Amino Acid Incorporation by Cleaving Ova of *Arbacia punctulata*

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Introduction

Asters, prespindle organelles, and the mitotic apparatus (MA) of *Arbacia punctulata* ova do not incorporate appreciable amounts of amino acids during early cleavage stages (Mazia 1961, Kane 1967, Wilt *et al.* 1967, and Rinaldi 1967). Evidently, these microscopically visible organelles, during early cleavage stages, obtain their proteins in a preformed state from the mature ovum and are not necessarily dependent on new protein synthesis following cleavage stimulation. The experiments of Went (1959) support this perspective since extracts of unfertilized sea urchin ova exhibited at least one and/or more antigens of the MA. Conversely, MA extracts produced only antigens which were present in the unstimulated ovum. Kane (1967) found sufficient amounts of protein, isolated with 0.6 M KCl comprising the microtubules and fibers of the MA, in the unfertilized ova to support early cleavage stages. Wilt, Sakai, and Mazia (1967) discovered that 10% of the labeled amino acids representing new protein synthesis in early cleavage to be localized in proteins isolated from the MA.

In a previous investigation there was noted (Rinaldi 1967) a low incorporation of amino acids in both anucleate and nucleate egg fragments by the asters, prespindle organelles, and the MA. The present investigation results from improvements on the histological techniques allowing a more precise establishment for the locus of proteins synthesized within the cell. Thin 1μ and 2μ sections were used to enhance the coincidence between cytological details in the cells and recording of the short-pathed β rays (average path is 1μ) from the tritium labeled amino acid. Second, microphotographic techniques permitted more precise determinations of the areas of the organelles and of the cells and also facilitated counting of the exposed silver grains. In addition two inhibitors of protein synthesis, puromycin and cycloheximide were used to determine whether those cells that cleave in the presence of inhibiting doses of these drugs incorporated similar amounts of the label as a normal (untreated) cell.

Materials and methods

Sea urchins were obtained at Duke University Marine Laboratory, Beaufort, North Carolina. The electrical stimulation method was used to obtain ova (Harvey 1956). All ova were treated with four to five drops of 0.1 N HCl per 100 cc of sea water to remove the jelly coat. The ova were centrifuged in 0.85 M sucrose in tap water to which was added 5 cc of sea water per 100 cc utilizing a Beckman Model L centrifuge. Centrifugation for three minutes at 15,300 to 17,500 RPM separated the ova into two major fractions, red halves (non-nucleates) and white halves (nucleates). The white halves were selected for this study and were fertilized or parthenogenetically stimulated. Following activation, the egg fragments were incubated continuously with DL-leucine-4, 5- H^3 (final concentration 1.23 m μ M/ml and a specific activity of 6.25 μ C/ml). Polyethylene bags and wet paper towels provided a disposable moisture chamber which prevented evaporation of the small (2 ml) samples.

An apparatus employing a millipore filter retained all the labeled cells and permitted fixation, dehydration, and embedding in the same container (Rinaldi *et al.* 1967). Ova were fixed with Bouin's fluid or buffered 10% formaldehyde. All ova were rinsed in concentrated amounts of nonradioactive leucine, and then treated with 5% TCA at either 25° or 90°C for a minimum of 15 minutes, dehydrated, stained with fast green, and embedded in araldite. A Porter-Blum ultramicrotome MT-2 was used to obtain 1 and 2 μ sections. These sections were placed on gelatin-subbed slides and dipped in Kodak NTB-2 nuclear emulsion. After drying, and exposure periods of one to three days at 0°C, the autoradiographs were developed with cooled (15°C) photographic solutions. The appearance of cellular organelles was enhanced by staining in 70% alcohol solutions (15 minutes each) of light green, methyl green, and toluidine blue; destaining 10 minutes in acid alcohol; and finally restaining in nuclear fast red.

Selected sections of the ova were photographed using Zeiss oil phase optics (95 $\times$ objective) in such a manner (Schlegel and Zoeten 1967) that the silver grains of the photographic emulsion appear as white spots and the cellular organelles of the ova both were in focus.

The areas of asters, MA whole cells and samples of the background were determined by cutting out and weighing images of the organelle cell or background from photographic paper (Kodak F-5) and comparing them to known area weights from the same photographic paper. Experiments were done to test this comparative method of finding the area of the cells, and it proved to be an accurate method.

Puromycin was obtained from Nutritional Biochemical Corporation and the concentration employed was 100 μ gm/ml (1.8×10^{-4} M). Cycloheximide was obtained from Sigma Chemical Company and used at a concentration of

300 to 3000 $\mu\text{g}/\text{ml}$ (10^{-3}M and 10^{-2}M). The protein inhibitors were applied ten minutes before fertilization and were incubated continuously with the ova. Immediately following fertilization 30 μl of DL-leucine-4, 5- H^3 were added to 4 ml of the sample. The specific activity was 3.75 $\mu\text{C}/\text{ml}$ at a concentration of $9.75 \times 10^{-5}\text{mg}/\text{ml}$. Ten minutes before fixation the ova were washed in an excess of non-labeled leucine and were prepared as described above.

Results

Table 1 presents an analysis of 136 ova used in this investigation. Note the consistently low incorporation of the label by the background surrounding the cells. There appeared no fundamental difference in the autoradiographic

Table 1. A comparison between 1μ and 2μ cell sections and their organelles with respect to the amount of labeled leucine uptake. The average area per cell section was $1620\mu^2$ with the maximum being $2045\mu^2$ and the minimum being $1122\mu^2$. The asters had an average area of $107\mu^2$ with a maximum of $148\mu^2$ and a minimum of $74\mu^2$. The MA areas averaged $174\mu^2$ with a maximum of $365\mu^2$ and a minimum of $31\mu^2$

	1μ		2μ	
	Number of samples	Average number of 10^{-2} silver grains ($\times 10^{-2}/\mu^2$)	Number of samples	Average number of silver grains ($\times 10^{-2}/\mu^2$)
Cell sections (including organelles)	25	$5.4 \pm 2.6^*$	52	5.1 ± 7.0
	26	4.1 ± 1.3	34	6.2 ± 7.0
Asters	13	5.4 ± 2.6	39	7.1 ± 4.7
	21	4.3 ± 2.6	9	3.3 ± 2.5
MA's	12	8.3 ± 7.3	19	7.8 ± 2.8
	7	4.9 ± 0.7	17	9.9 ± 9.1
Background	25	0.64 ± 0.3	50	0.87 ± 0.5
	26	0.6 ± 0.2	33	1.0 ± 0.9

$$* \sqrt{\frac{(x-\bar{x})^2}{n-1}}$$

data between the 1μ sections and the 2μ sections. However, all 1μ sections were exposed to the NTB-2 emulsion for a minimum of 36 hours and a maximum of 72 hours; whereas the 2μ sections were exposed from 24 to 36 hours. A useful feature of Table 1 is to compare rates of amino acid incorporation by the cellular organelles with its surrounding cell. In the case of the 1μ sections comprising selected sections from 51 cells, the number of silver grains per μ^2 for asters and MA are identical to those for the whole cell, that is a range of $5.4 (\times 10^{-2})$ silver grains per μ^2 to $4.1 (\times 10^{-2})$ silver grains per μ^2 . The only exceptions were the 12 MA which averaged $8.3 \pm 7.3 (\times 10^{-2})$ silver grains per μ^2 .

In 2μ sections the values recorded for silver grains per μ^2 in the whole cells ranged from 5.11 to 6.2 ($\times 10^{-2}$). The average for asters is within this range, $5.2 \pm 3.6 (\times 10^{-2})$. The MA contain about one and one half times as many silver grains per μ^2 as the whole cell. In view of the large standard deviations of these samples, the range of silver grains in either the asters or the MA is not substantially different from that of the silver grains recorded per μ^2 of the whole cell.

Protein synthesis in those cells treated with a protein inhibitor incorporates labeled amino acid at the same rate as the untreated cells (Table 2). The attention here is on those cells that cleaved in this evaluation of the tracer's uptake. Following fertilization, initially, there is only a slight incorporation of the label until aster formation and MA formation. Then the

Table 2. Comparison of H³ leucine by cleaving ova incubated in puromycin and cycloheximide with untreated cleaving Ova

Time after fertilization	Cycloheximide	Puromycin	Control
0 minute	no label	no label	no label
8 minutes	no incorporation	no incorporation	no incorporation
30 minutes	slight label (10-20 silver grains per cell)	slight label	slight label
55 minutes	slight label	slight label	labeled as high as 50 grains per section
75 minutes asters appeared	labeled as high as 50 silver grains per section)	labeled	labeled
120 minutes cleavage figures	heavily labeled (80+ silver grains per section)	heavily labeled	heavily labeled
180 minutes first cleavage	8/100 cleaved-heavily labeled	6/100 cleaved-heavily labeled	97/100 cleaved-heavily labeled
240 minutes 4 cell stage	1/100 reaching 4 cell stage	3/100 reaching 4 cell stage	85/100 reaching 4 cell stage

incorporation becomes pronounced (approximately 50 silver grains per cell section). Further, upon completion of cleavage the cells were more heavily labeled (80 grains or more). These cleaving cells are influenced by the inhibitors as indicated by the pronounced depression of the cleavage index. The cleavage is 6 to 8% for the first cleavage compared to the normal cleavage index for the control untreated cells of 96%. Those cells that did reach the two-cell stage were further inhibited from progressing to the 4-cell stage. However, the cells (3 in puromycin and 1 in cycloheximide) that did reach the 4-cell stage incorporated relatively as much H³ leucine as the control 4-cell stages.

Discussion

Recent investigations have supported the possibility that the MA is formed from proteins present in the egg cell before fertilization. Kane (4) isolated MA with 12% hexylene glycol and dissolved them in 0.6 M KCl. One half of the total proteins of the MA was soluble. The isolated proteins constitute 0.5% of the total cell protein. Sufficient amounts of this 22S protein are present in unfertilized ova to serve as a reservoir for early cleavage stages. Wilt *et al.*, (1967) found that amino acid incorporation into isolated purified solubilized MA is equal to or only slightly greater than incorporation into bulk cell protein. They concluded that synthesis of solubilized structural MA protein does not represent a heavy investment of the protein synthesis of the cell prior to first cell division. Mangan *et al.* (1965) have found a similar uptake of labeled protein and noted the fact that only 11% of the label was present in the MA. They concluded that this probably represented both structural and enzymatic proteins during the synthesis of the MA.

Inoue and Sato (1967) used colchicine to dissolve the MA and following removal of this alkaloid the MA would reappear. Further, the MA appeared in the presence of puromycin, chloramphenicol, or actinomycin. Surprisingly the MA was formed more quickly with puromycin treatment. These authors concluded that the MA is assembled from a preexisting reservoir of proteins and would not be dependent on *de novo* protein synthesis.

It would appear, therefore, that the MA and asters do not require substantial new synthesis for the formation of most structural elements and for normal assembly. But some of the enzymatic processes may be catalytic components necessary and indispensable for mitosis. The effects on cleavage by puromycin and cycloheximide support the concept that some protein synthesis is required for cleavage. Although the cleavage was depressed by 94% in the presence of protein inhibitors those cells that cleaved (6%) exhibited a synthesis of proteins in the MA and asters equal to that of the cytoplasm. However, the protein synthesis was depressed in all cells comparable to the control cells analyzed. Therefore, it appears that some critical level or type of protein synthesis is necessary for the cells to complete mitosis.

Summary

Fertilized *Arbacia* ova were incubated with H^3 -leucine from 30 minutes to 4 hours and thin sections (1 and 2μ) analyzed autoradiographically. The asters and MA incorporated H^3 -leucine at approximately the same rate than the whole cell. Puromycin and cycloheximide, depressed mitosis (94%). However, those cells that cleaved contained newly synthesized proteins.

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