Proc. Natl. Acad. Sci. USA
Vol. 74, No. 4, pp. 1610–1613, April 1977
Cell Biology

Synthesis and activation of mitotic Ca^{2+}-adenosinetriphosphatase during the cell cycle of mouse mastocytoma cells

(mitosis/Ca^{2+}-regulation/cell synchronization)

CHRISTIAN PETZELT AND DORIS AUEL

Institute for Cell Research, German Cancer Research Center, P.O. Box 101949, D-6900 Heidelberg, West Germany

Communicated by Daniel Mazia, January 24, 1977

ABSTRACT **The activity of a Ca^{2+}-adenosinetriphosphatase (ATP phosphohydrolase, EC 3.6.1.3) increases during the mitotic phase of synchronized mouse mastocytoma P-815X2 cells. The enzyme is synthesized mainly during a distinct period of the interphase and is activated at mitosis. It is thought to regulate the formation of the mitotic apparatus by controlling the concentration of Ca^{2+} ions at the site of formation of the mitotic spindle.**

Ca^{2+} ions have been implicated as regulating factors in mitosis (1). They can interfere with microtubule assembly (2–4) and a system capable of controlling the concentration of these ions at the place of spindle formation has been tentatively identified (1, 5).

In sea urchins a Ca^{2+}-adenosinetriphosphatase (ATPase; ATP phosphohydrolase, EC 3.6.1.3) exists which shows in early development cyclic fluctuations during the cell cycle; one peak of activity occurs in the first half of the cell cycle and another one at the time of mitosis (6, 7). Whereas the meaning of the first peak is as yet unclear, some evidence has accumulated that the activity increase of the Ca^{2+}-ATPase at mitosis is involved in the regulation of mitosis. If the length of the cell cycle is altered experimentally, the increase of the enzymatic activity still remains linked with mitosis (7, 8). In parthenogenetically activated eggs the activity of the enzyme rises whenever a spindle-like figure is formed (5). If only parts of the cell cycle are turned on, which in sea urchins can be performed by NH_3 treatment of unfertilized eggs (9, 10), the Ca^{2+}-ATPase cycle is also turned on, reaching its maximum of activity when the chromosomes show a mitosis-like configuration (11).

These and the following results suggest that the enzyme is indeed involved in the regulation of mitosis. Therefore, we propose to name this enzyme the "mitotic Ca^{2+}-ATPase."

The experiments so far described were carried out on sea urchin eggs, in which cell divisions follow each other without cell growth during interphases. There, fluctuations of an enzyme activity are likely to represent the activation of pre-existing proteins rather than production of additional enzyme molecules. The same Ca^{2+}-ATPase has also been found in growing cells (mouse fibroblasts) and a cyclic change of activity with a peak at mitosis has been observed (12). The purified enzyme promotes the polymerization of brain tubulin onto isolated mitotic apparatus from L cells (12). The interaction of tubulin from nonneuronal cells with isolated spindles has been reported to be enhanced in the presence of the enzyme (13). In the present work we measure the synthesis and the activity of the mitotic Ca^{2+}-ATPase through the entire cell cycle in synchronized mouse mastocytoma cells. The synthesis of new enzyme proteins takes place during interphase. The peak of the enzymatic activity occurs during mitosis, as in sea urchin eggs, and must reflect the activation of preexisting enzyme molecules.

MATERIAL AND METHODS

Cells. Mouse mastocytoma P-815X2 cells (14) were grown in medium I supplemented with 10% horse serum (15). The sea urchins *Echinus esculentus* were kept routinely in artificial sea water. To obtain and fertilize the eggs, common procedures were followed (16).

Synchronization. Exponentially growing cultures were transferred into isoleucine-glutamine-free medium (17) and after 24 hr resuspended in complete medium. The grade of synchrony was determined by establishing growth curves, determining the mitotic index, and pulse-labeling the cells with [^{3}H]thymidine.

Isolation of the Mitotic Apparatus of Sea Urchin Eggs. As described in ref. 18, eggs at the first metaphase were stored in 30% (vol/vol) ethanol/H_2O at −10°; after 24 hr they were transferred to 30% ethanol, 0.1% (vol/vol) Triton X-100 at −10° and stored until use. For the isolation the ethanol/Triton mixture was removed, five volumes of distilled water were added, and the egg–water suspension was warmed up to +10°. By agitation on a vortex mixer the cells were broken and the mitotic apparatus was set free. After several washes with 30% ethanol at 0°, the enzyme was prepared from the isolated spindles as shown in the purification scheme (Fig. 1).

Preparation of the Ca^{2+}-ATPase. At the times indicated, the cells (about 10^8 cells) were centrifuged (2000 × *g*, 10 min), washed in 0.9% NaCl in H_2O, and either used immediately for the enzyme preparation or stored in 30% ethanol in H_2O at −10°. The two methods produce identical results. Details of the purification procedure and of the biochemical parameters of the enzyme will be described elsewhere (Petzelt, unpublished); an outline of the purification is shown in Fig. 1. The activity of the Ca^{2+}-ATPase was determined as described earlier (6), and the amount of liberated inorganic phosphate was measured according to Eibl and Lands (42) and the protein concentration according to Lowry *et al.* (19) with bovine serum albumin as standard.

Labeling. The rate of DNA synthesis was determined by incubating 500 μl of the cell suspension with 1 μl of [*methyl*-^{3}H]thymidine (specific activity 18.4 mCi/mmol, 1 mCi/ml) for 20 min at 37° and the radioactivity of the trichloroacetic-acid-insoluble material was measured in a scintillation counter. Proteins were labeled by a 2 hr pulse with ^{14}C-labeled amino acids (uniformly ^{14}C-labeled protein hydrolysate, 58 Ci/g-atom of carbon, 50 μCi/ml). Thereafter, the cells were centrifuged and the enzyme was prepared as described above. The radioactivity of the enzyme fraction is expressed as dpm/mg of protein. The counting efficiency for ^{14}C was 85%.

Gel Electrophoresis. Sodium dodecyl sulfate gel electrophoresis of the Ca^{2+}-ATPase was carried out on slab gels using the 10% acrylamide gel system described in ref. 20. The native

Cell suspension or suspension of isolated mitotic apparatus

500 × g, 5 min → Supernatant / Pellet

Suspend in 0.9% NaCl.

500 × g, 5 min → Supernatant / Pellet

Homogenize in 10 volumes of 50 mM Mops/1 mM DTE/1.5 M glycerol, pH 6.2.

$3 \times 10^4 \times g$, 30 min → Supernatant / Pellet

Homogenize in 10 volumes of 50 mM Mops/1 mM DTE/1.5 M glycerol, pH 8.2; 20 min on vortex.

$3 \times 10^4 \times g$, 30 min → Supernatant / Pellet

Homogenize in 10 volumes of 5 mM Mops/1 mM DTE/1.5 M glycerol, pH 8.2; 20 min on vortex.

$3 \times 10^4 \times g$, 30 min → Supernatant / Pellet

Homogenize in 10 volumes of 50 mM Mops/1 mM DTE/1.5 M glycerol, pH 8.2. Triton X-100:protein = 1:1 (wt/wt); 30 min on vortex.

$3 \times 10^4 \times g$, 30 min → Pellet / Supernatant

Dialyze overnight versus 0.1 M Pipes/0.5 mM $MgCl_2$/4 M glycerol, pH 6.6.

$3 \times 10^4 \times g$, 30 min → Pellet / Supernatant

Enzyme solution

FIG. 1. Purification scheme of the Ca^{2+}-ATPase. All steps are carried out at 4°. Mops, 4-morpholinepropanesulfonic acid; DTE, dithioerythritol; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

proteins were separated in basically the same gel system but instead of sodium dodecyl sulfate 0.1% Triton X-100 was included in the stacking and separating gel and in the buffer and an 8% acrylamide gel was used. At least duplicate samples were run on each slab gel. After the run the gel was sliced longitudinally and one half was stained with Coomassie blue. The other part was used to locate the enzyme on the gel by incubating it in 100 ml of 1.25 mM ATP/2.5 mM $CaCl_2$/0.5 mM EDTA/0.5 mM $MgCl_2$/2 mM $Pb(CH_3COO)_2$/20 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (Hepps) at pH 8.40 for 60 min at 37°. The P_i formed by the enzyme reacts with Pb^{2+} to form $Pb_3(PO_4)_2$. After several washes with H_2O the $Pb_3(PO_4)_2$ was visualized as PbS by the addition of 0.1% $(NH_4)_2S$ in H_2O. The band stained by this procedure was cut out and solubilized in Protosol and the radioactivity was measured in a scintillation counter.

Table 1. Labeling procedure of the synchronized mastocytoma cells

Flask	Application of the labeled precursors, hr: ^{14}C-Labeled amino acids	Application of the labeled precursors, hr: [^{3}H]Thymidine
1	0–2	1.5–1.8
2	2–4	3.5–3.8
3	4–6	5.5–5.8
4	6–8	7.5–7.8
5	8–10	9.5–9.8
6	10–12	11.5–11.8
7	12–14	13.5–13.8
8	14–16	15.5–15.8

The experiment was started 24 hr after the change from deficient to full medium. Times are measured from this start.

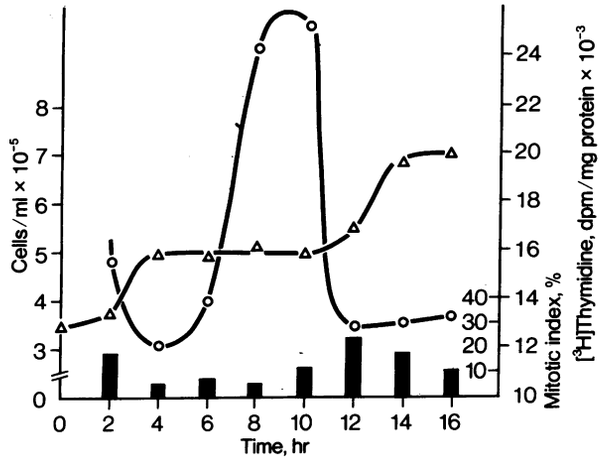

FIG. 2. Growth characteristics of the synchronized mastocytoma cells. The time 24 hr after the change to the complete medium was taken as time 0. Growth of the cell culture, (△); rate of [^{3}H]thymidine incorporation, (○); mitotic index (bars) is expressed as mitotic cells per 100 cells counted.

Experimental Setup. About 4×10^7 cells (2×10^5/ml) were synchronized as described above. After the cells were arrested in the G_1 phase by a 24 hr incubation in the isoleucine-glutamine-deficient medium, they were grown in full medium for 24 hr, allowing them to complete the first cycle. Thereafter, the suspensions were divided into eight parts and cultured in eight flasks preconditioned with medium for 48 hr.

Starting from this point the enzymatic activity was determined every 2 hr for a period of 16 hr. The labeling protocol is shown in Table 1.

RESULTS

Synchronization. The course of the growth curve and of the rate of [^{3}H]thymidine incorporation, and the mitotic index show that a reasonable degree of synchronization has been reached by the use of the isoleucine-glutamine-deficient medium (Fig. 2). The mitotic period lasts about 4 hr in the first mitotic phase studied. The S phase—documented by the increased incorporation rate of [^{3}H]thymidine—occurs during 5–6 hr, a time which is in agreement with the values described for this cell line (21).

Ca^{2+}-ATPase during the Cell Cycle. If the enzymatic activity of the purified enzyme fraction is followed during the cell cycle one can observe a rise of the activity whenever the mitotic phase is reached (Fig. 3). During the interphase the enzyme stays at a low level of activity. These results confirm our findings obtained with crude homogenates of mouse fibroblasts (L cells), where likewise an increased enzymatic activity was found during mitosis (12).

Rate of Enzyme Synthesis during the Cell Cycle. The cyclic variations in the activity of the Ca^{2+}-ATPase may be the result of an activation of preexisting enzyme molecules or *de novo* synthesis of the enzyme during mitosis. To decide this question cells were labeled every 2 hr with a 2 hr pulse of ^{14}C-labeled amino acids and the Ca^{2+}-ATPase fraction was prepared. The rate of the incorporation of the labeled amino acids should be

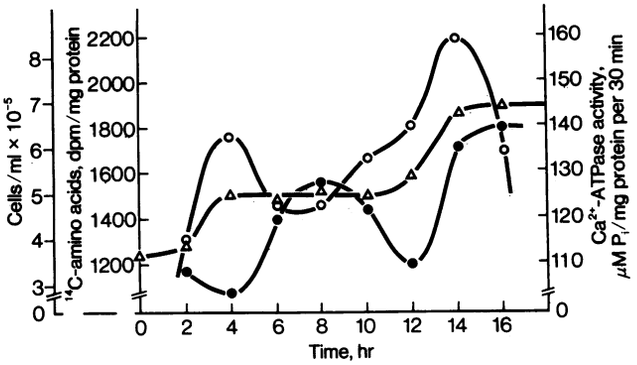

FIG. 3. Course of the Ca^{2+}-ATPase activity (○) and the rate of ^{14}C-labeled amino acid incorporation into the enzyme fraction (●) during the cell cycle. The growth curve of the synchronized cell population (△) is taken from Fig. 2.

a measure of the rate of enzyme synthesis. If an activation of the enzyme is the cause of the increased enzymatic activity during mitosis, no increase in the synthesis of the enzyme should be found at that time. If *de novo* enzyme synthesis is the cause of the activity peak, an increased incorporation of the radioactive amino acids into the enzyme fraction should occur during mitosis. This has not been found, and we therefore conclude that the enzyme is activated at mitosis (Fig. 3). On the other hand, there is a distinct maximum of enzyme synthesis during interphase which occurs approximately at the beginning of the S-phase. That the maximum of enzyme synthesis in the second cell cycle studied is not as clear as in the first synchronous

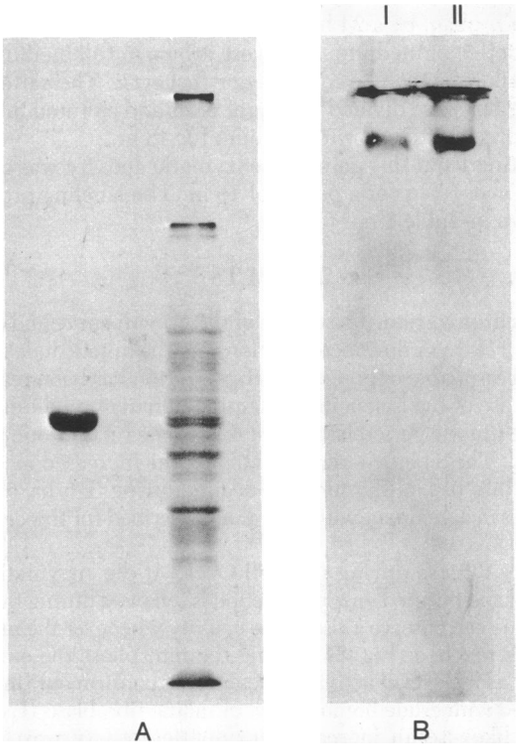

FIG. 4. (*A*) Separation of the denatured Ca^{2+}-ATPase fraction on a sodium dodecyl sulfate/polyacrylamide gel. The marker on the left is brain tubulin purified by two polymerization cycles and chromatography on DEAE-cellulose. (*B*) Separation of the native Ca^{2+}-ATPase fraction on polyacrylamide gel. The enzyme was visualized on the gel by the deposition of PbS as described in the *text*. No difference is observed in the electrophoretic mobilities of the enzymes from isolated sea urchin mitotic apparatus (I) and from mastocytoma cells (II).

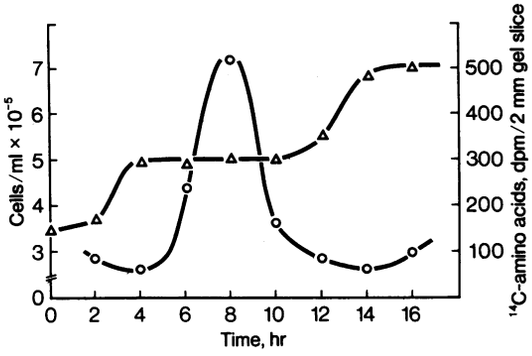

FIG. 5. Cell-cycle-dependent incorporation of ^{14}C-labeled amino acids into the Ca^{2+}-ATPase. After separation of the native Ca^{2+}-ATPase fraction on polyacrylamide gel (○) the gel slice positively staining with PbS (see *Materials and Methods*) was used for measurement of the incorporated radioactivity. Identical amounts of protein were applied each time onto the gel. The growth curve is taken from Fig. 2 (△).

cycle is probably caused by the rapidly lost degree of synchrony of the cell culture.

Two arguments may be raised against these findings. As the sodium dodecyl sulfate gel shows (see Fig. 4*A*), our enzyme preparation is as yet not pure; therefore, the cyclic variations in the rate of enzyme synthesis may also be caused by differences in the rate of synthesis of the other contaminating proteins, thereby simulating an increased incorporation of the radioactive amino acids into the enzyme. Furthermore, the ATPase itself might be irrelevant with respect to mitosis and the fluctuations of the enzymatic activity could be an accompanying finding unrelated to mitosis. To weigh these arguments the native enzyme was separated and visualized by specific staining and the incorporation of radioactive amino acids into the enzyme band was measured on a polyacrylamide gel. It is evident from Fig. 5 that by plotting the radioactivity per gel slice versus the cell cycle one obtains the same curve as by using the whole enzyme fraction (see Fig. 3). Therefore, the fluctuations in the amount of incorporated amino acids into the enzyme fraction during the cell cycle represent fluctuations in the enzyme synthesis itself and are not caused by syntheses of other proteins.

At the same time a Ca^{2+}-ATPase fraction was obtained from isolated mitotic apparatus of sea urchin eggs, where the enzyme has been described as occurring (18) and where it should exert its function. This preparation was run on the same gel. Fig. 4*B* shows that the Ca^{2+}-ATPase from mouse cells and the one from isolated mitotic apparatus from sea urchins have identical electrophoretic mobilities.

DISCUSSION

Several lines of evidence indicate that Ca^{2+} ions have an important regulatory function for the establishment and maintenance of a mitotic apparatus. Timourian *et al.* (22) showed that calcium is concentrated in the spindle during mitosis. Kiehart and Inoué (23) could change the course of mitosis by a local application of Ca^{2+} ions onto the living spindle. Fuller *et al.* (24) described the regulatory function of Ca^{2+} ions in the polymerization of tubulin from mammalian cells. Calcium can cause breakdown of microtubules *in vitro* and *in vivo* (2, 3, 25). These results indicate that a Ca^{2+}-regulating system that is active during mitosis exists in the cell. The Ca^{2+}-ATPase described in this paper is probably one of the main components of this system. Its solubility properties indicate that the enzyme is membrane bound. It is concentrated in the isolated mitotic

apparatus (18) and most probably localized in the vesicles that have been described to exist in the spindle (26, 27). The enzyme has functional similarities to the Ca^{2+}-ATPase of the sarcoplasmic reticulum. It is inhibited *in vitro* by SH-oxidizing agents like *p*-chloromercuribenzoate (Petzelt, unpublished) and diamide (28). Rebhun *et al.* have described that the blockage of mitosis by caffeine and diamide is presumably caused by an inhibition of this Ca^{2+}-ATPase system (28, 29).

Preliminary investigations have shown that the enzyme occurs in all cells possessing microtubules ranging from the slime mold *Physarum*, yeast, plant cells (wheat germ, gingko buds), and a variety of animal cells up to nondividing cells with a high microtubule content like brain cells (Petzelt, unpublished). We do not know yet if the cyclic change of activity during the cell cycle is a characteristic property of all cells capable of traversing the cell cycle; we have examined so far only the course of the Ca^{2+}-ATPase activity in the cell cycle of the sea urchin egg, mouse fibroblasts, mouse mastocytoma cells, and HeLa cells. In these cells the Ca^{2+}-ATPase cycle has been demonstrated. The present paper shows that the enzyme is synthesized during a distinct period of the interphase, with no concomitant increase of activity, and is activated at a time when the cells go through mitosis. Although electrophoresis in neutral gels still would not remove those proteins that have comigrated with the enzyme on the gel, the removal of the bulk of the other proteins having different electrophoretic mobilities makes it highly probable that the incorporated ^{14}C-labeled amino acids are indeed incorporated into the enzyme.

Many enzymes are synthesized during only a short period of the cell cycle (30). It is quite unusual, however, that an enzyme is synthesized well in advance of its use in the cell, as it seems to be the case with the Ca^{2+}ATPase. We know that RNA and protein synthesis is necessary for the entry of the cell into mitosis (31–34). Sensitive periods during the cell cycle are known for inhibitors of RNA and protein formation (35, 36), for radiation-induced effects (37, 38) and for heat treatment (49). These periods indicate that at these points events may occur that are necessary for the beginning or completion of mitosis. On the other hand, the protein of the microtubules is synthesized continuously during the cell cycle in sea urchins (40) as well as in most animal cells (41). We may now conceive the preparation of the cell for mitosis as a coordination of continuous syntheses like that of tubulin and periodic ones, like the synthesis of the Ca^{2+}-ATPase. At mitosis, then, one or more factors have to activate the constituents of the mitotic apparatus such as the Ca^{2+}-ATPase, the microtubule-organizing centers, and probably also the tubulin itself in order to allow the formation of the mitotic spindle.

We thank Christine Roesel and Kurt Marian for their technical assistance and Dr. Karl-Heinz Doenges for providing the tubulin marker.

1. Mazia, D. (1975) *Ann. N.Y. Acad. Sci.* **253,** 7–13.
2. Weisenberg, R. C. (1972) *Science* **177,** 1104–1105.
3. Rosenfeld, A. C., Zackroff, R. V. & Weisenberg, R. C. (1976) *FEBS Lett.* **65,** 144–147.
4. Hayashi, M. & Matsumura, F. (1975) *FEBS Lett.* **58,** 222–225.
5. Petzelt, C. & von Ledebur-Villiger, M. (1973) *Exp. Cell Res.* **81,** 87–94.
6. Petzelt, C. (1972) *Exp. Cell Res.* **70,** 333–339.
7. Petzelt, C. (1972) *Exp. Cell Res.* **74,** 156–162.
8. Petzelt, C. (1974) *Exp. Cell Res.* **86,** 404–408.
9. Mazia, D. (1974) in *Cell Cycle Control,* eds. Padilla, G. M., Cameron, I. L. & Zimmerman, A. (Academic Press, New York), pp. 265–272.
10. Mazia, D. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 690–693.
11. Petzelt, C. (1976) *Exp. Cell Res.* **102,** 200–204.
12. Petzelt, C. (1974) *J. Cell Biol.* **63,** 267a.
13. Petzelt, C. (1975) *J. Cell Biol.* **67,** 332a.
14. Schindler, R., Day, M. & Fischer, G. A. (1959) *Cancer Res.* **19,** 47–51.
15. Schaer, J. C. & Schindler, R. (1967) *Biochim. Biophys. Acta* **147,** 154–161.
16. Hinegardner, R. (1975) in *The Sea Urchin Embryo,* ed. Czihak, G. (Springer-Verlag, Berlin-Heidelberg-New York), pp. 10–25.
17. Tobey, R. A. (1973) in *Methods in Cell Biology,* ed. Prescott, D. M. (Academic Press, New York, London), Vol. VI, pp. 68–112.
18. Mazia, D., Petzelt, C., Williams, R. O. & Meza, I. (1972) *Exp. Cell Res.* **70,** 325–332.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
20. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
21. Schindler, R. & Schaer, I. C. (1973) in *Methods in Cell Biology,* ed. Prescott, D. M. (Academic Press, New York), pp. 43–65.
22. Timourian, M., Jotz, M. & Clothier, G. E. (1974) *Exp. Cell Res.* **83,** 380–386.
23. Kiehart, D. P. & Inoué, S. (1975) *Biol. Bull.* **149,** 433.
24. Fuller, G. M., Ellison, J., McGill, M. & Brinkley, B. R. (1975) *J. Cell Biol.* **67,** 126a.
25. Kiehart, D. P. & Inoué, S. (1976) *J. Cell Biol.* **70,** 230a.
26. Luykx, P. (1970) *Int. Rev. Cytol. Suppl.* **2,** 1–173.
27. Harris, P. (1975) *Exp. Cell Res.* **94,** 409–425.
28. Nath, J. & Rebhun, L. I. (1976) *J. Cell Biol.* **68,** 440–450.
29. Rebhun, L. I., Nath, J. & Remillard, S. (1975) in *Cell Motility,* eds. Goldwin, R., Pollard, P. & Rosenbaum, J. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 4, pp. 1343–1366.
30. Mitchison, J. M. (1971) *The Biology of the Cell Cycle* (Cambridge University Press, New York-London), pp. 159–180.
31. Bibring, T. & Cousineau, G. H. (1964) *Nature* **204,** 805–807.
32. Byfield, J. E. & Scherbaum, O. H. (1967) *Science* **156,** 1504–1505.
33. Jacobson, B. S., Salmon, R. J. & Lansky, L. L. (1964) *Exp. Cell Res.* **36,** 1–13.
34. Parchman, L. G. & Stern, H. (1969) *Chromosoma* **26,** 298–311.
35. Gross, P. R. & Cousineau, G. H. (1966) *Exp. Cell Res.* **33,** 368–395.
36. Wilt, F. H., Sakai, H. & Mazia, D. (1967) *J. Mol. Biol.* **27,** 1–7.
37. Rustad, R. C. (1960) *Exp. Cell Res.* **21,** 596–602.
38. Sachsenmaier, W., Bohnert, E., Clausnitzer, B. & Nygaard, O. F. (1970) *FEBS Lett.* **10,** 185–189.
39. Zeuthen, E. (1970) *Exp. Cell Res.* **61,** 311–325.
40. Raff, R. A., Brandis, J. W., Green, L. H., Kaumeyer, J. F. & Raff, E. (1975) *Ann. N.Y. Acad. Sci.* **253,** 304–317.
41. Lawrence, J. H. & Wheatley, D. N. (1975) *Cytobios* **13,** 167–179.
42. Eibl, H. & Lands, W. E. M. (1969) *Anal. Biochem.* **30,** 51–57.