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11 January 1965

Protein Synthesis and the Mitotic Apparatus

Abstract. *Sea urchin eggs, exposed to a radioactive amino acid during the period from fertilization to metaphase of the first cleavage, incorporate the label into proteins. Some radioactivity is localized in the region of the mitotic apparatus (MA). The specific activity of proteins from isolated mitotic apparatus is more than three times that of the proteins from the rest of the cell, although under the conditions used for isolation only 11 percent of the total incorporated label is in the MA. The radioactivity is found in proteins that do not contribute significantly to the bulk of the MA protein, but this description might possibly apply to the fibrous skeleton. Electron microscope autoradiograms reveal a close association of radioactivity with the fibers.*

Some protein synthesis is required in order for a mitosis to be completed in the developing sea urchin embryo (*1*). When fertilized eggs are exposed to labeled amino acids during the first and subsequent cleavage cycles, the mitotic apparatus and its associated structures become radioactive. This was shown by Gross and Cousineau (*2*) with autoradiograms from sections of the intact embryo and by Stafford and Iverson (*3*) with preparations of isolated mitotic apparatus (MA). Their preparations were highly radioactive although made from eggs whose exposure to label had been terminated and followed by an excess of unlabeled amino acid before metaphase.

These observations suggest that components of the MA are among the first products of the protein synthesis that are activated at fertilization (*4*), and the suggestion is strengthened by the finding in whole-egg autoradiograms that the spindle region is preferentially labeled (*2*). Thus the new proteins that associate with the MA might constitute an important fraction of those made in the period of early cleavage. Preferential localization is lost after the first few divisions; this implies that the "mitotic" proteins, if such indeed are being observed, become a less prominent class among those synthesized as development proceeds.

This interpretation has a number of important consequences for the analysis of mitosis, and a careful study of possible alternatives is therefore justified. Some of the more obvious ones were considered in the earlier papers (*2*, *3*), and have been discarded. Examination of one of the important remaining alternatives is the objective of experiments to be described.

Let us suppose that most of the protein in the region of the MA, *in situ* and as isolated, is associated specifically with the structure or function of that organelle. Contaminant proteins would make, by definition, a minor contribution to the mass. In consequence, the observed patterns of localization of radioactive protein would imply that some of the proteins of the mitotic apparatus must be newly synthesized during the cell cycle. But there is reason to believe that at least a large fraction of the protein in the MA is not specifically associated with its structure or function. Evidence from electron microscopy provides the main support for this statement (*5–7*), and more is provided by the experiment presented here. The region in which the MA is forming extrudes large cytoplasmic particulates—pigment granules, yolk particles, and mitochondria—but seems to retain, at least in the electron-microscope image, a normal sample of the ground cytoplasm, including ribosomes and smooth-surfaced vesicles. Thus, the concentration of large particulates (not likely to contain much newly synthesized protein) is perforce increased in the peripheral cytoplasm, while that of the ground cytoplasm, in which complete new proteins presumably first appear, is increased in the region of the MA. The earlier autoradiographic observations might therefore be explained on the basis of a passive concentration of new proteins having no structural or functional association with the MA itself.

The experiments which aid in evaluating this explanation are concerned with (i) the specific radioactivity of the proteins in the isolated MA as compared with the specific activity of the rest of the cell, (ii) with the chromatographic behavior of the proteins isolated from the MA, and (iii) with the localization of radioactivity in the isolated MA as indicated by the electron microscope. The results fit the original interpretation well, and can be reconciled with the alternative given only with some difficulty.

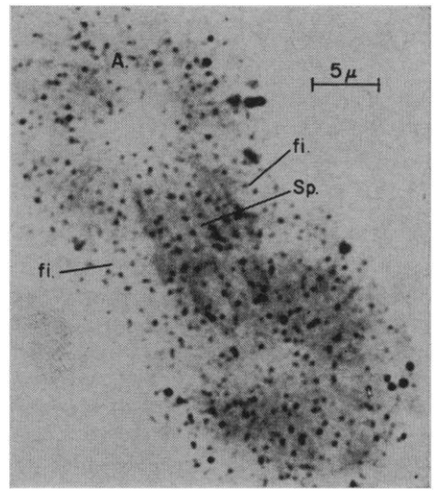

Fig. 1. Optical autoradiogram of a 1-μ section from the MA pellet. Stained with toluidine blue. Ilford L-4 emulsion exposed for 7 days. *A.*, aster; *Sp.*, spindle. The MA shown is at very early anaphase. Fibers (*fi.*) stain more darkly than the matrix, and silver grains tend to be distributed along their length.

The MA was isolated by a modification of the method of Kane (*8*). *Arbacia punctulata* were induced to shed their gametes (*9*). The eggs were fertilized, and their fertilization membranes were removed by a brief exposure to 1*M* urea, after which the zygotes were washed and resuspended

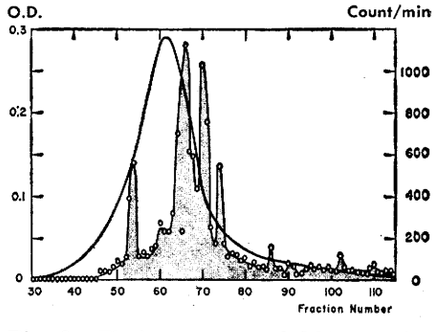

Fig. 2. Elution pattern of MA proteins dissolved in urea, from a column of Sephadex G-200. A part of the elution profile (85 of 130 fractions) containing more than 95 percent of optical density (O.D.) and counts, is represented. The continuous line represents optical density in the effluent. Circles and shaded region represent radioactive protein. Counts and O.D. have been adjusted to correspond, by correction for the dead space in tubing (0.75 ml). Fractions were collected in drops to 0.34 ml each.

in artificial free sea water that contained no calcium ion, the composition being, in grams per liter: NaCl, 26.5; KCl, 0.7; $MgSO_4$, 11.9; $NaHCO_3$, 0.5. This medium contained tritiated leucine, 10 μc/ml (*10*), and the embryos remained therein until 50 percent of them were at metaphase of the first cleavage (40 minutes after fertilization at 21°C).

After centrifugation and washing with the aforementioned sea water without isotope, the eggs were suspended in isolation medium consisting of 1*M* hexanediol, 1m*M* ethylenediaminetetraacetate, disodium salt (EDTA), and 10m*M* potassium phosphate buffer, at *p*H 6.3. The cytoplasm was dispersed and the MA's were released by vigorous shaking. The MA's were collected by centrifugation for 2 minutes at 500*g*. This low-force centrifugation results in a lowered yield of MA, but it prevents the sedimentation of contaminating small particulates, such as yolk. The first supernatant was saved. Pellets were washed in a large volume of fresh isolation medium and then again with a solution containing 0.05*M* KCl, 10m*M* potassium phosphate buffer, at *p*H 7.0, and 1*M* EDTA. Supernatants from these washings were combined with the original one. The cells were thus partitioned into two fractions, isolated MA (with any contaminants) being in the pellet, and everything else in the supernatant.

The two fractions were then processed for analysis and counting of the purified total protein. Parts of the pellet were fixed in 1 percent OsO_4 in 0.05*M* KCl at *p*H 6.3 or in OsO_4 isolation medium, and embedded in "Epon 812" (*11*) or in a mixture of methyl and butyl methacrylates (20:80) for sectioning on a Servall MT-2 microtome. Sections 1 μ thick were cut and used for optical microscopy and autoradiography. Silver or gold thin sections were used for electron microscopy and for autoradiography in the electron microscope. The light microscope preparations were coated with Ilford L-4 emulsion, exposed for 7 days, and examined after staining with toluidine blue. The preparations for electron microscopy were exposed for 10 days, and the procedure for autoradiography was essentially that of Hay and Revel (*12*). These preparations were examined with a Hitachi HU-11A electron microscope operating at 75 kv and with a 50-μ objective aperture.

An optical photomicrograph of one of the thick-section autoradiograms (Fig. 1) shows that the isolated MA's were highly radioactive. The fibers running longitudinally through the spindle and radiating outward from the centers in each aster are particularly well-differentiated in specimens of this type, since fibers take the toluidine-blue stain more strongly than does the surrounding matrix of ground cytoplasm. The autoradiograms (Fig. 1) give a strong impression of close association between the silver grains and fiber tracts; indeed, the grain pattern almost provides a diagram, in optical specimens as thin as this, of the fiber distribution.

In order to determine the way in which radioactivity is partitioned be-

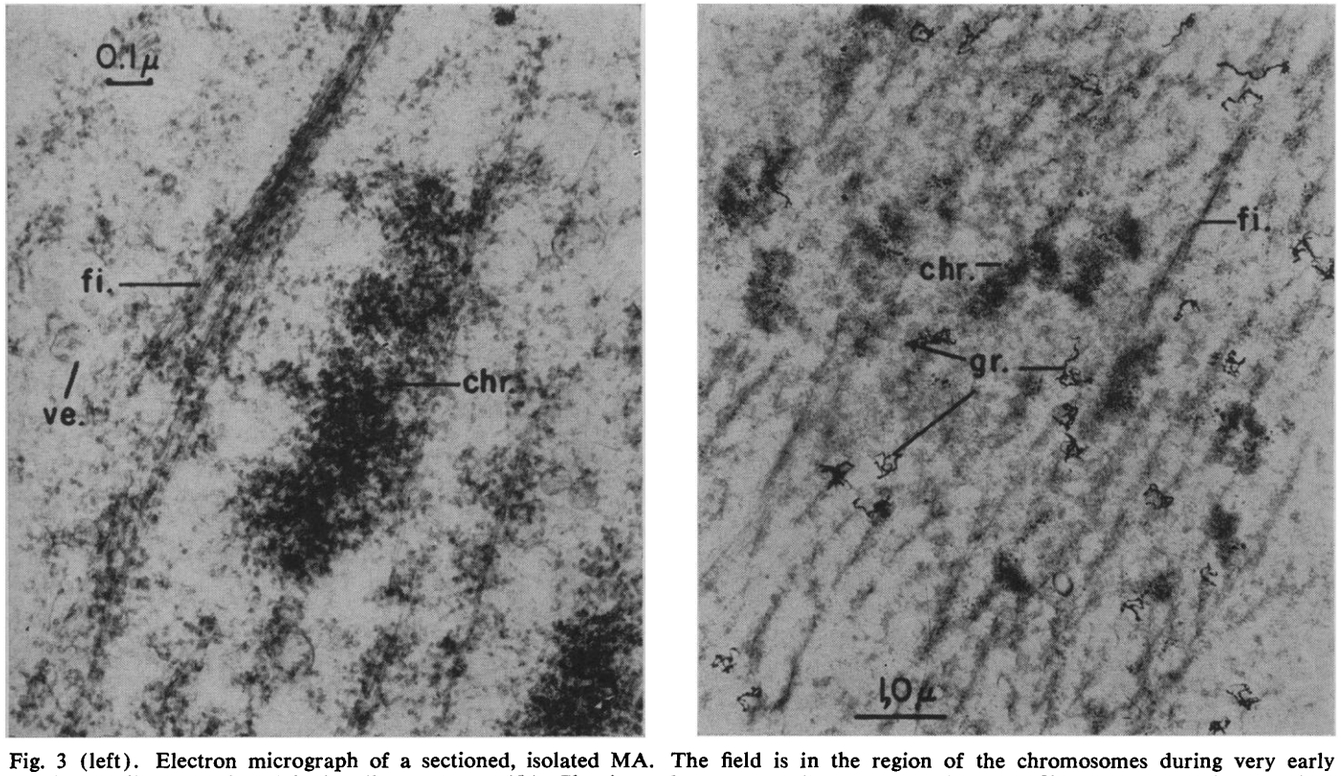

Fig. 3 (left). Electron micrograph of a sectioned, isolated MA. The field is in the region of the chromosomes during very early anaphase. Fibers are found in bundles or tracts (*fi.*) *Chr.* is a chromosome. Large spaces between fiber tracts contain abundant small particles, fibrillar reticulum, and vesicles (*ve.*). Organization of the interfibrillar matrix is not markedly different from that of the ground substance elsewhere in the cell. Fig. 4 (right). Electron-microscope autoradiogram, at low magnification, from a section of the MA in the region shown in Fig. 3. The figure is at early anaphase, and chromosomes (*chr.*) are aligned from upper left to lower right. Individual fibers cannot be distinguished at this magnification, but the fiber tracts (*fi.*) stand out, by virtue of their higher electron density, from the less-organized matrix. Silver grains (*gr.*) show as dense, tangled skeins. More than half of the grains are directly over fibers, or immediately adjacent to them. This is in spite of the fact that clearly nonfibrous regions account for most of the area in the field. Distances between fiber tracts are large enough so that, despite the large grain size, localization of radioactivity on or near fibers is apparent. Random distribution of grains in an equal area would place about 20 percent on fibers; this is determined by placement, with the aid of a random-number table, of circles having a diameter equal to the mean grain diameter.

tween the MA and the remainder of each cell, the two fractions described earlier were subjected to an exhaustive purification procedure, which included several cycles of washing with cold 5 percent trichloroacetic acid (TCA), boiling in 5 percent TCA, dissolving the precipitate in 1*M* NaOH, warming to 50°C, reprecipitating with excess cold TCA, and washing with a series of lipid solvents. The protein content of each fraction and of all samples for counting was determined by the method of Lowry *et al.* (*13*). The original solutions contained, for the supernatant proteins, 25.8 mg/ml, and for the corresponding MA proteins, 1 mg/ml. That 4 percent of the total protein was recoverable in the MA fraction is somewhat at variance with the result of Mazia and Roslansky (*14*), who found, with another isolation method, about 10 percent of the total cell protein in the isolated MA. The technique used by these authors is likely, however, to give a better yield and less extracted MA than the one used by us, and hence we consider the 10-percent figure a more reliable guide to the protein distribution that obtains in vivo.

About 11 percent of the radioactivity (Table 1) is recovered in the MA pellets and about 89 percent in the supernatant. Using somewhat different methods, Bibring and Cousineau have recently obtained 10 percent of the counts in the MA fraction (*15*). But in our experiments the specific activity of the MA pellet is more than three times that of the combined proteins of the rest of the cell. This difference in specific activity is probably a minimum value for two reasons: First, the yield of MA is likely to be a good deal less than 100 percent, since low-speed centrifugation leaves a fraction of the MA unsedimented, and the pellet contains a large number of single asters and partially dissolved MA; therefore, some of the radioactivity associated with the MA must remain in the supernatant under the conditions chosen (in behalf of clean MA in the pellet). Second, the contamination of the pellet, which is a few percent, is due mainly to unbroken eggs, which would, on the basis of the counting results, tend to lower the specific activity of the pellet proteins.

In order to reconcile these counting results with the proposal that the labeling is nonspecific and unrelated to the working parts of the MA, it would be necessary to abandon the notion that the proteins unique to the MA are a quantitatively predominant fraction of the isolated material. But the bulk-counting data give no information about whether a quantitatively major or minor component has been labeled, nor about whether that component is a part of the fibrous skeleton of the MA. The behavior of dissolved MA proteins in gel-filtration gives some useful information on this point.

Table 1. Radioactivity of isolated mitotic apparatus and supernatants. The indicated quantities of protein were dissolved in 1 ml of hydroxide of hyamine (Packard Instrument Co.). Samples diluted with 15 ml of toluene-based scintillation fluid; counted in a liquid scintillation counter, approximately 15-percent efficiency.

Sample	Protein content (mg)	Radioactivity	
		Total (10^4 count/min)	Specific activity (10^4 count/min per mg)
MA I	0.5	4.54	9.08
MA II	.5	4.23	8.46
Supernatant I	5.0	13.63	2.73
Supernatant II	5.0	12.90	2.58

The mild methods for "dissolving" the MA (high concentration of salt, changes in *p*H) fail to produce well-behaved molecular dispersions. Urea treatment, however, dissolves MA pellets effectively, and the solution can be used for a number of types of chromatographic and centrifugal separation. In our experiments, C^{14}-labeled isolated MA's were dissolved in 8*M* urea, and the solutions were centrifuged at 2500 rev/min (Servall SS-33 head) for 30 minutes to remove debris and any undissolved material. The supernatants were then placed on a column of Sephadex G-200 (3 × 14 cm) equilibrated with 0.2*M* KCl (*14*). The effluent from this column was passed through the 1-cm flow-cell of a Gilford recording spectrophotometer, and a continuous record of the optical density at 280 mμ was obtained. One hundred thirty fractions of 0.340 ml were collected. Each fraction was supplemented with carrier bovine serum albumin, precipitated with TCA, and purified as described earlier; the purified precipitate was collected on a Millipore filter and counted in a low-background gas-flow counter at an efficiency of 15 percent. All of the optical density and more than 95 percent of the counts placed on the column were recovered (Fig. 2). Two features of these results are important for our discussion. First, the radioactivity and the optical density are not coincident. Therefore, the new proteins are different from the bulk components of the isolated MA. Second, the radioactivity is eluted as a group of sharp and well-defined peaks. Since the combination of urea treatment and gel filtration tends to separate proteins as subunits and on the basis of molecular weight (*16*), it is possible that the entire pattern has been generated by the initial breakdown of a few labeled species into subunits that reaggregate in the column once the urea has been left behind in the gel. In any case, this result means that something other than the bulk of the MA accounts for the very high specific activity and that most of the radioactivity may be in a small number of species.

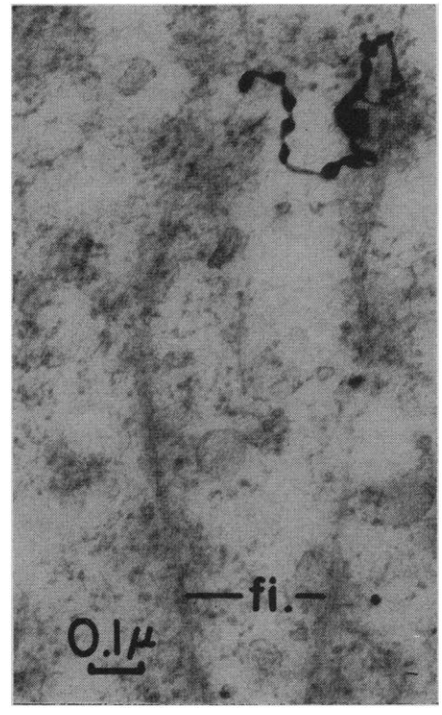

Fig. 5. A higher-power electron-microscope autoradiogram from a field such as that shown in Fig. 4. The characteristic association of silver grains with fibers (*fi.*) is represented. Particles and vesicles are in the matrix.

The pellets for electron microscopy were fixed in OsO_4 at *p*H 6.3. They were exposed to 1 percent phosphotungstic acid in 100 percent ethanol at the end of the dehydration series, (*6*). Additional contrast was obtained by a final staining of the exposed and developed autoradiograms with lead citrate (*17*) in 0.01*N* NaOH, a step which removes the emulsion but not the silver grains.

Figure 3, an ordinary electron micrograph of a section of an isolated spindle, is included to show the quality of the material from which the autoradio-

grams displayed at lower magnification were made. The appearance of the spindle has been described by Kane (*6*) and, for MA *in situ*, by Harris (*7*) and Gross *et al.* (*5*). The fibers, in the spindle region, run in bundles or tracts which are separated by space of a few thousand angstroms to several microns. In the spaces there are very large numbers of small particles and some vesicles. The particles resemble ribosomes and may contribute a major fraction of the total protein of the isolates.

The field of the low-power autoradiogram (Fig. 4) contains the metaphase chromosomes and a number of fiber tracts, the latter clearly distinguishable by their increased electron density with respect to the nonfibrous matrix. This field is representative in that the silver grains tend to lie either directly over or very close to the fiber tracts. The field shown contains 26 silver grains (or perhaps a few more if some of those shown are double). Seventeen of these, at least, are directly over fibers or fiber tracts. This means that about 62 percent of the radioactive disintegrations that occurred during the period of exposure had their sites in or immediately adjacent to fibers which occupy a very small fraction of the total area of the specimen. Figure 5 shows, at higher magnification, the association of a silver grain with a pair of fine fibers and, in addition, the appearance of the nonfibrous matrix in the vicinity.

The electron microscope autoradiograms therefore confirm the impression given by the optical autoradiograms shown in Fig. 1. The radioactivity in the isolated MA tends toward a distinct association with the fibers, which represent the structural skeleton of the organelle. It might be argued that the fibers present a surface for nonspecific adsorption of radioactivity from labeled proteins in the matrices. But in that case one would expect to find only some label on the fibers, and not a strong tendency for it to localize on them. With regard to low molecular weight precursors trapped in the preparations, the routine fixation and embedding treatments render considerably more of the label soluble (and hence remove it) than does treatment with the usual protein precipitants, such as TCA. A similar conclusion has been reached by Caro and Palade (*18*) for other specimens.

Taken together, these results support the original interpretation of the acquisition of radioactivity by the MA during exposure to labeled amino acids, namely that proteins associated with the structure or function of this organelle are synthesized during cleavage in the sea urchin egg, as they must be in tissue cells (*19*), and that these proteins are an important fraction of the total made during early cleavage. They also justify an enlarged effort to separate and purify the proteins of the isolated MA by various methods, so that an accurate identification of the preexisting and newly synthesized species can be made (*20*).

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16. Sephadex G–200 from Pharmacia Fine Chemicals, Inc. The Sephadex column cannot be equilibrated with urea directly, because in this solvent the gel grains swell excessively and flow rates fall almost to zero. With the method described, the urea occupies only a small region of the column and the protein moves ahead into a urea-free zone. Under such conditions hemoglobin is eluted as two peaks. The MA proteins, labeled and unlabeled, are eluted several "bed volumes" earlier than a hemoglobin marker.
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20. These data make no direct contribution to the question of how the total protein prepared from the isolated MA relates to the protein of the fibers. The most recent review of this topic is by A. Zimmerman in *The Cell in Mitosis*, L. Levine, Ed. (Academic Press, New York, 1963). The discussion following his paper is also relevant. Indirectly, our labeling data suggest that the protein of the fibers may be different from the bulk protein of the isolates, but this is by no means proven. The fibers may be in equilibrium with a matrix precursor protein.
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Agrobacterium tumefaciens: Thermal Inactivation of Tumor-Inducing Ability

Abstract. *Treating cultures of* Agrobacterium tumefaciens *at 39° to 48°C reduces the infectivity of the bacteria without necessarily affecting viability. Destruction of the capacity to initiate tumor growth follows first-order kinetics from which rate constants for thermal inactivation are derived. From these rates, values for heat of activation of 56.7 kcal mole^{-1} and entropy of activation of 107 cal mole^{-1} deg^{-1} are obtained. A particular protein or nucleoprotein active in the process of infection may be inactivated by the treatment.*

Treatment of plants infected with *Agrobacterium tumefaciens* (Smith and Town.) Conn, the organism that induces crown-gall tumors, at temperatures of 32° to 35°C inhibits tumor formation (*1, 2*). Braun (*3*) has calculated an activation energy for this process of 80 kcal mole^{-1} or greater. We used a quantitative assay, similar to the ones for plant viruses, for the infectivity of *A. tumefaciens* (*4*) to reexamine the effect of temperature treatments on the virulence of the bacterium without the complications involved in subjecting both bacterium and host to the treatments.

Shake cultures of *A. tumefaciens* strain B6 were grown for 48 hours at 27° ± 1°C in a medium consisting of Bacto nutrient broth and 0.1 percent yeast extract (*5*). Portions (50 ml) of such cultures in 250-ml erlenmeyer flasks were heated for various periods in a constant-temperature water bath. Temperatures ranged between 39° and 48°C. Samples (2 to 3 ml) were removed at various times and pipetted into sterile tubes held in crushed ice. Portions of each sample were then diluted for assaying infectivity and for making counts of viable cells. At least three different cultures were treated at each temperature. The infectivity of each sample was measured on 12 to 18 primary pinto bean leaves (*4*). Plate counts of viable cells were made from one or two dilutions of a serial tenfold dilution series; three plates were used to measure each dilution. The inoculation of both leaves and plates was usually