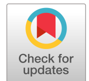

PROTEIN SYNTHESIS IN SEA URCHIN EGGS: A "LATE" RESPONSE TO FERTILIZATION*

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Communicated by C. B. Van Niel, February 6, 1967

Fertilization of echinoderm eggs results in a complex series of metabolic activations, resulting in greater than 30 changes within the first ten minutes after insemination. The most prominent of these are modifications in structure,[1] increases in respiration rate[2] and coenzyme content,[3] increases in substrate uptake,[4–6] transient proteolytic activity,[7] and a decrease in external pH.[8] These changes are possibly related to enzymic activations leading to the synthesis of lipids, proteins, and nucleic acids required for cell division and differentiation.

Studies on the temporal sequence of these diverse physiological reactions could provide insights into the mechanisms and interrelationships of these changes. Thus far, such temporal data are available on the light-scattering and external pH changes[9] (resulting from structural changes in the cell cortex), activation of NAD kinase[3] (resulting in NADP and NADPH synthesis), and activation of respiration.[2]

The present report concerns the temporal relationships of the above events to the postfertilization increase in protein synthesis, and also attempts to resolve contradictory results regarding amino acid incorporation in unfertilized eggs. Previous studies have shown that the rate of protein synthesis is low or negligible in unfertilized eggs, and increases markedly after fertilization.[6, 10–12] This increased rate is apparently dependent on mRNA already present in unfertilized egg cytoplasm, since it is unaffected by either actinomycin D[13] or enucleation.[14] Current hypotheses regarding activation mechanisms of this increased protein synthesis, which need not be mutually exclusive, implicate (1) structural changes in ribosomes resulting from protease activation at fertilization,[15] (2) synthesis of a factor(s) controlling mRNA translation rate,[16] or (3) energy-dependent processes involved in mRNA attachment.[17]

In the present study, effects of changes in cellular amino acid permeability following fertilization[5, 6] were minimized by "preloading" unfertilized eggs with radioactive amino acid, and incorporation kinetics measured by sampling at close intervals following fertilization. The results indicate that the activation of protein synthesis is actually a "late" response to fertilization, since increased synthesis does not begin until six to ten minutes after insemination. The results also show that unfertilized eggs transport and concentrate leucine and valine, and incorporate these amino acids into protein; that a sizeable amount of added leucine is converted to compounds not involved with protein synthesis; and that the rate of this conversion is accelerated by fertilization.

Materials and Methods.—Handling of gametes: Shedding of gametes of *Lytechinus pictus* (Pacific Bio-Marine Co.) was induced by intracoelomic injection of 0.5 M KCl, and cell counts were made by the dilution–capillary tube method.[18] The eggs, maintained at 16°C, were washed 4–5 times by decantation with millipore-filtered sea water, and then "preloaded" with C^{14}-amino acid before fertilization by incubation in μM solutions of the isotope for 4–10 min. Exogenous isotope was then removed by four washes with sea water and gentle centrifugation in a hand centrifuge, and insemination effected by addition of 10 μl undiluted sperm per 10 ml of egg suspension.

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Isotope incorporation: One-half-ml samples of a suspension of preloaded eggs were added to 5.0 ml 5.5% TCA containing 0.05 *M* unlabeled amino acid. The eggs were centrifuged, and 1.0 ml supernatant liquid was removed for isotopic analysis of the TCA-soluble fraction as described by Berg.[19] For preparation of TCA-insoluble protein 1 mg bovine serum albumin was added to the TCA-egg suspension, which was then heated at 90°C for 20 min, filtered through Gelman A or Whatman GF/B glass fiber filters, and the residue washed four times with 5% TCA–0.05 *M* C^{12}-amino acid, twice with ethanol:ether:chloroform (2:2:1), and twice with ether. The filters were then glued to planchets and counted in a Nuclear-Chicago gas flow counter with mica window.

Autoradiography: Preloaded eggs were fixed in alcohol:acetic acid (3:1), washed three times with 95% alcohol, taken through toluene, and embedded in Tissuemat. Sections of 5 μ thickness were spread on gelatin-subbed slides, rehydrated through toluene and alcohol to water, immersed for 10 min in boiling 5% TCA, washed thoroughly, dipped in Ilford K5 nuclear emulsion, and left for 3 weeks at 5°C before development.

Chromatography: The ether-extracted TCA-soluble fraction was chromatographed on Whatman no. 1 paper by ascending chromatography for 16 hr with 2,4 lutidine:collidine:water, 1:1:1, plus 1% diethylamine,[20] and radioactivity measured with a Nuclear-Chicago actigraph radiochromatogram scanner. Acid hydrolysates of the TCA-soluble and insoluble fractions were prepared by boiling in 6 *N* HCl under reflux conditions, the solution was dried in a flash evaporator, and the redissolved residue chromatographed as above.

Isotopes: Specific activities of the uniformly labeled C^{14}-amino acids (International Chemical and Nuclear Corp.) were: C^{14}-leucine, 210 mc/mM; C^{14}-valine, 190 mc/mM; C^{14}-phenylalanine, 300 mc/mM.

Results.—Amino acid uptake and incorporation in unfertilized eggs: Unfertilized eggs, incubated for five minutes in 0.5–7.3 $\times$ 10^{-6} *M* C^{14}-labeled leucine or valine, respectively contain 8.1–1.3 times more amino acid, on a volume basis, than was originally present in the external medium. Data not herein presented show that the transport system approaches saturation at micromolar levels, that leucine is transported at a greater rate than is valine, and that the uptake rate is initially linear.

In all experiments, unfertilized eggs were found to incorporate appreciable amounts of C^{14}-labeled leucine, valine, or phenylalanine into TCA-insoluble protein (Figs. 1 and 5). This incorporation does not result from a small population of exceptionally active cells, since autoradiographs of unfertilized eggs showed that *all* cells incorporated C^{14}-leucine into hot TCA-insoluble material.

Protein synthesis following fertilization: Figure 1 depicts the results of an experiment in which cumulative incorporation of C^{14}-leucine into protein was measured at close intervals following fertilization. The data show a ten-minute lag period between sperm addition and the first increase in rate of protein synthesis, the rate then increasing for seven to ten minutes to a constant rate five times that of unfertilized eggs. Similar kinetics were found in nine separate experiments, using eggs from seven different females and either C^{14}-labeled leucine, valine, or phenylalanine. In these experiments the relative rate of protein synthesis had increased 5 to 15-fold 20 minutes after fertilization. The lag period between insemination and increased protein synthesis varied between six and ten minutes, with an average of 7.8 ± 1.2 minutes.

The same lag period is observed when cumulative protein synthesis is measured with different methods of protein precipitation. In one experiment, the kinetics of C^{14}-valine incorporation were compared in samples which were either heated or not heated in 5 per cent TCA. The former method hydrolyzes sRNA, releasing amino acyl-sRNA and polypeptidyl-sRNA from the TCA-insoluble residue.[21]

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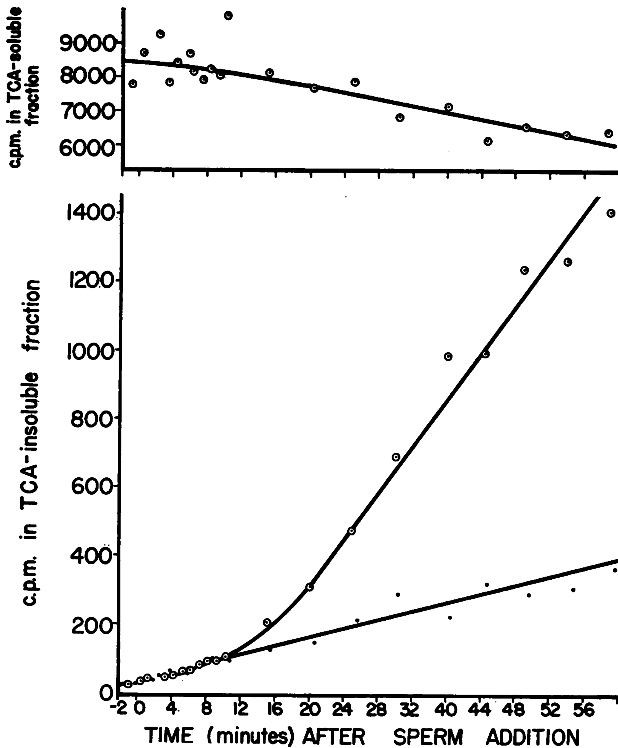

Fig. 1.—Cumulative incorporation of C^{14}-leucine by unfertilized (*dots*) and fertilized (*circles*) eggs (3880 cells/sample). Upper part of figure shows cpm/sample in the TCA-soluble fraction of fertilized eggs.

Conversely, elimination of the heating step should result in retention in the TCA-insoluble residue of these sRNA-linked compounds. The results of this experiment, shown in Figure 2, indicate similar kinetics in both cases. The same is also true if TCA-soluble basic polypeptides are precipitated with the TCA-tungstic acid procedure of Gardner *et al.*[22]

Auxiliary metabolism of leucine following fertilization: Data in the upper part of Figure 1 show that although the TCA-soluble fraction decreases in parallel with the increase in TCA-soluble material, this decrease is not stoichiometric (2200-cpm decrease in TCA-soluble vs. 1400-cpm increase in TCA-insoluble material). This lack of stoichiometry, also found with valine, suggests a significant conversion of these amino acids to substances other than protein. Chromatographic analyses show this to be true, and indicate that this conversion is of sufficient magnitude to affect the measurement of rate of protein synthesis.

Figure 3 depicts a radiochromatographic tracing of the TCA-soluble fraction of fertilized eggs which were preloaded before fertilization with C^{14}-leucine. The major peak coincides chromatographically with leucine, whereas the substances responsible for the two minor peaks, *X1* and *X2*, have not yet been identified. These compounds do not migrate with α-ketoisocaproic acid, nor are they peptides, as their chromatographic behavior is unaltered by 16 hours' boiling in 6 N HCl.

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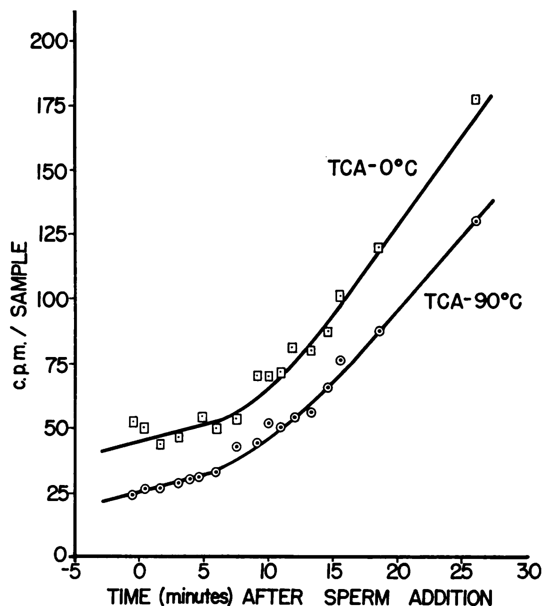

FIG. 2.—Cumulative incorporation of C^{14}-valine by fertilized eggs into hot (*circles*) and cold (*squares*) TCA-insoluble material (2720 cells/sample).

They are not protein constituents, as only C^{14}-leucine is released upon acid hydrolysis of labeled protein.

Figure 4 shows that these compounds gradually appear in unfertilized eggs, and that their rate of formation is accelerated by fertilization (2.5–3.4 times in two experiments). Unfertilized eggs, preloaded and then incubated for two hours at 16°C, convert 30 per cent of the TCA-soluble C^{14}-leucine into *X1* and *X2*. If these eggs are now fertilized, the duration of the period between sperm addition and increased protein synthesis is the same as in eggs fertilized immediately after loading with C^{14}-leucine, and free of *X1* and *X2* at the time of fertilization. Hence it appears that these two unidentified substances are not intermediate products in the synthesis of protein from leucine, and that their accelerated rate of formation after fertilization is unrelated to the postfertilization increase in rate of protein synthesis.

Analysis of rate of protein synthesis: Because of the extensive leucine conversion, analysis of the rate of leucine incorporation must be corrected for the actual amount of C^{14}-leucine in the TCA-soluble fraction. In two experiments, measurements were made of radioactivity of the TCA-soluble and TCA-insoluble fractions, and the amount of leucine actually present in the TCA-soluble fraction was determined by radiochromatography. The results of one of these experiments are shown in Figure 5. The bottom curve represents leucine incorporation into protein, the upper one the amount of C^{14}-leucine determined by radiochromatography to be present in the TCA-soluble fraction. Figure 6 shows an analysis of the rate of incorporation calculated from Figure 5 by (1) slope analysis (curve *A*) and (2) by calculation of the percentage of the C^{14}-leucine incorporated into protein per two-minute interval (curve *B*). For comparison with other fertilization reactions, the figure also depicts the previously determined temporal sequence of four other fertilization-induced changes.[9]

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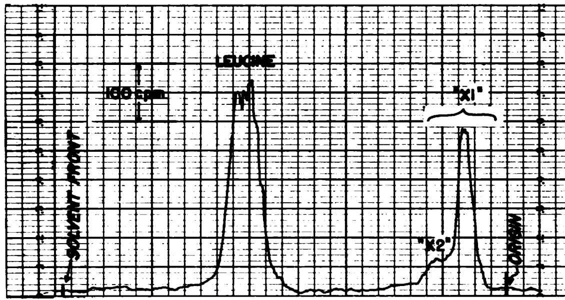

Fig. 3.—Recorder tracing of radiochromatogram of TCA-soluble fraction of eggs preloaded with C^{14}-leucine and sampled at 30 min after fertilization.

Discussion.—The reported experiments demonstrate that *unfertilized* eggs of *L. pictus* incorporate leucine and valine into protein. Furthermore, autoradiographic data show that this protein synthesis occurs in all cells of the population, so that the observed incorporation cannot be attributed to the presence of immature oöcytes.

These findings confirm and extend recent data of Tyler *et al.*[10] obtained with the same species. They differ, however, from results with other species of sea urchins, whose unfertilized eggs reportedly synthesize little or no protein, and whose rate of protein synthesis increases more than 100-fold upon fertilization.[11, 12] These conflicting results might reflect species differences, or might result from not taking into account the lower permeability and incorporation rates of unfertilized eggs. Since fertilization results in four to eightfold increases in amino acid uptake,[5, 23] and 5 to 15-fold increases in protein synthesis rate, measurements of synthesis which do not consider these permeability changes could indicate apparent increases of 20 to 120-fold.

Protein synthesis has also been reported in unfertilized echiuroid,[24] polychaete,[25] and amphibian[26] eggs. The metabolic significance of this synthesis is presently unclear (cf. ref. 26). Kavanau, over 15 years ago, observed a depletion of free amino acids, and a concomitant increase in protein, in unfertilized sea urchin eggs stored for 24 hours after ovulation.[27] He suggested that this might represent a continuation of the ripening process, dependent in the female animal on utilization of exogenous amino acids of the body fluids, but proceeding after ovulation through utilization of the endogenous amino acid pool.

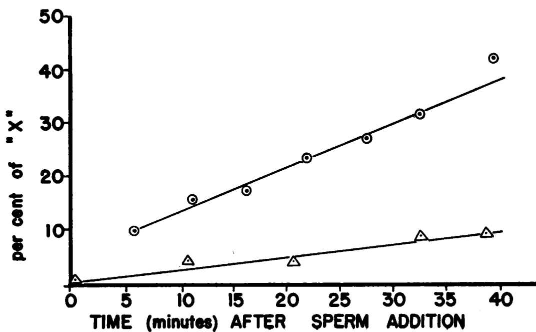

Fig. 4.—Percentage of total radioactivity of the TCA-soluble fraction found in X1 and X2 in unfertilized (*triangles*) and fertilized (*circles*) eggs.

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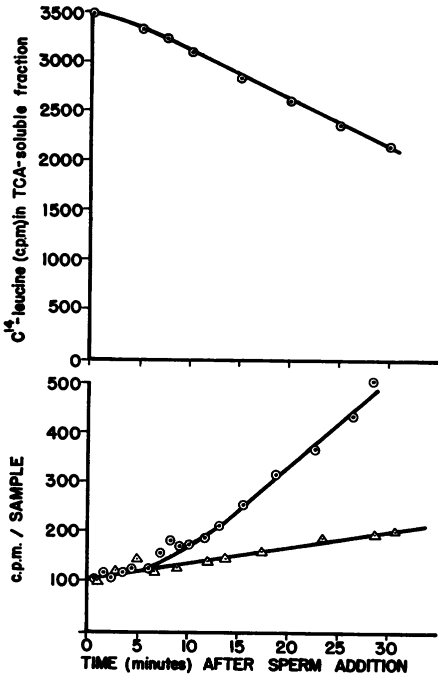

FIG. 5.—*Lower figure:* Cumulative incorporation of C^{14}-leucine in unfertilized (*triangles*) and fertilized (*circles*) eggs (14,000 cells/sample). *Upper figure:* Actual cpm of C^{14}-leucine in TCA-soluble fraction of fertilized eggs.

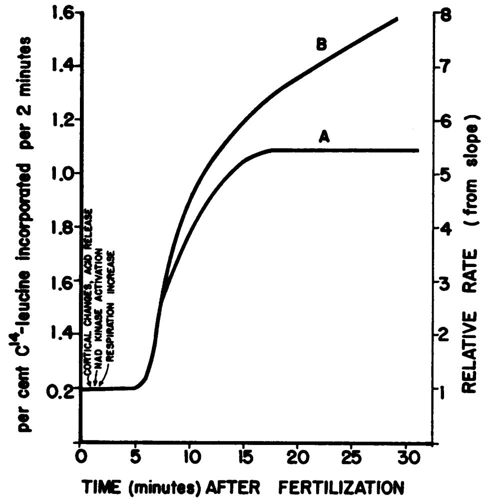

FIG. 6.—Relative rates of protein synthesis. Procedure as described in text. Temporal sequence of other changes determined in separate experiments (ref. 9).

Estimates of relative rates of protein synthesis are complicated by the observed conversion of leucine to other products. The increased rate of conversion after fertilization, of considerable interest in itself, suggests that fertilization increases amino acid catabolism (cf. ref. 27).

Two alternative modes of analyzing relative rate of protein synthesis were presented in Figure 6. The first (*A*), derived from slope analysis of the cumulative formation of radioactive protein, would be valid if amino acid compartmentation exists, and if the pool involved in protein synthesis is small and essentially saturated by added C^{14}-leucine. The second alternative (*B*), calculated from the percentage of C^{14}-leucine incorporated per two-minute interval, would be valid if C^{14}-leucine were always in equilibrium with leucine utilized for protein synthesis (whether compartmentation existed or not). Since the later phases of these kinetics correspond almost exactly to the kinetics observed in *in vitro* systems prepared at various times after fertilization,[11] this latter approach probably reflects more accurately the *in vivo* rate.

The most interesting aspect of these experiments is the finding of a temporal lag between the cortical events and increased protein synthesis. This lag indicates that the structural and metabolic changes following fertilization are not simultaneously activated. Rather, the changes associated with cortical granule breakdown, acid excretion, NAD kinase activation, and increased respiration appear to be "early"

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responses to fertilization, whereas increased protein synthesis appears to be a "late" response. The results presently suggest no obvious correlations of these early events with the increased rate of protein synthesis. Furthermore, the absence of correlation with increased respiratory activity implies that the postfertilization burst in O_2 consumption[2] does not result from the energy demands of protein synthesis. This absence of correlation is further strengthened by the lack of immediate effect of puromycin on respiration[28] and ATP levels.[23]

The results of this kinetic study are pertinent to proposed mechanisms of increased protein synthesis after fertilization. The five to nine-minute lag between cortical changes and increased protein synthesis does not appear to support the hypothesis that protease activation at the time of cortical granule breakdown[7] is the sole factor increasing protein synthesis.[15] Rather, the observed lag suggests a chain-type reaction system, where a number of reactions must occur (or new products accumulate) before increased mRNA readout can be initiated. Clues to the kinetic behavior of these other reactions, inferred from the kinetics of protein synthesis (Fig. 6*B*) indicate that in addition to the lag phase there is also an acceleration phase during which protein synthesis increases towards its full postfertilization rate.

The present results, while not eliminating involvement of proteases, suggest that multiple factors,[29] such as the synthesis of a rate-controlling substance in fertilized eggs[16] or the involvement of an energy-linked process in mRNA-ribosome attachment,[17] are controlling translation rate. Whatever the factors, one proof of their operation *in vivo* should be their temporal conformation to both the lag and acceleration phases.

I wish to thank Miss Sigrid Elsaesser for expert technical assistance, and Dr. Meredith Gould for preparing the autoradiographs. I also appreciate the many pertinent comments of Drs. John P. Phillips, C. B. Van Niel, and H. Hilgard. The valuable criticisms of Dr. Van Niel and Dr. Norman Wessels aided greatly in the preparation of this manuscript.

* Supported by a grant from the National Science Foundation (NSF GB-4206).

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