeled RNA from lampbrush stage oocytes for hybridization with homologous DNA, it was concluded that the RNA synthesized throughout oogenesis is conserved and sequestered in the mature oocyte.

Gross, Malkin and Hubbard (1965) investigated *in vivo* RNA synthesis by oocytes of the sea urchin *Arbacia punctulata* during their final week of maturation. They showed that in sea urchins, too, growing oocytes synthesize primarily 28S and 18S ribosomal RNA and that the labeled RNAs are preserved in the mature egg. Apart from labeled ribosomal RNA, Gross, Malkin and Hubbard (1965) showed that some 4S RNA becomes labeled during oogenesis. In addition they found small quantities of labeled RNA of higher specific radioactivity than the ribosomal RNA. This labeled RNA sedimented heterogeneously in a sucrose density-gradient and was eluted from a methylated albumin-kieselguhr column at higher ionic strength than was the labeled ribosomal RNA. Furthermore, about 1.5% of the labeled RNA phenol-extracted from unfertilized eggs hybridized with homologous DNA even in the presence of a 350% excess of non-radioactive ribosomal RNA.

The present experiments, as well as others to be presented elsewhere (Piatigorsky, in preparation), are in accord with those cited above with respect to the relatively larger amounts of 28S and 18S RNA than of 4S and heterogeneously sedimenting RNAs that are made by the growing oocytes. These labeled RNAs are conserved in mature unfertilized eggs for prolonged periods of time. The intense labeling of the oocyte nucleolus with labeled RNA-precursors in sea urchins (Ficq, 1964; Piatigorsky, Ozaki and Tyler, 1967), starfish (Ficq, 1953, 1955; Vincent, 1954), amphibians, Ficq, 1961, 1964; Ozban, Tandler and Sirlin, 1964), polychaete worms (Tweedell, 1966) and some insects (Zalokar, 1965) is consistent with large quantities of ribosomal RNA being synthetized by the nucleolus (Perry, 1965) of the oocyte.

The present tests show that the incorporation of H^3-uridine into heterogeneously sedimenting RNA does not exceed one-tenth to one-fifth of the total. This value is based on the assumption that labeled messenger RNAs are present in the 28S and 18S regions at the same level as outside these regions. Gross, Malkin and Hubbard (1965) utilized a comparable procedure to estimate a maximum value of label incorporated into heterogeneously sedimenting RNA during the final stages of oogenesis in sea urchins. Their determinations indicated that 10 to 15% of the total label sedimented heterogeneously. These percentages of label in the various types of RNA do not necessarily reflect mass ratios since consideration of possible specific radioactivity differences have been neglected. Nevertheless, it would seem that the 10 to 20% of heterogeneously sedimenting radioactivity in the extracted RNAs would easily account for the 4 to 5% of the total RNA of unfertilized eggs possessing template potential with respect to the *in vitro* incorporation of labeled amino acids into protein (Slater and Spiegelman, 1966b).

Summary

The present experiments provide data on the results of labeling the RNA of sea urchin eggs during oogenesis, by injection of C^{14}- and H^3-uridine into the perivisceral cavity of previously spawned females. Not more than 5% of the label was

found in the surrounding sea water during the first three weeks after injection. Mature eggs were obtained from animals kept for various periods of time extending to three months after injection. Optimum labeling of the RNA generally occurred at one month, at which time the eggs contained on the average some 20% of the injected label of which 95%, on the average, was in the form of macromolecules. Additional assessment, in eight of the 28 experiments of the percentage of the cold-acid-precipitable label that was in RNA gave minimum values ranging from 86 to 96%. Sucrose density-gradient centrifugation profiles of the extracted RNA showed the label to be mostly (70 to 80%) ribosomal, with about 1.5 times as much 28S as 18S RNA, and about 5 to 10% transfer RNA (4S). The heterogeneously sedimenting labeled RNA, possibly messenger RNA, would amount to an upper limit of 10 to 20% of the total.

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