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Continuity of Protein Synthesis Through Cleavage Metaphase

Abstract. *Protein synthesis continues without a decline in rate throughout the period of chromosome condensation and of cytokinesis in the first two cleavages of sea urchin embryos. The natural synchrony of the egg populations and the conditions of measurement allowed even a partial inhibition of synthesis to be observed. Our results do not explain the mechanism of inhibition of protein synthesis that occurs at metaphase in cultured mammalian cells, but it shows that such a change in rate is neither universal nor obligatory.*

The suppression of synthesis of macromolecules during mitosis in cultured mammalian cells has been known for several years (*1*). Protein synthesis, in particular, either stops or is strongly inhibited (*2–4*); the decreased rate of synthesis is correlated in time with a loss in polyribosomes. Some investigators have been reluctant to attribute this change to the ordinary decay of messenger RNA (in the absence of resynthesis during metaphase), because the duration of mitosis is but a small fraction of the assumed half-life of the messenger (*5*). Salb and Marcus (*2*) suggest that a material which is sensitive to trypsin and which renders ribosomes nonfunctional is released from the nucleus during metaphase.

If inhibition of protein synthesis were observed at metaphase in cells differing radically from those in the cell cultures, with respect to intermitotic time, schedule of nucleic acid synthesis, and average half-life of messenger RNA, then the possibility that such an inhibition is an obligatory consequence of nuclear breakdown would be strengthened.

We have reexamined protein synthesis through the first and second cleavage cycles of the sea urchin embryo. This system is distinguished from mammalian cell cultures by: (i) a much smaller ratio of nuclear to cytoplasmic volume; (ii) a high degree of natural synchrony; (iii) a much shorter intermitotic time; (iv) an atypical pattern of DNA synthesis, including initiation during telophase; and (v) a very long half-life of messenger RNA relative to the intermitotic time. That protein synthesis is completely insensitive to demonstrably adequate doses of actinomycin D through the entire period from fertilization to the formation of the blastula (*6*) is evidence for a long half-life of messenger RNA.

Our results indicate that inhibition of protein synthesis is not a universal concomitant of mitosis, because in the cells of the sea urchin embryo neither chromosome condensation nor cytokinesis is accompanied by a significantly reduced incorporation of radioactive amino acids into proteins.

Gametes were obtained from *Strongylocentrotus purpuratus* (*7*). In the incorporation experiments, unfertilized eggs were studied, as controls, in parallel with the developing embryos to detect background incorporation and possible microbial contamination. Unfertilized eggs normally take up and incorporate negligible quantities of labeled amino acid, compared with zygotes (*8, 9*). Eggs which were known from trials earlier in the same day to give 95 percent fertilization or better, and to cleave with maximum synchrony, were suspended (10^3 cells/ml) in Millipore-filtered sea water. Immediately after fertilization, and at the same times as all subsequent samplings for incubation with radioactive precursor were taken, samples of the suspension were removed and fixed in sea water containing 2 percent formalin. The fixed material was later examined microscopically to help assess the synchrony of the cytologic changes accompanying cleavage.

We exposed the eggs to radioactive amino acids by adding 1 ml of well-stirred egg suspension to an equal volume of sterile sea water containing L-leucine-C^{14} (222 mc/mmole) at an activity of 0.5 μc/ml (*10*). Five minutes later, the mixture was poured into a chimney funnel and the eggs were collected by filtration on a Millipore filter (type HA, 0.45-μ pore size). The eggs were then rinsed by suction with two 5-ml portions of sea water containing 0.75 mg of unlabeled L-leucine per milliliter. This was followed by a rinse with sea water. The collection procedure required 1 minute. In addition to the use of unfertilized eggs, samples of incubated medium collected on filters were used to determine background radioactivity. (Modifications made to test the contribution of C^{14}-aminoacyl sRNA to the radioactivity are described in the legend to Table 1.)

The cells do not break under this treatment; in suspensions of low density such as we used, they dry quickly on filter papers and do not subsequently detach, even when the papers are wetted again for further treatment. The pools of the low-molecular weight precursor appear to be retained within the cells (*11, 12*).

The filter papers were affixed to stainless steel planchets by tacking them onto discs of "Parafilm"; this procedure allows them to be taken off undamaged for further processing. The radioactivity on the planchets was counted in a low-background GM counter. After counting, we removed the papers and washed them on the chimney funnel with several 5-ml portions of 5 percent trichloroacetic acid (TCA) containing 0.5 mg of unlabeled L-leucine per milliliter. This was followed by a wash with distilled water. We dried the papers, attached each to the planchet from which it had been removed, and again counted the radio-

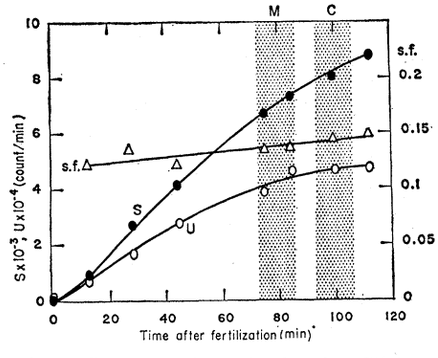

Fig. 1. Incorporation of L-leucine-C^{14} into *Strongylocentrotus* zygotes during the first cleavage cycle. The curve marked *U* (open circles) represents total uptake of label. *S* (filled circles) represents the radioactivity in acid-insoluble material. On the ordinate scale, *s.f.* (triangles) is the fraction of the total uptake found as radioactivity in acid-insoluble material, *M* indicates the time of metaphase, and *C* that of furrowing. Over the interval covered by the shaded bands, about 90 percent of the cells in the population were judged by microscopic observation and counting to have been in the stage indicated. Ordinate: radioactivity (count/min) incorporated during a 5-minute exposure.

Table 1. Rates of protein synthesis during the first cleavage cycle of *Arbacia* embryos. A suspension of *Arbacia* zygotes was sampled at the times indicated. Label was administered as described in the text. Incorporation was stopped after 5 minutes by addition of an equal volume of cold 10 percent TCA containing 0.5 mg of unlabeled leucine per milliliter. After 2½ hours at 0°C, embryos were packed by centrifugation and washed twice with TCA-leucine. Half of the samples were then resuspended in 5 percent TCA and kept at 90°C for 15 minutes. The others were chilled. All samples were finally washed with cold 5 percent TCA-leucine and collected on Millipore filters for counting, as described above. Data given in the table are counts per minute ± probable error of 2×10^3 embryos exposed to labeled leucine for 5 minutes. Temperature of the incubation was 23.5°C.

Stage	Time (min)	Cold TCA (count/min $\times10^{-3}$)	Hot TCA (count/min $\times10^{-3}$)
Prophase, "Streak"	20	4.56 ± .05	4.37 ± .05
Metaphase	36	8.35 ± .06	7.12 ± .06
Furrowing	53	9.59 ± .07	8.81 ± .06

activity. The difference between the results of this counting and those of the series before treatment with TCA represents the radioactivity of the acid-soluble pool accumulated over 5 minutes. The fraction of the total uptake becoming acid insoluble may reflect the rate of protein synthesis somewhat more accurately than do the counts of the acid-insoluble material, because the permeability of the eggs changes considerably, at least during the first cleavage cycle (*12*), while the pool sizes probably change more slowly (*13*). With the specific activity of precursor chosen, this "synthetic fraction" did not exceed 0.15, except toward the end of the second cleavage cycle, when it rose rapidly to more than 0.3.

Rates of DNA synthesis during the first cleavage cycle were assessed by allowing samples of the egg suspensions to incorporate thymidine-2-C^{14} [25 mc/mmole, 0.5 μc/ml (*10*)] for 5 minutes. The cells were collected as described, but they were washed immediately with TCA containing excess, unlabeled thymidine and then with water. The radioactivity of the filters was counted, and the filters were then removed from the planchets for individual incubation with deoxyribonuclease [10 μg/ml, in "tris," 0.01*M*, $MgCl_2$ 0.01*M*, *p*H 7.0 (*10*)] for 2 hours at 25°C. The contents of each incubation vessel were filtered through the corresponding paper at the end of this time, and the paper was again washed with the mixture of TCA and thymidine in water and then dried and counted. The difference between the first and second series of counts represents the labeled thymidine which was incorporated into a deoxyribonuclease-sensitive, acid-insoluble product, presumably DNA synthesized during the incubation.

Direct observation of the cells and enumeration of stages represented in the samples fixed at the times of incubation show that populations incubated during nominal metaphase contained 70 to 90 percent of the cells in this stage during the interval of exposure to labeled precursor.

Total amino acid uptake and rates of protein synthesis during the first cleavage cycle are plotted in Fig. 1, on which are shown the intervals of metaphase and of cytokinesis (furrowing). The rate of total uptake, which reflects the "permeability" of the eggs to exogenous amino acid, increases through the first cycle and approaches a constant value toward the end of the cycle. [After the second cleavage, the penetration rates for valine and cytidine remain essentially constant throughout early development (*12*).] The rate of incorporation of label into proteins appears to increase, but the shape of its function is very close to that of the total uptake. The fraction of the total uptake that becomes acid-insoluble, which is represented by points "s.f.," changes only slowly through the cycle. This finding indicates that immediately after fertilization, protein synthesis is turned on and proceeds at a rate which is a significant fraction of the maximum first-cycle rate. The rate of protein synthesis in unfertilized eggs appears to be very low in comparison (*9*). Inferences about rates cannot, unfortunately, be more exact than this, because early data on pool sizes (*13*) may be open to question (*14*). The concentration of free valine in the *Spisula solidissima* egg, measured under conditions that minimize autolysis, fluctuates 15 to 20 percent between formation of the second polar body and first cleavage (*14*).

The rate of protein synthesis in a system prepared from *Arbacia punctulata* eggs in vitro doubles between 30 minutes (prophase-metaphase) and 60 minutes (first cleavage) (*15*). Close study of the rates of protein synthesis in vivo during the period just after fertilization has not been reported; the protease activation in the early minutes of development (*16*) may result in a transient increase in the size of the amino acid pool. Although two of our samples were exposed to the isotopic label over intervals through which almost all of their cells were in metaphase, in no case was there a measurable decrease in the rate of incorporation. In fact, the rate of protein synthesis continues to increase slowly, but steadily, through both mitosis and cytokinesis (which begins at telophase).

The method used for these experiments is convenient, but its result is open to question on grounds that the radioactivity fixed by cold TCA may in large part be due to C^{14}-amino acyl sRNA. If this were so, failure to find a decrease at metaphase would not reflect on the rate of protein synthesis. To test this possibility, a comparison of the radioactivity of materials precipitated by cold and hot TCA was made. Hot TCA, which removes radioactivity due to charged

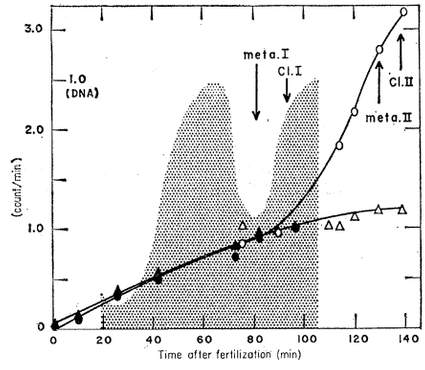

Fig. 2. Relative rates of total uptake and incorporation into polymer for L-leucine-C^{14}, and of incorporation into DNA for thymidine-2-C^{14}. The data for leucine represent two different experiments (open and filled symbols), covering the first and second division cycles. All rates (count/min incorporated during a 5-minute exposure) are normalized to 1.0 at the time 50 percent of the cells showed their first cleavage furrow (*cl. I*) and are plotted against the values 0 to 3.0 on the ordinate. Triangles, total uptake; circles, counts in TCA-insoluble material. First and second metaphases and furrowing periods are indicated by "meta." or "cl." followed by the appropriate number. DNA synthesis is represented by the shaded region; its rates are normalized to 1.0 at the observed maximum and are referred to the inner ordinate. Data points (not shown) were at the same times as those indicated for amino acid uptake, and the upper boundary of the shaded region is the curve drawn through such points. The rate change at "meta. I" is large. The area under the shaded region may not accurately represent the amount of DNA synthesis, since the penetration rate for thymidine was not measured, and it may increase during the first cleavage cycle.

sRNA, slightly reduces fixed radioactivity at each stage, but does so neither selectively nor sufficiently to indicate that the rates of protein synthesis decrease at metaphase (Table 1).

The first cleavage cycle is unusual, for, during the early part of it, sperm penetration and nuclear fusion must take place. The second and subsequent cleavages are, however, normal mitotic divisions (except that the cells are not "growing" in the sense of increasing in mass). The total uptake of amino acid and the protein synthesis during the second cycle were also measured. In Fig. 2 are plotted data points for two separate experiments, all normalized to a rate of 1.0 at the time that 50 percent of the cells showed the first cleavage furrow. Also represented is the relative rate of DNA synthesis during the first cycle, as it is reflected in the incorporation of C^{14}-thymidine. The rate of DNA synthesis is normalized to 1.0 at the observed maximum.

The DNA synthetic period ("S") of the first interphase is discrete, to the extent, at least, of a very sharp drop in the incorporation rate at metaphase. If we assume that DNA synthesis in any one cell stops at metaphase, these data indicate that the departure from perfect synchrony and the length of the incubation period interact in such a way as to cause a decrease in the observed incorporation rate of about 60 percent of that during the synthetic period.

A reduction of the same magnitude in the rate of protein synthesis and in the number of polyribosomes is seen in HeLa cells (*2–4*). We might therefore expect a similar decrease in the observed rate of protein synthesis of sea urchin blastomeres if it behaved as does that of DNA, but there is no detectable decline in protein synthesis and no detectable decrease in the rate of penetration by the amino acid.

During the first cell cycle, changes in the rate of protein synthesis follow changes in the permeability rather closely, which suggests that penetration may limit the number of counts incorporated into protein (that is, that proteins are being synthesized from a pool of leucine of increasing specific activity). During the second cycle, the permeability becomes almost constant, and there is evidence that thereafter it remains so at least to the gastrula stage (*12*). The rate of protein synthesis, however, increases very greatly during the second cleavage.

The suppression of protein synthesis at metaphase in mammalian cell cultures has been attributed to stopping of transcription at the time of chromosomal condensation, which allows the population of functioning cytoplasmic messengers to decay. Polypeptide assembly is assumed to be limited by the availability of template RNA, and therefore the rate of protein synthesis decreases until transcription starts again, perhaps in the G_1 period of the next interphase. This explanation is favored by the observation that cells arrested in metaphase manifest an increasingly severe inhibition of H^3-histidine incorporation (*1*).

Scharff and Robbins (*3*) and Salb and Marcus (*2*) reject this explanation on the ground that the half-life of messenger RNA, estimated from the decay of polyribosomes after treatment with actinomycin (*5*), is much longer than the duration of normal mitosis. In addition, there is evidence that ribosomes from metaphase populations are less competent to function in vitro than are those from interphase cells (*2*). The metaphase ribosomes can be "restored" by a trypsin treatment; this is interpreted (*2*) as indicating that the nucleus, as it breaks down in prometaphase, releases an inhibitor of ribosomes which is sensitive to trypsin. However, the claim that mitotic HeLa cells support the replication of normal yields of polio virus (*4*) does not corroborate this explanation.

The inhibition of protein synthesis during metaphase of HeLa cells is in any case a fact; our results show that it is not so in another cell type. Continuity of protein synthesis at metaphase of sea urchin blastomeres neither supports nor denies the explanations offered for inhibition in the cell cultures. The exceptionally long half-life of messenger RNA in the embryo (*8*) may account for the observed continuity; this supports the idea that it is decay of the messenger that causes the metaphase inhibition in exponentially growing cells.

On the other hand, the ratio of the nuclear to the cytoplasmic volume is very much smaller in a blastomere than it is in a HeLa cell. Agents released from the nuclei during early cleavage may have a reduced effect on the relatively enormous population of cytoplasmic protein-synthesizing units. However it is clear that the rates of protein synthesis in sea urchin blastomeres have no obvious relation to the cytological changes of mitosis, and therefore the cessation of synthesis is not an obligatory result of the entry of cells into karyokinesis.

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Zinc and Cobalt: Effect on the Iron Metabolism of Ustilago sphaerogena

Abstract. *The ferrichrome content of cells of the smut fungus* Ustilago sphaerogena *during growth in a medium deficient in zinc increases with increase in the cobalt of the medium from 0 to 3 × 10^{-5}M. The addition of zinc to such cultures prevents the accumulation of ferrichrome. The results suggest that zinc is involved in the utilization of iron via a process which can be blocked by cobalt.*

Healy *et al.* (*1*) noted a striking similarity between cobalt toxicity and iron deficiency in the pattern of enzymatic activities in *Neurospora crassa*, and Padmanaban and Sarma (*2*) reported the production of a new "iron-binding compound" by the same species during growth under similar conditions. We have found that when the smut fun-