SYNTHESIS OF A COLCHICINE-BINDING PROTEIN DURING THE HELA CELL LIFE CYCLE

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Cells or tissues exposed to colchicine-^{3}H bind the isotope in direct relation to the morphologically visible number of microtubules that they manifest in the electron microscope (1). Those species, such as *Physarum polycephalum*, which have no discernible microtubules do not bind colchicine. Homogenization of tissues followed by sucrose density gradient analysis have demonstrated that the colchicine-binding activity runs as a 6S protein (1). When the central pair of microtubules in sea urchin sperm tails is selectively solubilized with EDTA, the extracted protein is rich in colchicine-binding activity, migrates as a single peak on acrylamide gels and, like the colchicine-binding activity from tissue homogenates, has a sedimentation coefficient of 6S (2). The apparently selective binding of colchicine to microtubule protein provides a convenient means of identifying it in heterogeneous preparations. We have thus used a combination of colchicine-^{3}H binding and leucine-^{14}C incorporation to trace the synthesis of the colchicine-binding protein during the cell life cycle in synchronized HeLa cells. Since the mitotic spindle is composed of microtubules, we make the tentative assumption that the pattern of synthesis demonstrated for colchicine-binding protein is applicable to the mitotic spindle.

MATERIALS AND METHODS

HeLa cells of the S_3 strain were routinely maintained in spinner culture as previously described. For synchronization, they were placed in monolayer culture containing only the Ca^{++} present in the serum supplement (3). Mitotic cells were selectively detached by gentle rocking as previously described (3) and placed in spinner culture. Aliquots of 10^7 cells were removed from spinner at 0, 3, 7, 9, and 15 hr after collection, washed in leucine-free medium and pulsed for 20 min with 15 μc of leucine-^{14}C (New England Nuclear Corp., Boston) in 20 ml of leucine-free medium. These time points were chosen so that the populations were in the mitotic, G_1, early S, mid-S, and late S-G_2 phases of their life cycle, respectively (3). Following exposure to the label, the cells were pelleted, washed, swollen in 2 ml of 0.01 M PO_4 buffer containing 0.001 M Mg^{++} at pH 7.2, and homogenized in a Dounce-type homogenizer. Nuclei were removed by centrifugation at 1000 g for 10 min, and particulate matter was removed by spinning at 100,000 g for 40 min in the 40 rotor of the Spinco Model L2 ultracentrifuge. The supernatant fluid was exposed to 2 μc/ml of colchicine-^{3}H at 37°C according to Borisy and Taylor (1). A 0.3-ml volume of the incubation mixture was placed directly on 4.5% acrylamide gels made according to Maizel but minus the detergent, sodium dodecylsulfate. Electrophoresis was carried out at pH 7.2 (7 ma/gel)

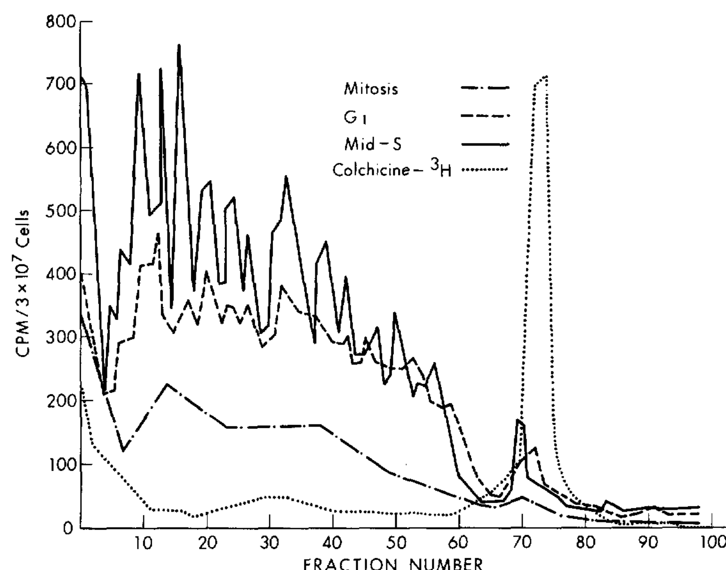

FIGURE 1 Acrylamide gel electropherogram of 100,000 *g* supernate from HeLa cells in G_1, mid-S, and mitosis pulsed with leucine-^{14}C and then labeled in vitro with colchicine-^{3}H. Only the colchicine label from the G_1 sample is shown. The colchicine label from the S sample was similarly exactly superimposed on the most rapidly migrating leucine peak but is not plotted, to avoid confusion.

for 20–30 hr, determined arbitrarily for each batch of acrylamide. The electrophoresis buffer was 0.1 M PO_4, 0.5 M urea. Gels were fractionated into scintillation vials with the Maizel autogeldivider (4) and counted in the Beckman Model 200 B liquid scintillation counter.

In some experiments, the colchicine-binding band detected on the gel was isolated by fractionating the gel in PO_4-Mg^{++} buffer at pH 7, 0°C, and then counting a small aliquot of each fraction to localize colchicine-^{3}H activity. The gel was spun off, and those tubes containing the peak activity in the supernate were placed in dialysis tubing and concentrated against Carbowax flakes (Union Carbide Co., New York). The eluted protein was run on 5–20% sucrose gradients in the SW 65 head at 2°C according to Borisy (1), while the 100,000 *g* supernate that had been incubated with colchicine-^{3}H but had not been run on gels was run on parallel gradients. An excess of unlabeled colchicine (10^{-3} M) was added to all gels and gradients for preventing any exchange of label after the initial incubation.

RESULTS AND DISCUSSION

Fig. 1 shows the distribution on gels of colchicine-^{3}H and leucine-^{14}C from the 100,000 *g* supernate of populations in the G_1, mid-S, and mitotic phases of their life cycle. The time points of early and late S were similar to that of mid-S and are not plotted, so as to avoid confusion. Likewise, the colchicine-^{3}H, while present in all samples, is plotted only once. The bound colchicine is localized in a single sharp peak superimposed on the most rapidly migrating leucine peak. This fortuitously clean separation from the numerous, more slowly migrating leucine-labeled proteins and the superposition of the colchicine-^{3}H activity indicate that both the colchicine and the leucine are in the same protein. The incorporation of labeled leucine into the colchicine-binding peak throughout interphase establishes that synthesis of this protein is continuous. The plot derived from the mitotic population suggests that the rapidly migrating leucine (colchicine-binding) peak probably is not synthesized at this time. The small hump that does appear could be due to cells that have passed into G_1 during the 20-min pulse; however, this point remains uncertain since it is difficult to complete a pulse of reasonable duration without some of the cells slipping into G_1. Fig. 1 also shows that the amount of colchicine-binding protein synthesized during the various stages of interphase is approximately proportional to the other proteins synthesized; we have consistently noted that there does not seem to be any preferential time for its synthesis even though this might have

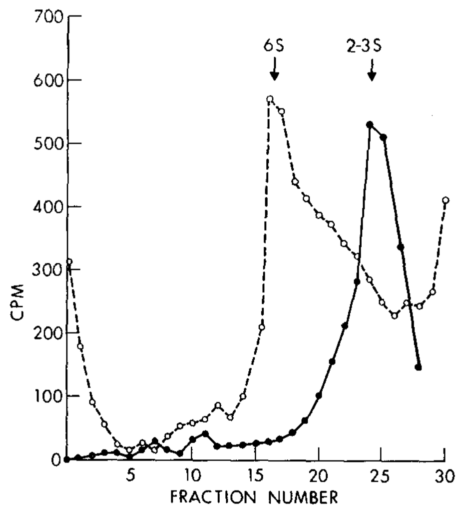

FIGURE 2 Sedimentation patterns of leucine-^{14}C label in colchicine-binding protein eluted from acrylamide gels and of colchicine-^{3}H-labeled 100,000 g-supernate run on 5–20% sucrose density gradients. The eluted protein (....) runs at 2–3 S, while the 100,000 g supernate run directly (—o—o—o—o—) contains a colchicine-binding protein that runs at 6S.

been expected in G_2 when preparation to form the mitotic spindle is made. Since the number of bound colchicine counts is a function of the amount of colchicine-binding protein present, it might be expected that the height of the colchicine peak would reflect cumulative synthesis during the cell cycle. This had been generally true, but the variable degree of dissociation of bound counts during electrophoresis has resulted in occasional inconsistencies. Thus, quantification with leucine label is preferable with colchicine serving as marker.

Only one colchicine-binding peak appears on acrylamide gels prepared and run as described; however, its rapid migration compared to the other labeled proteins raises the question of whether it was in the same state of aggregation as the 6S protein observed by others on sucrose density gradient analysis. Fig. 2 shows that when the rapidly migrating, colchicine-binding protein was isolated from the acrylamide gel and run on sucrose gradients, it migrated as a 2-3S protein rather than 6S. The colchicine-to-protein bond is a relatively unstable one (1), and most of the colchicine-^{3}H counts that run with the rapidly moving leucine label in the gel dissociated during the gel fractionation and density gradient spin; however, this problem was circumvented by the presence of the leucine-^{14}C which served to identify the position to which the protein of interest had migrated.

Protein with a sedimentation coefficient of 6S readily migrates into 4.5% acrylamide gels. Therefore, the fact that the single colchicine-binding protein isolated from our gels is a 2-3S protein rather than 6S indicates either that the 6S form has been converted to 2-3S during electrophoresis or that the 2-3S form is the native form and that a degree of aggregation obtains during centrifugation. This aggregation, if it were to occur, would necessarily be characteristic of only the initial spin which takes place in the presence of cytoplasm, since Fig. 2 shows that it is not manifest when the gel-purified product is subsequently subjected to the same conditions of centrifugation. The basis for this paradoxical behavior is not clear; however, work presently in progress with microtubule protein purified by vinblastine precipitation (5, 6) suggests that the 6S and 2-3S forms are indeed interconvertible under certain conditions.

If one assumes the identify of colchicine-binding protein and microtubule protein, it follows that close upon the termination of one mitosis the cell undertakes accumulation of microtubule protein which it presumably will use in the next mitosis.

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Addendum: Since submitting this paper, we have confirmed the continuous interphase synthesis of microtubule protein by isolating it from pulse-labeled, synchronized cells via vinblastine precipitation (5).

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