SURFACE FUNCTIONS DURING MITOSIS

II. Quantitation of Pinocytosis and Kinetic Characterization of the Mitotic Cycle with a New Fluorescence Technique

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ABSTRACT

The profound depression of fluid pinocytosis observed in mitotic cells (Berlin, R. D., et al. 1978. *Cell.* **15**:327–341) is documented by quantitative microspectrofluorimetry of fluorescein-labeled dextran uptake in single cells. In J774.2 macrophages, fluid pinocytosis is reduced 30-fold during mitosis. The depression develops within 30 s of entry into prophase and recovers with equal rapidity upon emergence from telophase into G_1.

This characteristic pattern of fluid pinocytosis forms the basis of a new method for detailed kinetic analysis of the duration of mitosis and its phases. The analysis is applied to the J774.2 macrophage cell line but should be generally applicable to other lines. Effects of ouabain and colchicine on the length of mitosis and its phases are evaluated, revealing a selective prolongation of metaphase by ouabain and suggesting a role for microtubules in the transition from G_2 into mitosis.

We recently showed that pinocytosis and phagocytosis are severely depressed during mitosis in macrophage and other cultured cell lines (1). The reduction in pinocytosis was noted in prophase cells, but its exact onset was not measured precisely. Furthermore, the extent of the reduction was difficult to determine. In particular, our estimate of fluid pinocytosis relied on the direct observation of accumulated product formed by the reaction of pinocytized horseradish peroxidase (HRP) with diaminobenzidine and H_2O_2. Thus, it remained possible that the uptake of HRP (or other marker) continued during mitosis but occurred in small pinocytic vesicles that could not readily be seen.

In this report, we first establish the quantitative pattern of fluid pinocytosis by microspectrofluorimetric analysis of the uptake by single cells of fluorescein-labeled dextran (fluorescein-dextran). The data confirm our basic findings of a marked depression of pinocytosis during mitosis. Careful kinetic studies show that this depression is established within less than a minute after the earliest recognizable stages of prophase.

These observations suggested a kinetic approach to the analysis of the duration of mitosis and its component phases. Because pinocytosis decreases sharply as cells enter mitosis, interphase cells can be pulse-labeled by pinocytosis, and their entry and passage through mitosis can be directly observed at subsequent timed intervals. The method should be generally applicable inasmuch as the

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decrease of pinocytosis occurs in all cell types examined so far.

We report the application of this procedure to the J774.2 mouse macrophage cell line and in the analysis of the effects of ouabain and colchicine on mitosis. The method is shown to be capable of pinpointing a selective prolongation of metaphase in the case of ouabain. Colchicine effects are shown to include a delay of movement into mitosis as well as the well-known "metaphase arrest."

MATERIALS AND METHODS

Cells

J774.2 macrophages were kindly provided by Drs. O. Rosen and B. Bloom, Albert Einstein School of Medicine, New York. They were selected from a line originally developed by Dr. P. Ralph, Sloan Kettering Institute, New York (8). The cells were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 20% horse serum as previously described (1). For studies in cell suspension, J774.2 macrophages were cultured on sterile plastic petri plates. As shown by Muschel et al. (4), macrophages are readily collected from these plates by gentle pipetting. Cell monolayers were grown on 13-mm diameter glass coverslips in 35-mm diameter Falcon dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). J774.2 cells adhere tightly and assume a fibroblastic cell shape on glass and Falconized surface.

Fluorescein-Dextran Uptake

J774.2 cells on monolayers or in suspension were incubated at 37°C in complete medium supplemented with fluorescein-dextran (Sigma FD-70, average molecular weight 62,000; Sigma Chemical Co., St. Louis, Mo.). After various periods of dextran uptake, cell monolayers were either rinsed three times in phosphate-buffered saline (PBS) and immediately fixed with 4% paraformaldehyde for 10 min or rinsed three times in complete medium at 37°C, further incubated in medium without dextran for various lengths of time, and finally rinsed in PBS and fixed. Cells labeled with fluorescein-dextran in suspension were treated similarly, except the washes included 3-s centrifugation in an Eppendorf microcentrifuge with resuspension of the cell pellets in 1-ml portions of medium, buffer, or fixative, as appropriate.

Identification of Mitotic Cells

Fixed cells were rinsed in PBS, incubated for 10 min at 37°C with 1 μg/ml Hoechst 33258, and rinsed again with PBS. Hoechst 33258 binds to DNA and permits ready observation of all stages of mitosis (1).

Microscopy

Phase and fluorescence observations were made with a Zeiss Photomicroscope III equipped with a III RS epi-illuminator and mercury lamp source. Hoechst 33258 and fluorescein emission spectra were optically separated with a band pass excitation filter BP, 390–440 nm, with an FT 460 dichroic mirror and LP 475 barrier filter (Hoechst) and standard Zeiss filter combinations for fluorescein. Phase-contrast was observed through a green interference filter (BP 546/10). The cells were photographed on Kodak Tri-X-Pan film.

Microspectrophotometry

A Zeiss photometer head with S20 phototube was employed. The III RS epi-illuminator of this microscope was equipped with the same optics as described above.

Cells labeled in suspension with fluorescein-dextran and Hoechst 33258 as described above were taken up in a small volume of 50% glycerol in PBS, and a droplet was spread under the coverglass. The cells were identified from the pattern of Hoechst 33258 fluorescence and centered within a circular measuring aperture by phase-contrast microscopy with light transmitted through a 546-nm interference filter. With all light incident to the specimen blocked, an optical pathway to the photomultiplier was established, the shutter to the incident beam was opened, and the intensity was recorded within 2–3 s. A blank value taken with the aperture centered on an "empty" space was recorded periodically and subtracted from the cell readings. For interphase cells, these blanks gave intensities <2–3% of the cell values. However, blanks were often comparable to values of mitotic cells. The amplifier was set during the collection of the standardized curves illustrated in Figs. 1 and 2 and was held constant through later experiments. This precaution ensured that recordings were linear with fluorescein-dextran uptake.

The procedure was the same for cells grown as monolayers on glass coverslips, except a circular measuring diaphragm could usually not be employed for cells spread on monolayers. Instead, an adjustable rectangular diaphragm was used to frame each cell. Fluorescence over the cell and fluorescence from a corresponding blank of identical dimensions were measured, and the difference was recorded as cell fluorescence intensity. The results confirmed studies on suspended cells. However, because of its tedious nature and because of difficulties in framing some of the larger cells without unacceptably high blank values, the method was extensively employed only to compare early G_1 with mean mitotic and interphase populations.

RESULTS

Quantitation of Pinocytosis

We think that fluorescent high molecular weight dextrans provide perhaps the most convenient and useful markers now available for observing fluid pinocytosis. As noted elsewhere, fluorescein-dextran resists biological degradation: for example, Sephadex chromatographic analysis of medium and cell extracts prepared after incubation of J774.2 cells with fluorescein-dextran for 24 h reveals no small molecular weight products. Fluorescein-dextran is commercially available and may be sensitively quantified by conventional fluorescence spectroscopy. Rhodamine conjugates are also easily prepared, making double-label studies feasible. Most importantly, previous kinetic studies in macrophage cell suspensions have shown fluorescein-dextran uptake to be linear with both concentration and time.[1] These kinetics are consistent

[1] Walter, R. J., R. D. Berlin, J. Pfeiffer, and J. M. Oliver. The polarization of endocytosis and receptor topography on cultured macrophages. *J. Cell Biol.* In press.

with internalization by fluid but not adsorptive pinocytosis.

Because it was critical in this study to determine pinocytosis at precise stages within mitosis, we have taken advantage of another property of fluorescein-dextran: the ready measurement of its fluorescence intensity in single cells by use of a microscope photometer. In practice, individual cells were identified for mitotic phase after staining with Hoechst 33258 as previously described (1). Fluorescein-dextran within the same cells was then measured by microspectrofluorimetry, and the results were expressed in relative units. As determined by this single-cell technique, the mean fluorescence intensity of interphase cells increased linearly with time (Fig. 1) and dextran concentration (Fig. 2). No surface fluorescence was observed. Fluorescent dextrans thus satisfy all the criteria for suitable markers of fluid phase pinocytosis and lend themselves to quantitation in single cells.

Pinocytosis in the Mitotic Cell

Having established the kinetics of dextran uptake by interphase cells, we quantified the extent of its pinocytosis for various mitotic phases. Fig. 3 shows relative values for interphase and mitosis (except prophase as explained below) determined in single macrophages that had been labeled in suspension for 2 min by pinocytosis of 5 mg/ml fluorescein-dextran, washed, and fixed. Fixation in suspension assured that all cells were in a similar rounded configuration. The results show a nearly 30-fold decrease in dextran uptake in mitotic as compared to interphase cells. This decrease is evident from prometaphase through telophase. Similar measurements using cell monolayers showed that early G_1 cells, identified as small paired cells

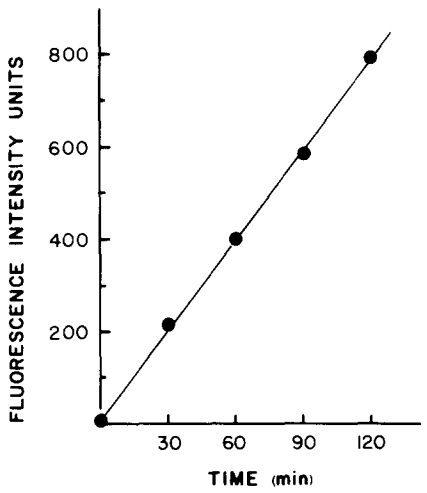

FIGURE 1 Uptake of fluorescein-dextran by J774.2 macrophages as a function of time. Cells in suspension were incubated for the indicated intervals in 1 mg/ml fluorescein-dextran and their fluorescence intensities measured as described in Materials and Methods. Each point represents an average of 50 cells.

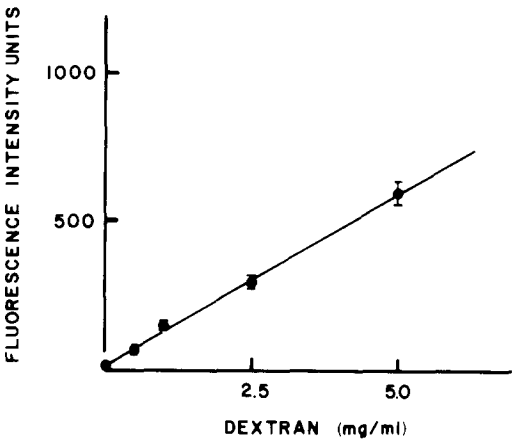

FIGURE 2 Uptake of fluorescein-dextran as a function of concentration. Cells were incubated for 30 min in the presence of 0.5, 1.0, 2.5, and 5.0 mg/ml fluorescein-dextran, washed, and analyzed as described in Materials and Methods.

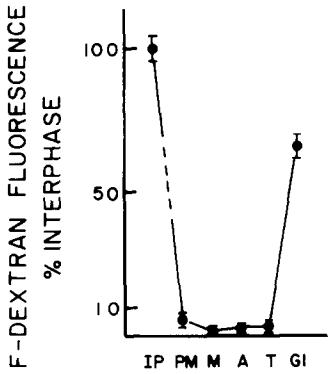

FIGURE 3 Fluorescein-dextran uptake by interphase and mitotic macrophages: IP, interphase; PM, prometaphase; M, metaphase; A, anaphase; T, telophase; G_1, early G_1 cells (½ of pair). Suspended cells were incubated for 2 min with 5 mg/ml fluorescein-dextran in complete medium and processed as described in Materials and Methods. Results are expressed as percent of mean interphase values. Bars represent ±2 SE. The mitotic cells were measured in suspension; G_1 cells, on monolayers. The results were compared to interphase cells also in suspension or monolayer.

with decondensed chromatin within a smooth nuclear envelope and often connected by a cytoplasmic bridge with midbody (see Fig. 7 *b*), attained values >50% of the values obtained for the remaining interphase population.

These quantitative differences are so large that mitotic cells should be readily distinguished from all cells in interphase by direct, visual observation. Fig. 4 shows a mixed population of interphase and mitotic cells after a 2.5-min incubation in 10 mg/ml fluorescein-dextran. The Hoechst 33258 (chromatin) pattern is displayed in Fig. 4 *a*. It includes a typical metaphase nucleus, as well as late telophase and interphase nuclei. By phase contrast microscopy (Fig. 4 *b*), both small, round (far left) and spread interphase cells are found. The pattern of fluorescein-dextran fluorescence (Fig. 4 *c*) shows the mitotic cells to be virtually empty, whereas interphase cells, small, large, or spread, are brightly fluorescent.

Initiation of the Pinocytic Block

The data of Fig. 3 indicate that pinocytosis is blocked at some point before prometaphase but do not define this point. For this determination, it is first necessary to define a beginning of prophase. This may be undefinable in the sense that chromosome condensation commences at some immeasurable time within interphase but presumably well before mitosis proper. We have, therefore, arbitrarily defined readily discernible stages as "late G_2" and "prophase." Late G_2 cells (Fig. 5 *A* and *B*) have large nuclei and very small, regular foci of Hoechst 33258 fluorescence. The cells represent the earliest detectable stages of chromosome condensation by light microscopy. On monolayers, late G_2 cells are usually (Fig. 5 *A*) but not always (Fig. 5 *B*) well spread and have nucleoli and intact nuclear envelopes. Prophase cells may also be well spread (Fig. 5 *C*) and have apparently intact nucleoli and nuclear envelopes, but these characteristics are variable (Fig. 5 *D*). However, all prophases are readily distinguished from late G_2 by their more advanced state of chromosome condensation, identified by larger and sharper fluorescent foci (cf. Fig. 5 *A* and *B* with *C* and *D*). Prophase is considered to have ended and prometaphase to have begun when an asymmetrical distribution of chromosomes first indicates their movement toward the metaphase plate (metakinesis). By this stage, all cells are rounded, and nucleoli and nuclear envelopes are gone.

When J774.2 macrophages are exposed to fluorescein-dextran for 1 min, all late G_2 cells are strongly labeled. In contrast, only a fraction of prophase cells are clearly labeled, indicating that pinocytosis is sharply decreased in early prophase. However, because the period of exposure to fluorescein-dextran must be finite, a fraction of labeled prophase cells that is derived from the G_2 population will be present. Hence, the precise transition point cannot be determined from a single labeling period.

To define the position of the transition, we exposed monolayers of dividing macrophages to fluorescein-dextran for brief periods of increasing duration, rapidly quenched pinocytosis by plunging the monolayer into fixative, and rinsed and subsequently scored prophase cells visually as positive or negative for fluorescein-dextran uptake. In Fig. 6, the percent of positive prophase cells is plotted as a function of the (increasing) duration of exposure to fluorescein-dextran. Two important conclusions may be drawn from this experiment. It is seen firstly that relatively few prophase cells are labeled during a 30-s labeling period. Thus, marked reduction of pinocytosis develops by the earliest stages of prophase. Secondly, it is clear that the transition from active to nearly zero pinocytosis is exceedingly rapid. Because labeled prophase cells, which must be derived from earlier pinocytic G_2 cells, first appear after only 17 s (extrapolating to zero level), the transition from active pinocytosis to the virtual absence of pinocytosis occurs within this 17-s interval. This transition is the more dramatic in view of the especially active pinocytic rates shown by late G_2 cells (on the average, 2.4 times the mean interphase population as shown by microspectofluorimetry: mean of six observations).

The telophase-G_1 transition was also analyzed on cell monolayers. For this purpose, telophase cells were identified as incompletely separated cells (although separation is occasionally difficult to discern) by their bright, fluorescent, usually kidney-shaped nuclei having sharp margins but sometimes showing protruding chromosomes or tightly packed parallel chromosomes (Fig. 7 *A*). Early G_1 cells (Fig. 7 *B*) are separate, though sometimes tightly apposed. A midbody is frequently, but not always evident by phase-contrast microscopy. Early G_1 nuclei tend to be rounded, and the fluorescence intensity due to Hoechst 33258 is uneven as a result of the progressive decondensation of chromatin and the reappearance of nucleoli. Monolayers exposed for 30 s to 10 mg/ml

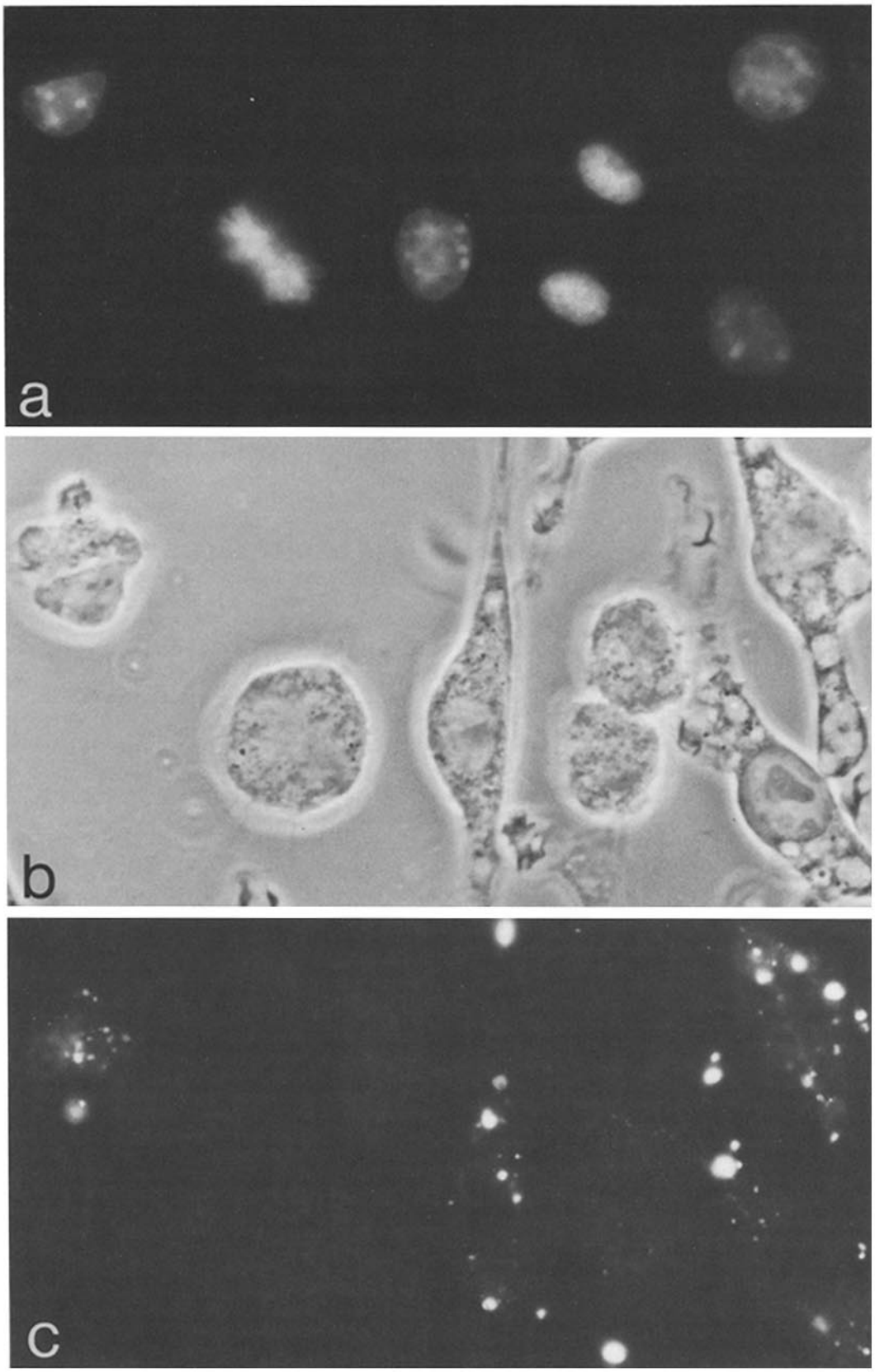

FIGURE 4 A representative field of a monolayer of nonsynchronized J774.2 macrophages incubated with 10 mg/ml fluorescein-dextran for 2 min at 37°C, fixed and stained with Hoechst 33258 as described in Materials and Methods. (*a*) Hoechst fluorescence. (*b*) Phase-contrast. (*c*) Fluorescein-dextran. × 1,150.

fluorescein-dextran show virtually all positive G_1 cells and all negative telophase cells. This indicates the onset of pinocytic capacity with emergence from telophase.

When monolayers are incubated with fluorescein-dextran for 60 s, rinsed quickly, and reincubated in medium without dextran, early G_1 cells derived from the unlabeled telophase population are observed. The entire early G_1 population becomes unlabeled in about 15 min, and some unlabeled G_1 cells are seen by 30 s. Their presence indicates a transition from the nonpinocytic to pinocytic state within that 30-s period. We did not attempt more exact analysis of this evidently rapid process.

Duration of Mitosis: Principles of Measurement

Because the depression and recovery of pinocytosis are precisely coterminous with the beginning and end of mitosis, an approach to the determination of the duration of mitosis is suggested. The approach may be explained as follows (Fig. 8).

As a first approximation, let us consider all cells to have identical durations of mitosis. After the initial uptake of dextran (which, for the moment, we will take to occur instantaneously), all interphase cells, including the emerging postmitotic populations, which we designate "early G_1," will be labeled; all mitotics will be unlabeled (Fig. 8, top line). Subsequently, labeled interphase cells move into mitosis, and unlabeled mitotics move into early G_1. Soon the entire early G_1 is populated by unlabeled cells and will remain so until cells that had been labeled in interphase pass completely through mitosis (i.e., until all mitotic cells are labeled) and begin to appear as labeled early G_1 cells. Thus, the duration of mitosis may be measured as either: (*a*) the time required after pulse to label the entire mitotic population, or (*b*) the interval between initial pulse-label and (re)appearance of labeled early G_1 cells. The time resolution of these measurements is obviously limited by the duration of the pulse-labeling and the sampling interval.

In practice, the values obtained by methods *a* and *b* differ somewhat and provide different information. The time required to label the entire population includes the time required to label cells with the slowest mitotic times, whereas the G_1 relabeling method gives the shortest mitotic time. The difference between the values obtained by the two methods indicates the range (and variations) of mitotic durations.

Because the various mitotic phases are readily distinguished by the chromosome stain, measurement of individual mitotic phases obeys the same principles as measurement of the entire period of mitosis. Immediately after the wave of labeled cells reaches the phase in question, it will gradually become labeled. The entire phase will become labeled after an interval sufficient for all of its unlabeled members to have progressed into the next phase. Thus, the duration of a given phase is the interval between initial and complete labeling of the phase population. Because the progression through mitosis is unidirectional, the time of the initial labeling of any phase is equal to the time at which the labeling of the immediately preceding phase is complete; i.e., phase duration is equal to the interval between the complete labeling of two sequential phases. Should the duration of a phase vary among cells, there will be some labeled cells appearing in the next phase before its antecedent is completely labeled. The latter phenomenon would provide information on the variation of phase times within the population. In addition, the presence of unlabeled mitotics after prolonged incubation would identify cells that are not progressing through mitosis; i.e., that are in mitotic arrest.

Duration of Mitosis: Experiments

In practice, nonsynchronized cell populations on coverslips are pulse-labeled by brief exposure to fluorescein-dextran, gently rinsed, incubated for various intervals, fixed, stained to reveal mitotic phase, and scored for the presence or absence of dextran. Details of the procedures are found in the appropriate figures and in Materials and Methods.

With 5 min-sampling intervals, the time required for complete labeling of all mitotics was 51.4 ± 1.2 (SE seven observations) min. The first labeled G_1 were observed at 45 ± 1.6 (SE six observations) min. Assuming a statistically normal population, these values indicate a mean of ~48 min.

The same technique was applied to estimate the duration of mitotic phases. Estimates based on the interval between initial and total labeling of a given phase were (mean, six experiments): prophase 3.8 ± 0.65 min (SE); prometaphase 4.2 ± 0.48 (SE); metaphase 30.2 ± 2.2 (SE), and telo-

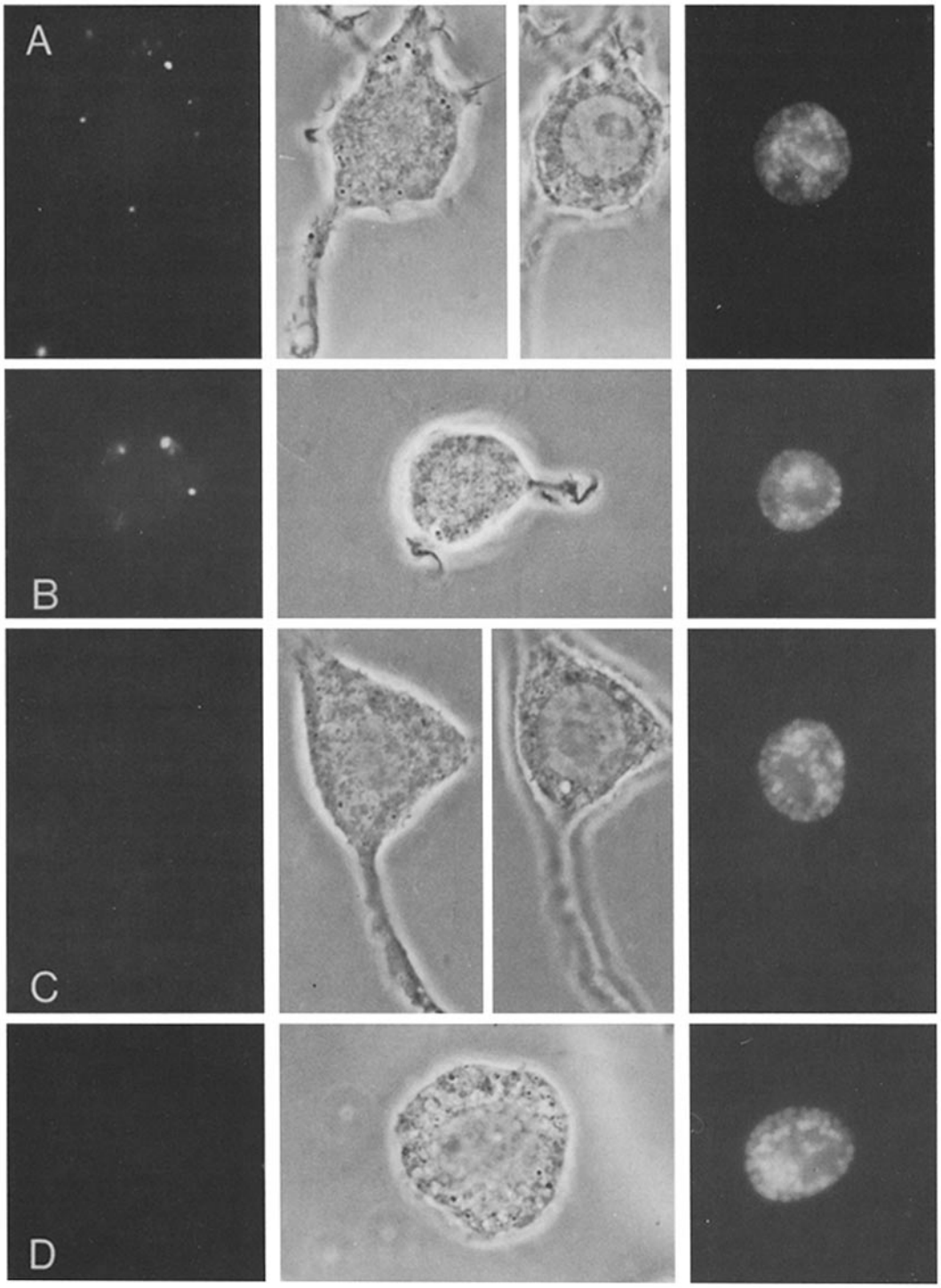

FIGURE 5 Morphologic characteristics of late G_2 (*A* and *B*) and prophase (*C* and *D*) cells. (*left*) Fluorescein-dextran. (*center*) Phase contrast. (*right*) Hoechst 33258. In *A* and *C*, phase-contrast images are shown at two focal planes. On the left of these, the full extension is shown near the coverslip surface. On the right, the nuclear outline is brought into focus above the surface plane to show the persistence of nucleoli and apparently intact nuclear envelopes through early prophase. The nuclear envelope appears to be breaking down in the rounded prophase cell (*D*). *A* and *D* are the most common patterns, respectively, for G_2 and prophase cells. Note that pinocytic activity is correlated with the extent of chromatin decondensation. A 50-s incubation with fluorescein-dextran (5 mg/ml) was chosen to facilitate photography, although, as is shown in Fig. 6, by 50 s many prophase cells contain fluorescein-dextran derived from late G_2 pinocytic activity. × 1,350.

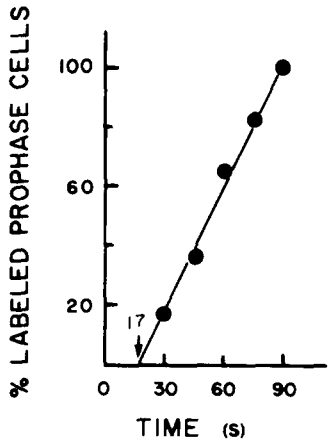

FIGURE 6 Labeling of prophase cells with increasing length of exposure to fluorescein-dextran. Cell monolayers were incubated for the intervals indicated with 10 mg/ml fluorescein-dextran, fixed, washed, and scored visually for fluorescein-dextran positive or negative prophase cells. This result is representative of 3 experiments, which gave time-intercepts of 17, 22, and 25 s.

phase 17.2 ± 1.6 (SE). Insufficient anaphase cells were counted for precise determinations, although it seems comparable to prophase in duration.

These estimates of duration of mitosis or of mitotic stages are probably as precise as those obtained by any other method and are comparable to direct time-lapse observations of single cells (see, for example, the classic studies of Bucher [2]). Indeed, our estimates may be more precise because the fluorescent stain techniques result in less ambiguity in defining phases than the observation of living cells by phase-contrast or Nomarski optics. On the other hand, the approach is limited by the frequency or interval of sampling, which determines resolution, and the observer's patience in deciding when sufficient cells have been evaluated to indicate "beginning" and "complete" labeling.

The latter ambiguities can be removed by determining the fraction of labeled cells in the phase in question and extrapolating to zero or 100% labeling. Thus, if a more precise measure of the total period of mitosis is required, the fraction of labeled telophase cells may be determined at frequent intervals and the time of complete labeling found by extrapolation. This alternative approach can also be applied to individual mitotic phases, as is shown for metaphase in Fig. 9. Precise extrapolation can be made, and it gives a metaphase duration of 41.6 min. In addition, the striking linearity of the increase in labeled metaphase cells with time indicates that the entire metaphase population is advancing at a nearly identical rate.

The significantly longer estimate of metaphase duration by this technique, as compared to the method described above, which is based on first to last labeling (30.2 min), may, in part, reflect differences in culture conditions on different days (cf. the metaphase duration in another untreated culture, Fig. 10). However, it is most likely that the minimal times for first and complete metaphase labeling were over and underestimated, respectively, by amounts that are similar to the sampling interval.

Application of the Method to Determining the Effects of Ouabain on Mitosis

At intermediate concentrations of ouabain, the growth rate of J774.2 is slowed, and a variable increase in the proportion of cells in prophase and metaphase is observed.[2] As a result of the loss of mitotic cells from monolayers, the relative counts of different mitotic phases are not a reliable index of their relative durations. To examine the ouabain effect in more detail, we therefore estimated the duration of phases by determining the beginning and the total labeling of all phases. Mitosis with ouabain was slightly prolonged: 58 vs. 55 min for total telophase labeling; the shortest mitosis was 50 vs. 45 min. The main difference is that metaphase is prolonged.

This feature was examined in detail by the extrapolation technique. Fig. 10 shows the percent of labeled metaphase cells with and without ouabain treatment. The duration of metaphase is increased from 33 to 41 min, thus accounting for the prolonged mitosis. A slight delay in the onset of labeling indicates prolongation of the prophase-prometaphase period as well. These changes could probably not be analyzed sufficiently by any other procedure and may form the basis for further analysis of ouabain or other drug effects.

We emphasize that ouabain does not cause metaphase arrest. With prolonged labeling, all metaphases (and other phases) become labeled; i.e., all cells progress through metaphase. Thus, once committed to enter mitosis, both normal and ouabain-treated J774 are apparently able to complete the process.

[2] Melmed, R. N., and R. D. Berlin. Regulation of cell volume. Submitted for publication.

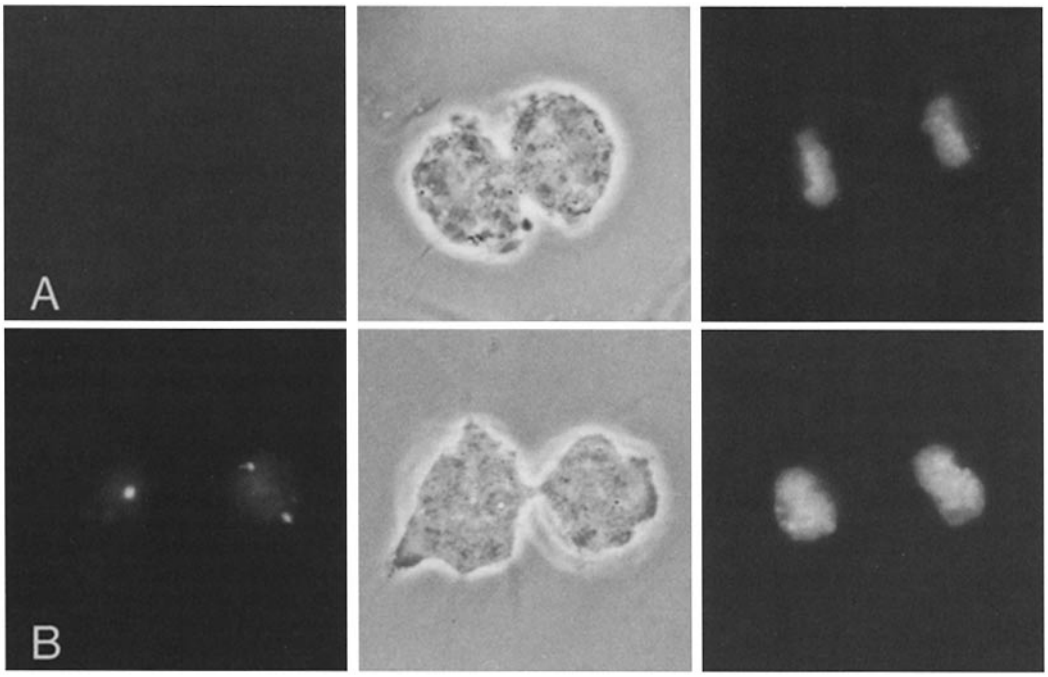

FIGURE 7 Morphologic characteristics of telophase, *A*. Early G_1, *B*. (*left*) Fluorescein-dextran. (*center*) Phase-contrast. (*right*) Hoechst 33258. Monolayers were incubated 30 s in 10 mg/ml fluorescein-dextran, fixed, and processed as described in Materials and Methods. In *A*, the advanced cleavage furrow and the small condensed nuclei, in which a few chromosome structures are discernible, identifies the cell as being in telophase. This cell contains no fluorescein-dextran. The chromatin of the nuclei of the small, early G_1 cells (*B*) is well dispersed by comparison. The cells illustrated are beginning to spread out over the surface. Both cells of the pair show fluorescence due to dextran. Early G_1 cells also appear frequently as pairs of small rounded cells with a clear midbody. × 1,350.

Metaphase Arrest by Colchicine

The breakdown of the mitotic spindle and the consequent failure of cells to undergo anaphase after colchicine treatment are well known. Accumulation of cells arrested in mitosis by colchicine has been used by Puck and others in elegant procedures for the analysis of cell cycle kinetics (see, for example, Puck and Steffen [5]). However, kinetic measurements of mitosis itself, after brief colchicine treatment by the technique described in the present paper, reveals its action to be considerably more complex than simply an inhibition of anaphase movements.

When J774.2 cells are treated with 10^{-6} M colchicine for 45 min and then labeled with 5 mg/ml fluorescein-dextran for 4 min, all interphase cells and prophase cells, but no other mitotics, are labeled (colchicine inhibits fluid pinocytosis in J774.2 to some degree[1] but does not affect its qualitative use in this case). If these cells are washed free of dextran and incubation is continued in medium with 10^{-6} M colchicine, the usual rapid labeling of the mitotic pool does not occur. Rather, <5% of mitotic cells are labeled after 45 min of incubation, and only 20% after 70 min. Because the period of colchicine administration was 90 and 115 min, respectively, even assuming complete mitotic arrest from the time of colchicine addition, the number of cells in the mitotic pool could not have increased more than two- to 2.5-fold. Accordingly, had entry into mitosis occurred at the normal rate, by 45 min roughly a third of the mitotics, not 5%, should have been labeled. We must conclude that, in addition to arresting cells in mitosis, colchicine also slows their entry into the mitotic cycle.

DISCUSSION

The quantitative measurements of pinocytosis during mitosis reported here establish that fluid pinocytosis is decreased more than 30-fold during this period of the cell cycle. Moreover, this pro-

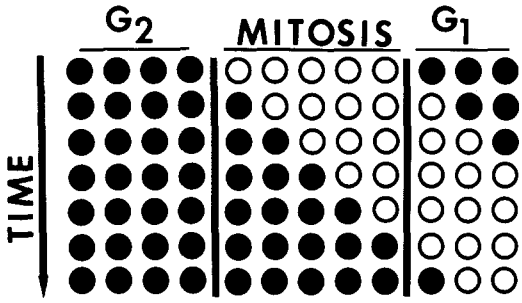

FIGURE 8 Principle of a new method for determining the duration of mitosis. In this diagrammatic representation, the solid circles represent cells labeled very briefly with fluorescein-dextran. The open circles are unlabeled cells. With time, all cells progress through the cell cycle, resulting in a shift of cells from left to right ($G_2 \rightarrow M \rightarrow G_1$), illustrated in successive lines from top to bottom. The top line illustrates the initial distribution. Label is restricted to G_2 and early G_1 cells. Note that all other interphase cells would also be initially labeled so that the G_2 population shown is continually replenished with labeled cells. With time, labeled G_2 cells move into mitosis. Labeled early G_1 cells move further into G_1, whereas new G_1 cells are derived from unlabeled mitotics. This emerging G_1 population is soon entirely unlabeled (in J774.2 the recognizable early G_1 period is about 15 min). The mitotic population, on the other hand, is labeled progressively. The time required for complete labeling of mitotic cells is equal to the duration of mitosis. At virtually the same time, labeled mitotics will begin to enter early G_1 and repopulate it with labeled cells.

found change is induced in seconds at the transition between late G_2 and prophase. The recovery of pinocytosis in early G_1 is also extremely rapid. Such rapid and extensive changes in pinocytosis, we believe, indicate its specific physiologic control. This is of particular interest in view of the general resistance of fluid pinocytosis to inhibition by various drugs, for example, cytochalasin B, or by low temperature (9).

The data reported here and previously (1) are not in complete agreement with recent studies by Quintart et al. (7) on variations of endocytosis during the cell cycle of cultured hepatoma cells. These authors claimed a significant, although moderate, reduction in pinocytosis that began in late S phase and increased progressively through G_2 and mitosis. We suggest that their results are severely limited by the techniques employed. Quintart and co-workers quantified pinocytosis by horseradish peroxidase uptake after 1 h of incubation. However, this is comparable to the duration of mitosis itself. Furthermore, their data were obtained by biochemical assay in cell populations that had been arrested by colchicine and then allowed to progress in only approximate synchrony through a division cycle. Consequently, the lesser magnitude and slow onset of the depression they observed in "mitotic cells" can be related to pinocytosis by substantial numbers of contaminating nonmitotic cells. These data reinforce the importance of cytological analysis in determining the timing of precisely and rapidly controlled physiological events. In particular, our direct observations in J774 cells localize the decrease in pinocytosis to early prophase and show that fluid pinocytosis in late G_2 cells occurs at rates that are actually greater than the mean interphase population.

The use of fluorescein-dextran to observe and quantify the depression of pinocytosis during mi-

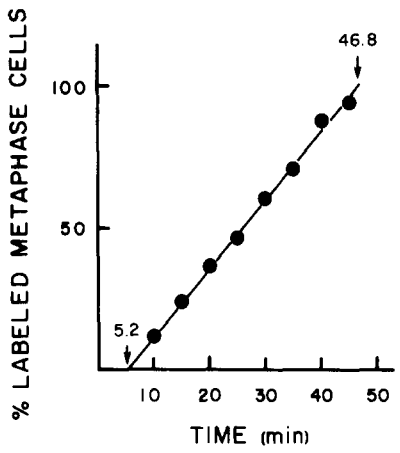

FIGURE 9 Increase of labeled metaphase cells after pulse-labeling with fluorescein-dextran. The percent fluorescent metaphase cells observed is plotted vs. time, taking the beginning of the 5 min pulse-label with 5 mg/ml fluorescein-dextran as zero time. At the conclusion of the labeling period, the monolayers were carefully rinsed in 37°C medium and maintained in a 5% CO_2 atmosphere. At intervals, replicate monolayers were removed, fixed, and stained with Hoechst 33258 and a minimum of 50 metaphase cells scored for the presence or absence of label. Transition through prophase and prometaphase is rapid, the first labeled cells appearing after an (extrapolated) period of about 5 min. The linearity of accumulated positive metaphases indicates that the progression of individual cells is of nearly constant duration over the period of observation. In this experiment, complete metaphase labeling was attained at 46.8 min (extrapolated). The duration of metaphase was thus (46.8 − 5.2) 41.6 min.

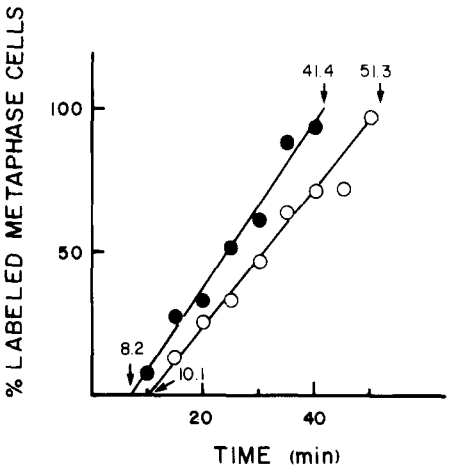

FIGURE 10 Appearance of labeled metaphase cells after pulse-labeling with fluorescein-dextran. (○) Control. (●) Ouabain treated. The experiment was performed as described for Fig. 8, except that the treated monolayers were incubated in 100 μM ouabain for 3 h before a 5-min labeling with 5 mg/ml fluorescein-dextran and subsequent incubation in the presence of ouabain. 100 μM ouabain inhibits rubidium influx by approximately 40% in J774.2 macrophages.[2] 90% of Rb^+ influx is inhibitable at higher concentrations. In this experiment, control metaphase was complete in (41.4 − 8.2) 33.2 min, and in (51.3 − 10.1) 41.2 min in the ouabain-treated cells. Although variability in control metaphase duration was seen from day to day, a consistent prolongation of ~30% was seen with ouabain treatment.

tosis was basic to the development of a technique to measure the duration of mitosis and its component phases. The success of this approach was equally dependent on the use of the dye, Hoechst 33258, which can be optically isolated and separately observed in fluorescein-labeled cells, to identify mitotic cells of different phases. As illustrated here and previously (1), the stain yields readily differentiated images. What is less obvious from these images (although discernible in Fig. 4) is that the image in mitotic cells has extremely high contrast (the DNA is not diffused throughout the nucleus), and, thus, the mitotic cells are brilliant. This makes possible the rapid identification and enumeration of mitotic phases before observation of fluorescein-dextran uptake by microspectrophotometry or by visual examination.

A major advantage of our technique is that only a fraction of mitotic cells need be observed to determine the length of mitosis or its phases. In contrast, techniques dependent on measurement of accumulated mitotic cells require observation of the whole population and so are often difficult to apply to cells growing on monolayers where mitotic cells are readily detached and lost.

Of course, previous investigators have introduced other pulse-labels before mitosis and have followed these labels into and through mitosis to determine its duration. In particular, a pulse of tritiated thymidine introduced in S phase can be followed through G_2 and mitosis (see, for example, Mendelsohn and Takahashi [3]). This technique was first applied by Quastler and Sherman (6), and it provides important information on cell cycle kinetics, particularly in vivo. However autoradiography is tedious to apply, and silver grains deposited over the nuclei obscure subtle morphological features of mitotic phases. More importantly, the timing of mitosis by thymidine autoradiography is imprecise because the labeling time and variations in the duration of G_2 are long compared with the length of mitosis itself. Using the pinocytosis labeling method on the other hand, the pulse is placed immediately before mitosis so that variations in other parts of the cell cycle become irrelevant.

The resolution of our technique is sufficiently high to allow observation of selective changes in individual mitotic phases. For example, we were able to establish prolongation in both prometaphase and metaphase by ouabain treatment. Moreover, application to colchicine-treated cells raises new questions about the action of colchicine on mitotic cells. Thus, it was readily confirmed that cells become arrested in mitosis by colchicine. However, it was also clear that colchicine delays the entry of J774.2 cells into mitosis. Although this was not reported in earlier studies of cell cycle kinetics using colchicine, it should be pointed out that in many studies the cell populations have been rapidly growing, often transformed lines cultured in suspension. In contrast, J774 macrophages, while rapidly growing, are relatively well differentiated and adaptable to monolayers. In his cinematographic studies of the effects of colchicine on dividing fibroblasts, Bucher (2) showed 40 years ago that in the 2–3 h after colchicine, a cardinal feature of its action was a marked decline in the number of cells entering mitosis. New mitoses subsequently rose back to precolchicine levels. These results, and ours, suggest that a premitotic population may expand after colchicine so that even at a reduced probability of a G_2-mitosis transition, the number of new mitotics will even-

tually tend toward normal. More importantly, these data suggest a role for microtubules in the regulation of the transition into mitosis.

Finally, we emphasize the general applicability of this technique. Essentially all mammalian cells exhibit pinocytosis and so can accumulate fluorescein-dextran. Many cells are comparable in pinocytic activity to J774.2 macrophages. In addition, an increase in the concentration of fluorescein-dextran used for labeling, coupled with the potential for extremely sensitive fluorimetric measurement, should allow examination of cell lines with significantly lower rates of pinocytosis. Moreover, we have shown previously that the basic phenomenon of decreased endocytosis during mitosis is not a unique property of J774.2 macrophages but is readily observed in other cultured cell lines; for example, 3T3 fibroblasts and Chinese hamster ovary cells, which are thus clearly suited for mitotic analyses similar to those reported here.

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