Radioautographic Evidence for the Incorporation of Leucine–Carbon-14 into the Mitotic Apparatus

Abstract. *Mitotic apparatus isolated at metaphase from sea urchin eggs, and placed in sea water containing leucine–carbon-14 after fertilization, has been shown by radioautographs to have taken up carbon-14 in such a way that it is not removable by repeated washing, by "chasing" with leucine–carbon-12, or by treatment with hot trichloroacetic acid.*

The development of techniques for isolation of the mitotic apparatus has opened the question of its origin to a direct investigation. The nonchromosomal proteins of the mitotic apparatus (M.A.) may originate at the time of cell division by: (i) formation *de novo* from precursors of low molecular weight; (ii) formation from preexisting macromolecules by rearrangement; (iii) formation by association of protein synthesized *de novo* with preexisting macromolecular proteins.

In growing systems that double in mass between each division, at least half of the material of the mitotic apparatus is presumably synthesized *de novo* prior to division (*1*). However, in sea urchin eggs, where little or no growth occurs in the short interval between fertilization and the onset of the first division, the mitotic apparatus of the first cleavage is thought to originate largely by rearrangement of existing precursor macromolecules. Went (*2*) has presented evidence in favor of the "precursor" concept by demonstrating the presence in unfertilized sea urchin (*Strongylocentrotus purpuratus*) eggs of antigens that match those of the isolated and dissolved mitotic apparatus. However, Hultin (*3*) has suggested that in eggs of the sea urchin, *Paracentrotus lividus*, part of the mitotic apparatus is synthesized after fertilization, the third suggested origin. He found that incorporation of the amino acid valine-C^{14} began shortly after fertilization. Puromycin both inhibited this uptake of valine and blocked development at the streak stage in a manner suggesting interference with formation of the mitotic apparatus. From his studies it is not certain into which proteins the valine was incorporated.

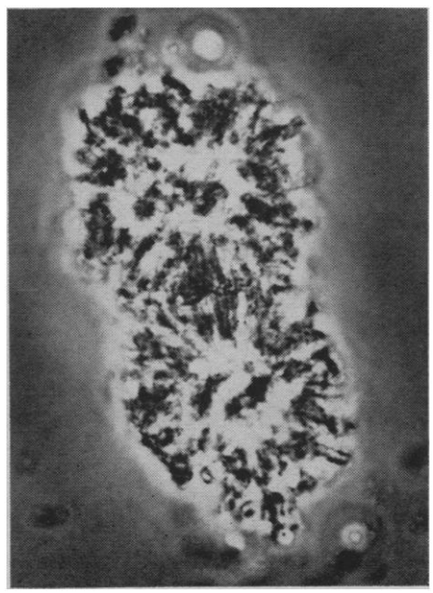

Fig. 1. Phase contrast view of *Lytechinus variegatus* mitotic apparatus isolated in hexanediol (× 1500).

Our radioautographic studies of the material isolated from sea urchin eggs at metaphase show that leucine-C^{14} added after fertilization is incorporated into some of the proteins comprising the mitotic apparatus.

Eggs were obtained (*4*) from *Lytechinus variegatus* by injection of 0.5*M* KCl, and from *Echinometra lucunter* by filtering the gonads through cheesecloth. The eggs were washed three times with sea water, treated with trypsin (*4*, p. 181) to prevent formation of the fertilization membrane, and washed by centrifugation with sea water to remove the trypsin. The eggs were then fertilized, and incubated in $10^{-7}M$ leucine-C^{14} (10.2 mc/m*M*) at 30°C until the metaphase was visible. After isolation (Fig. 1) by Kane's technique (*5*), stabilization and washing with $5 \times 10^{-4}M$ calcium chloride (*6*), and three successive 2-hour washings with excess leucine-C^{12}, the M.A. was applied to gelatinized slides and radioautographed with Ilford K-5 emulsion.

Radioautographs (Fig. 2) show that leucine-C^{14} was incorporated into the mitotic apparatus. Because of drying of this structure and viewing through the radioautographic emulsion, the precise origin of the tracks cannot be determined. The adsorption of leucine-C^{14} to these proteins is considered unlikely as it was not removed by repeated washings with nonradioactive leucine. Since particles resembling ribosomes have been observed within the mitotic apparatus of sea urchins (*7*), it is possible that the tracks we observed were from proteins being synthesized upon ribosomes within the apparatus, rather than from the structural proteins of the apparatus itself. Two experiments show that the radioactive material is probably not bound to ribosomes.

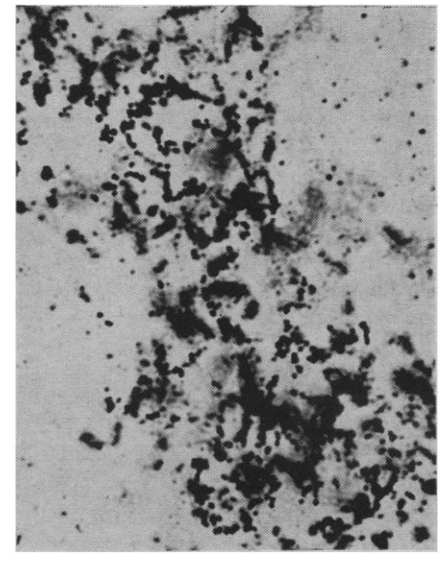

Fig. 2. Radioautograph of isolated *Lytechinus variegatus* mitotic apparatus after exposure for 11 days (× 2190).

In the first experiment, leucine-C^{14} was added to eggs of *E. lucunter* 5 minutes after fertilization. Fifteen min-

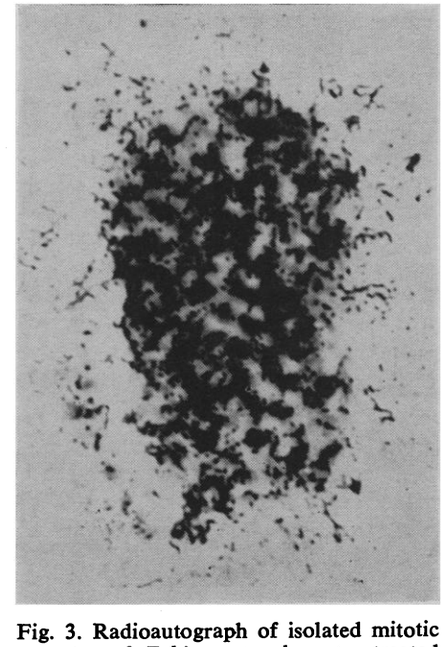

Fig. 3. Radioautograph of isolated mitotic appartus of *Echinometra lucunter* treated with hot trichloroacetic acid. Radioautograph exposed for 7 days (× 1950).

utes later the eggs were washed two times with excess leucine-C^{12} and resuspended in sea water containing excess leucine-C^{12} until the M.A. was isolated. If the labeled material observed in the previous experiments were due to adsorbed leucine-C^{14} or to independent nascent proteins formed upon ribosomes within it, the M.A. isolated in this experiment should not be labeled (*8*). Since the M.A. isolated after this treatment was still radioactive, we believe that the labeled protein was either part of the M.A. or that the proteins are retained on the ribosomes for a longer period in sea urchin eggs than in other systems. The latter possibility we consider unlikely because leucine-C^{14} was rapidly incorporated into proteins after its addition to the system.

If the transfer-ribonucleic acid binds the growing peptide chain to the ribosome by covalent bonds (*9*), then hydrolyzing the RNA by treatment with trichloroacetic acid should release nascent protein from the ribosome and allow the protein to be washed away. In the second experiment, the mitotic apparatus isolated from cells grown continuously in leucine-C^{14} was washed as described, and treated with trichloroacetic acid at 90°C for 15 minutes to remove nucleic acids. The M.A. fraction was then washed in the presence of excess leucine-C^{12}, two times in $5 \times 10^{-4}M$ $CaCl_2$, three times in distilled water, applied to albuminized slides,and radioautographed. The M.A. retained its achromatic structure, and the label (Fig. 3) remained associated with the M.A.

The mitotic apparatus may be considered as both a cell organelle and a region of the cell (*1*, p. 236). However, the M.A. probably consists primarily of material directly concerned with cell division, for during its formation particles such as mitochondria and yolk are pushed outside its boundaries (*10*) and proteins isolated from it are relatively few and are present as discrete, homogeneous components (*11*).

Although we cannot state that in our experiments leucine-C^{14} was incorporated into the structural protein of the M.A., we can say that leucine-C^{14} was incorporated into protein present in the M.A. isolated by our techniques, and that the labeled material was retained after treatments designed to remove free amino acids and protein bound to ribosomes. Our results are consistent with the idea that part of the M.A. proteins in sea urchin eggs is synthesized between fertilization and metaphase of the first division.

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Allelic Mapping in Yeast by X-ray–Induced Mitotic Reversion

Abstract. *A new method for determining the sequence of mutational sites is based on the linear dose-effect relation for x-ray induction of allelic recombination in* Saccharomyces cerevisiae. *Mutations at two loci have been mapped by this method. The use of x-ray simplifies allelic mapping and greatly increases its sensitivity.*

When two independently isolated mutant alleles of a given locus, a_1 and a_2, are placed in repulsion in the same diploid yeast cell (a_1/a_2), this heteroallelic diploid has a much higher frequency of reversion to wild type during mitotic division than either of the homoallelic combinations (a_1/a_1 or a_2/a_2). A process of allelic recombination in heteroallelic diploids has been suggested to account for this greater frequency (*1*). The effect can be stimulated both by ultraviolet light (*2*) and by x-rays (*3*). While in the case of ultraviolet light a nonlinear dose-effect relation is observed, with sublethal doses of x-rays the number of induced revertants is proportional to the dose. We have found that the value of the slope of the x-ray curve depends on the pair of alleles involved. The nature of this dependence provides the basis of a new method for determining the sequence of alleles within a gene. We have tested this method at two nutritional loci in *Saccharomyces cerevisiae:* ar_4 (arginine biosynthesis) and tr_5 (tryptophan synthetase).

The strains used in these experiments were cultured in 10 ml of rich liquid medium (2 percent yeast extract, 4 percent peptone, and 4 percent dextrose) to avoid preferential selection of revertants (prototrophs) which occur spontaneously during growth. Cultures were vigorously swirled in 50-ml erlenmeyer flasks for 3 days at 30°C. Between 10^6 and 10^8 washed cells, the number depending on the alleles and the dose, were plated (four plates per dose point) on synthetic medium lacking either arginine or tryptophan. For each dose about 200 cells were plated on each of four plates of complete medium to assay viability. X-rays from a beryllium-window tube (Machlett OEG 60) were delivered to the cells on the agar surface. The x-ray tube was operated at 50 kv (peak) and 20 ma without additional filtration, giving a dose rate of about 200 r/sec at the position of the cells. Visible colonies were counted after 3 to 5 days of incubation at 30°C. Less than 1 percent additional colonies appeared after 5 days of incubation.

Dose-frequency curves for diploids with various allelic combinations at the ar_4 locus are shown in Fig. 1*A*. The slopes of these curves are highly reproducible from one culture of the same strain to another, while the intercept is extremely variable because of the clonal distribution of spontaneous revertants (*4*).

The allelic map in Fig. 1*B* was constructed from the data in Fig. 1*A* on the basis of the assumption that the slope of each curve is proportional to the distance between the two alleles involved. The map intervals so determined are consistently additive.

Figure 2 is a map, constructed by the same method, of several alleles at the tr_5 locus. Again, the intervals are consistently additive, permitting the sequence to be unambiguously determined.

The sequence assigned to the ar_4 alleles has also been confirmed by an entirely independent method. A *cis-*