DNA Distribution in the Cell Cycle of *Euglena gracilis*. Cytofluorometry of Zinc Deficient Cells†

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ABSTRACT: Analysis of DNA content of intact cells by laser induced cytofluorometry permits dynamic studies of the cell cycle in the synchronously dividing eukaryote *Euglena gracilis*. In this manner, the effects of zinc deficiency and cadmium toxicity on in situ DNA synthesis and cell division of this organism have been studied. In the G-1 phase of the cell cycle, prior to initiation of DNA synthesis, the DNA content of synchronously growing *E. gracilis* is characteristic of cells with an unreplicated genome. In S phase there is a progressive increase in DNA content which leads to genome duplication as the cells enter G_2. In the subsequent mitosis all cells divide. Cytofluorometric definition of DNA content serves as a standard of reference to study variables which alter or block each stage of the cell cycle. Growth in zinc deficient media inhibits cell division. The DNA content of such zinc deficient cells is characteristic of a population of cells blocked in S/G_2 with a small fraction in G_1. Moreover, cells synchronized in G_1 and placed in zinc deficient media do not progress into S phase. Cadmium also inhibits cell division, and the DNA content of these blocked cells is three to four times greater than that of cells in G_1. Zinc is essential for the biochemical events of the premitotic state which include initiation of DNA synthesis, DNA synthesis, and progression from G-2 to mitosis. Cadmium-induced derangements of the cell cycle include alterations in regulation of cellular DNA content.

The eukaryotic cell cycle is an orderly sequence of biochemical processes involving RNA and DNA synthesis, chromosomal replication, formation of the mitotic apparatus, and mitosis resulting in cell division (Howard and Pelc, 1953). While premitotic events of the cycle have been defined in terms of the DNA content of cells, those in mitosis are based on structural changes in the nucleus, e.g., condensation of chromosomes, presence of mitotic apparatus, etc. Analysis of the premitotic events in whole cells has been limited by the lack of sensitive, specific approaches capable of quantitating DNA and changes in its content in situ. Autoradiography, chemical measurement of nucleic acids, and ultraviolet and fluorescence spectrophotometry are all suitable for this purpose but are either time-consuming and/or incapable of resolving details of dynamic events affecting the DNA content of a statistically significant number of individual cells. The advent of instrumentation and technology, such as flow cytofluorometry, for the rapid analysis of laser-induced fluorescence of DNA-dye complexes in intact cells has now made feasible the study of DNA metabolism and the effect of either essential or toxic substances on the cell cycle (Van Dilla et al., 1969; Crissman and Steinkamp, 1973; Kraemer et al., 1972; Tobey and Crissman, 1972, Yataganas et al., 1974, Krishan, 1975).

In our continuing effort to explore the role of metals in biological systems we have employed this flow cytofluorometric technique to study the effects of zinc or cadmium on the cell cycle of the eukaryote *Euglena gracilis*. Induction of zinc deficiency in this organism results in growth arrest associated with an increase in DNA and a decrease in RNA content, while the nuclear ultrastructure apparently remains normal (Wacker, 1962; Falchuk et al., 1975a). Cadmium excess not only causes growth arrest but is also associated with derangements in nuclear and cytoplasmic cleavage (Falchuk et al., 1975b). Importantly, each stage of the cycle of this organism can be defined and resolved in great detail, since the cell cycle can be synchronized by exposing log phase cultures to alternating periods of light and dark (Cook and James, 1960; Cook, 1961a,b; Edmunds, 1964). In this manner, large numbers of log phase cells can be studied as they progress through the cell cycle and their DNA distribution can be compared with that of cells whose growth is arrested by deficiency of zinc or excess of cadmium.

Methods

Euglena gracilis, strain Z, was grown in media previously described (Falchuk et al., 1975a). Zinc sufficient media (+Zn) contained 10 μM Zn^{2+}, while deficient media (−Zn) contained 0.1 μM Zn^{2+}. Cadmium, in a final concentration of 10 μM, was added to (−Zn) media to grow cadmium intoxicated cells (Falchuk et al., 1975b). Cells were grown at 22° with constant shaking. Log phase cultures of *E. gracilis* were synchronized by exposing the cultures to alternating light and dark periods lasting 14 and 10 hr, respectively. The intensity and time of light exposure are critical for synchronization. Lights were switched on and off automatically to regulate duration of light and dark periods. Light intensity was fixed at 35,000 lux by placing four fluorescent Westinghouse bulbs 3 ft above the culture flasks (Cook and James, 1960; Cook, 1961a,b; Edmunds, 1964). Nondividing populations of (+Zn) cells were obtained by allowing aliquots of synchronized (+Zn) cells to reach stationary phase or by incubating early stationary phase (+Zn) cells in (−Zn) media. Initiation of cell division was achieved by dilution of stationary phase cultures with fresh media or by increasing the zinc content of (−Zn) media to 10 μM.

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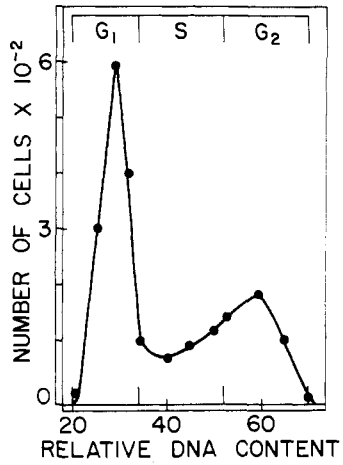

FIGURE 1: Histogram of DNA content from an unsynchronized log phase culture of *E. gracilis*. The first peak corresponds to the fraction of cells in G_1 with an unreplicated genome; the second peak to cells which have doubled their DNA content and are in G_2 or M phase of the cell cycle. The intervening channels correspond to cells which are in the process of DNA synthesis, e.g., are in S phase. As described in the text, the fluorescence intensity is proportional to DNA content. Thus, in relative terms, the DNA content of cells in peak II is double that of cells in peak I.

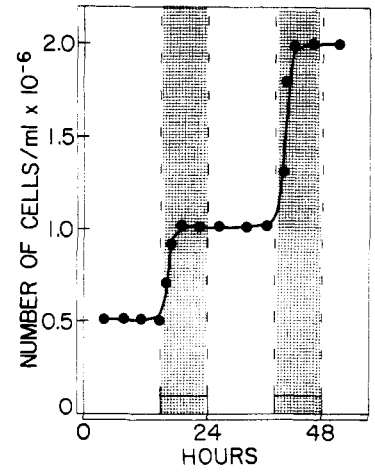

FIGURE 2: Synchronously dividing culture of *E. gracilis*. The culture was exposed to alternating periods of light and dark, lasting 14 and 10 hr, respectively. In the light periods cells do not divide. A burst of cell division occurs within 2–4 hr of entering the dark period.

Aliquots of cells for analysis of DNA content by flow cytofluorometry were collected by centrifugation at 150*g* for 5 min and fixed at 4° in 70% ethanol for 30 min. Subsequent washing in 30% ethanol and glass-distilled water was followed by incubation of the cells with RNase (1 mg/ml, pH 7.0 in 0.2 *M* phosphate buffer at 37°) for 30 min. At the end of incubation, the cells were washed with water and stained in ice-cold propidium diiodide solution (0.5 mg/ml in 1.12% sodium citrate) for 30 min. Excess stain was removed by washing two times with glass-distilled water (Crissman and Steinkamp, 1973). Propidium diiodide stained samples were analyzed in a cytofluorograph (Model 4801, Bio/physics System Inc., Mahopac, N.Y.). The flow system of this instrument allows passage of one cell at a time through a 100-μ orifice (restrictor valve) into a flow chamber where laminar flow is induced by a sheath of water. The cell traverses through exciting, monochromatic radiation from an argon ion laser beam tuned to emit at 488 nm. The resultant fluorescence of propidium diiodide–DNA complexes of the cell nucleus is converted to an electrical signal by a photomultiplier, the output being displayed on the horizontal axis of a cathode ray tube. The signal also enters a multichannel pulse height distribution analyzer (Model 2100, Bio/physics Inc., Mahopac, N.Y.) where the frequency distribution of the pulses as a function of the magnitude of the signal is stored in a memory unit and subsequently displayed as a histogram. One hundred channels are used, and the abscissa of the histogram reflects increasing values of the fluorescence signal. The numbers of cells recorded in each channel are registered simultaneously on a print-out tape system, allowing quantitation of the number of cells fluorescing at a characteristic intensity.

Propidium diiodide, the dye used in this study, stains both DNA and RNA. Incubation of mammalian cells with RNase, prior to staining, has been shown to obviate interference by RNA in DNA analysis (Crissman and Steinkamp, 1973, Krishan, 1975). This was shown to be true also for *E. gracilis* by processing two sets of cells, in parallel, with RNase and propidium diiodide on one hand and with Acriflavin–Feulgen stain alone on the other. The latter dye is known to be specific for DNA (Kasten, 1959; Kraemer et al., 1972). After hydrolysis for 20 min at room temperature, cells were stained with an 0.5% Acriflavin–SO_2 mixture for 1 hr and washed three times in 70% ethanol. Acriflavin–Feulgen stained cells and those stained with propidium diiodide after RNase digestion resulted in identical histograms of DNA content.

E. gracilis, on exposure to light, develop chlorophyll, which can also fluoresce under these conditions. To determine the extent to which this might interfere with the determination of DNA in *E. gracilis*, two unstained samples were analyzed. The first contained *E. gracilis* suspended in water. A broad peak of fluorescence was observed between channels 20 and 70 of the pulse height analyzer. The second sample was suspended in 70% ethanol for 30 min followed by a 5-min wash in 30% ethanol. The green color disappeared from the cell suspension and no fluorescence was observed, demonstrating that ethanol fixation and dehydration, conventionally employed in the preparation of cells, obviate interference by chlorophyll in DNA determination.

Results

Log phase cultures contain organisms in all different stages of the cell cycle. A histogram from a log phase culture of *E. gracilis* stained with propidium diiodide and analyzed in the cytofluorograph is shown in Figure 1. Channels of the pulse height distribution analyzer from numbers 20 to 70 contain the entire fluorescence distribution pattern of this population. Two major peaks are observed; the maximum of peak I coincides with channel number 28 and is separated by channels 35–52 from peak II, encompassing channels 53–70. Peak I corresponds to a cell population in G-1 with an unreplicated genome, whereas peak II corresponds to cells which have doubled their DNA content and are in G-2 or M phase of the cell cycle. The intervening channels apparently correspond to cells in which DNA synthesis is occurring, i.e., in S phase.[1] Cell division of synchronized organisms does not occur during the 14-hr light period, as shown by the growth curves (Figure 2). Within 2 hr

[1] The stages of the cell cycle are correlated with position in the channels by means of cells labeled with tritiated thymidine. The uptake signifies DNA synthesis. Autoradiography together with propidium diiodide staining then allows correlation of cells known to actively synthesize DNA with positions in the channels of the cytofluorometer.

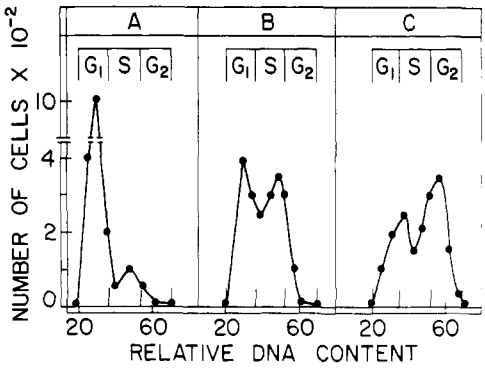

FIGURE 3: Histogram of DNA content of synchronized cells harvested during the light period at 4, 8, and 14 hr. At 4 hr most cells are in G_1 and have not initiated DNA synthesis (panel A). At 8 hr, as the fraction of cells synthesizing DNA increases, the height of the G_1 peak decreases associated with a concomitant increase in the height of S phase (panel B). At 14 hr, the majority of cells are in S or have reached G_2 and doubled their DNA content.

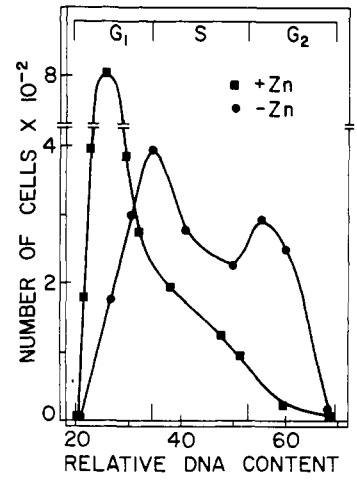

FIGURE 4: Comparison of the histograms of DNA content of zinc deficient with stationary phase zinc sufficient *E. gracilis*. In stationary phase, the majority of (+Zn) cells are in G_1, with a small fraction in S. In contrast, nondividing (−Zn) cells are mostly in S or G_2, with a fraction of cells in G_1.

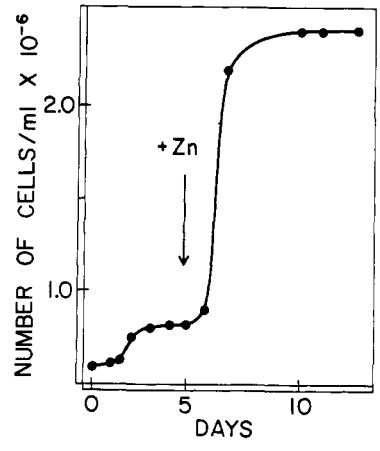

FIGURE 5: Growth characteristics of early stationary phase (+Zn) cells incubated in (−Zn) media. Following incubation, there is a small increase in cell density followed by a cessation of cell division. On raising the zinc content to 1×10^{-5} *M* there is a striking increase in cell division, the final cell density reaching that expected of a (+Zn) culture.

of entering the dark period, the number of cells per milliliter increases and then approximately doubles prior to the next light period. The cycle is repeated during the following dark period. Panels A, B, and C of Figure 3 show the cytofluorometric analysis of cells harvested at 4, 8, and 14 hr of light exposure, respectively, i.e., preceding cell division. Panel A shows the pattern of DNA content typical of cells in G-1. As the number of cells synthesizing DNA, i.e., the number in S phase, increases, the G-1 peak decreases while S increases (panel B). As the cells reach G-2, the DNA content has doubled, shifting the fluorescence pattern, as shown in panel C. Following cell division, the cells once again are in G-1 and are ready to initiate the cell cycle. The progressive increase in DNA content of the population of cells prior to division is demonstrated clearly and is consistent with chemical measurements of increasing DNA content of synchronized cells during similar periods (Edmunds, 1964). The histogram of DNA content characterizing synchronized cells, therefore, is consistent with the assignment of the channels defining G-1 and G-2 phases (Figure 1).

The DNA content of stationary, nondividing cells was examined next. Figure 4 compares the pattern of early stationary phase (+Zn) with that of (−Zn) cells, obtained when cell division ceases. The histogram of (+Zn) cells demonstrates that the majority, though not all, of the cells in stationary phase are in G-1, with a smaller number in S. In contrast, the pattern of nondividing (−Zn) cells is typical of that of S/G-2. (−Zn) cells have previously been shown to cease dividing on depletion of zinc in the growth media (Falchuk et al., 1975a). The resulting histogram of DNA content suggests that as the nonsynchronously growing cells in the (−Zn) media are deprived of zinc, those cells which are in S do not continue to G-2, while those which reach G-2 do not proceed to mitosis. Moreover, a small fraction in G-1 is also present. To further detail the effect of zinc deprivation on the G-1 to S transition, early stationary phase (+Zn) cells, known to be mostly in G-1 (Figure 4), were incubated in (−Zn) media. Figure 5 illustrates that following incubation in (−Zn) media there is a 25% increase in cell number followed by cessation of division. Addition of zinc to the medium confirms that the absence of zinc is responsible for the inhibition of cell division. Within 24 hr of the addition of zinc the cell numbers increase by 200%, reaching those expected for a (+Zn) culture. The cytofluorometric analysis of these cells (Figure 6) demonstrates that, prior to the addition of zinc, when cell division has ceased, almost all cells incubated in (−Zn) media are in G-1 phase. Hence, zinc deprivation of cells in G-1 blocks their progression into S. Addition of zinc to (−Zn) cells reverses the block of their cell cycle, restoring the normal pattern of dividing cells which becomes identical with that shown in Figure 1.

We have previously shown that the growth of *E. gracilis* in (−Zn) media to which cadmium is added (−Zn, +Cd) results in even greater inhibition of cell division than that observed in (−Zn) media (Falchuk et al., 1975b). Morphologic studies of these (−Zn, +Cd) cells have demonstrated multinucleation of greatly enlarged organisms. Cytofluorometric analysis of these cells is shown in Figure 7. The histogram of a population of (+Zn) cells exhibits both G-1 and S peaks, but in an analogous record of (−Zn, +Cd) cells the distribution of DNA is broad. The G-1 population is reduced, while the S and G-2 populations become predominant, just as observed in (−Zn) cells. In addition, and in contrast to (−Zn) cells, signals appear in all channels of the instrument, so that significant amounts of fluorescence

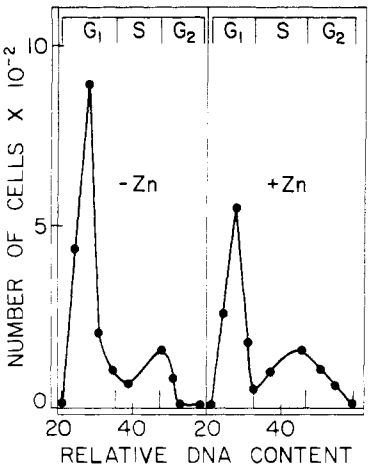

FIGURE 6: Comparison of the histograms of DNA content of *E. gracilis* incubated in (−Zn) medium prior to and following addition of zinc. Following cessation of cell division, the majority of the (−Zn) cells are in G_1 with a small fraction in S. On addition of zinc, the number of cells blocked in G_1 decreases and a histogram typical of dividing log phase cells results.

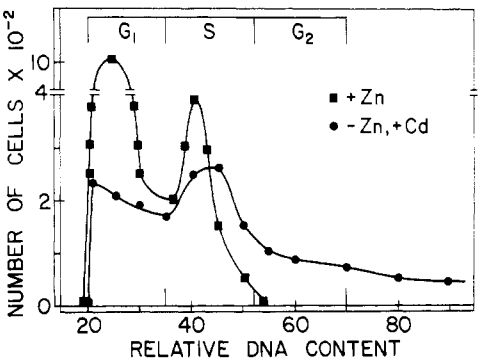

FIGURE 7: Comparison of the histograms of DNA content of stationary phase (+Zn) cells with that of (−Zn, +Cd) cells. Stationary (+Zn) cells characteristically are in G_1 with a small fraction in S. In contrast, the DNA content of cells grown in (−Zn, +Cd) media is increased. There are cells in S and G_2 and a fraction which contains three times or even higher the DNA content of a G_1 population. The histogram underestimates the DNA content of the enormous (−Zn, +Cd) cells since only the fraction of cells small enough to enter the 100-μ restrictor valve of the cytofluorograph can be analyzed (see text).

appear even in the higher channels, indicating the existence of cells containing triple, or even higher multiples of the normal amount of DNA. This histogram underestimates the width of the distribution of DNA in (−Zn, +Cd) cells since only those cells capable of passing the 100-μ orifice of the restrictor valve are analyzed and plotted. As previously shown (−Zn, +Cd) cells are larger and 85 times heavier than control cells. Hence, these results are consistent with the morphologic findings.

Discussion

Quantitative analysis of cell constituents in situ has been both an intellectual and technical challenge for nearly three decades owing to their exceedingly low absolute amounts which, moreover, vary markedly as a result of metabolic activity. Microscopic, histochemical, or autoradiographic techniques employed to study whole cells all have been limited either by lack of sensitivity, specifity, or capability to quantitate variations in the concentration of cell components as a function of time.

Thus, though both the light and electron microscopes permit qualitative identification of cellular components and of detailed structure of fixed, nonviable preparations, quantitative analysis has not been possible by these means. In contrast, quantitative studies with viable cells have been achieved using the characteristic absorption of nucleic acids at 260 nm (Dhére, 1906, Heyroth and Loofbourow, 1934, Loofbourow, 1940) to estimate DNA in single cells by means of ultraviolet microscopy (Caspersson, 1936, 1950; Loofbourow, 1950; Leuchtenberger et al., 1952; Walker and Yates, 1952; Davies and Walker, 1953). The method, however, is not specific for DNA, since under the conditions employed its spectral characteristics are indistinguishable from those of RNA, precursors, constituents, and degradation products of nucleic acids. Damage resulting from ultraviolet irradiation to living cells and the particular components being studied present further limitations in the use of the method (Freed, 1969).

Other approaches, particularly staining of viable cells with dyes which fluoresce in the visible range of the spectrum when excited, produce no apparent damage to cells and have proven more sensitive than methods based on absorption of ultraviolet irradiation. However, though fluorescent diaminoacridine dyes specifically stain nucleic acids and thereby provide the means for their quantitative analysis, they do not differentiate between DNA and RNA (Ellinger, 1940; Strugger, 1949; DeBruyn et al., 1950, 1953; West, 1969). Moreover, the $t_{1/2}$ of fluorescent decay subsequent to excitation of intracellularly bound dyes is of the order of seconds, demanding the design of complex instrumentation with rapid recording capabilities to permit quantitative analysis (West, 1969). Quantitation of fluorescence of viable, single cells stained with Acridine Orange has been achieved, albeit through laborious and time consuming methods. The instrumentation available requires selection of the particular cell to be examined from a suspension of cells on a glass slide by visual observation. This is followed by excitation of the intracellular dye and recording of the resulting fluorescent spectrum. The spectrum could be analyzed subsequently and the content of intracellular dye bound to nucleic acids quantitated (West, 1969). While this approach allows quantitative estimation of the nucleic acid content of viable cells, it cannot differentiate between DNA and RNA and, therefore, cannot be utilized to study the metabolism of either one alone. In contrast, analysis of Feulgen stained cells has been shown to be specific for DNA (Vendrely, 1971). However, statistically significant numbers of cells—stained either with fluorescent Acridine Orange or Feulgen dyes—cannot be analyzed within a period of time which is sufficiently short to examine metabolic events, as is necessary when dynamic changes of organelles are to be studied.

The flow cytofluorometric analysis employed in the present study obviates many of these limitations and now makes it possible to study dynamic variations in the composition of cell components in a specific and quantitative manner, taking advantage of sensitivity of fluorescence analysis. It processes cells by passing them extremely rapidly, single file, through a laser beam which excites their fluorescence and allows quantitation in a time interval sufficiently brief so that decay of the signal does not become a critical factor. In this manner, the fluorescent signals from each of 10,000 cells are recorded, stored, and analyzed in a matter of minutes, in the aggregate providing statistically significant results (Van Dilla et al., 1969; Kraemer et al., 1972; Tobey

and Crissman, 1972; Crissman and Steinkamp, 1973; Dean and Jebb, 1974; Yataganas et al., 1974; Yataganas and Clarkston, 1974; Krishan and Paika, 1974; Krishan, 1975). The approach is applicable to both living and fixed cells. The specificity and resolution of the resultant fluorescence permit precise quantitation of the distribution of DNA in the subpopulation of 10,000 cells. Moreover, samples can be removed from a stock suspension of cells and measured at short time intervals. Therefore, it has now become feasible to follow the redistribution of DNA in that population both as a function of time and cell metabolism.

Synchronization of a population, moreover, permits even more definitive studies of shifts in the distribution of DNA (Tobey et al., 1972; Krishan and Paika, 1974). In such cells the biochemical events required for division are presumably synchronized, causing nearly all cells to divide within a relatively short time interval. Therefore, synchronization can be used to define the events of the cell cycle itself, e.g., G-1, S, or G-2, in terms of the pattern of DNA content of the majority of the cells, while providing the means to follow this progression through each of the metabolic events of the cell cycle. Moreover, the shifts in the histogram of DNA content can be used as a standard of reference to study variables which alter or block progression from G-1 to S or to G-2. This is illustrated by comparing the histogram of the DNA content of synchronized cells with those of organisms whose division has been altered experimentally, through, e.g., zinc deficiency or cadmium toxicity.

The number of cells in a log phase culture increases linearly as a function of time. In contrast, in a synchronized culture increments in cell numbers occur only during discrete time intervals, e.g., the dark period for *E. gracilis*. The histogram of DNA content of cells from a log phase culture (Figure 1) shows most cells to be in G-1 with but a small fraction in S and G-2. Since division does not occur until cellular DNA has doubled, it is the small fraction of cells in G-2 which determines the continuous increase in numbers of cells. In contrast, in a synchronized culture (Figure 3) progression of the majority of cells from G-1 to S and then through G-2 occurs in unison and is restricted to the light period. On entering the dark period, the DNA content of the great majority of cells has doubled already and the cells are ready to enter mitosis, resulting in a burst of cell division (Figure 2).

The histogram of the DNA content of nonsynchronized, zinc-deficient cells (Figure 4) shows a striking decrease of the G-1 peak with increases in the peaks at both S and G-2. Though this histogram is remarkably similar to that of a population of synchronously dividing cells (Figure 3,c), the (−Zn) cells do not proceed to mitosis and, hence, do not divide. This indicates that a fraction of the (−Zn) cells is blocked in G-1, i.e., during initiation of DNA synthesis, a second fraction is blocked in S, in the course of DNA synthesis, while a third fraction is blocked in G-2, following DNA synthesis. The block in the step which results following incubation of log phase cells into (−Zn) media (Figure 5) most clearly demonstrates the effect of zinc deprivation on the progression from G-1 to S (Figure 6).

Though in both plant and mammalian cells the metabolic steps responsible for progression from G-1 to S, and from G-2 to M, are unknown, on-going protein synthesis is required (Gelfant, 1966; Brunori et al., 1966; Epifanova and Terskikh, 1969; Avanzi et al., 1969; van't Hof, 1974). Derangements in both RNA and protein metabolism induced by zinc deficiency might result in a block in G-1 or in G-2. Likewise, a requirement for zinc in DNA synthesis and metabolism could account for the block in S. Therefore, the results suggest that zinc is essential for biochemical processes essential to these premitotic events. The normal ultrastructure of the single nucleus found in a (−Zn) cell (Falchuk et al., 1975a) is consistent with this view. Zinc is, indeed, known to be involved both in nucleic acid and protein metabolism. Zinc deficiency in *E. gracilis* results in decreases of both RNA and protein content (Wacker, 1962). Both the DNA dependent RNA polymerase from *Escherichia coli* (Scrutton et al., 1971) and that from *E. gracilis* (B. Mazus, L. Ulpino, K. H. Falchuk, and B. L. Vallee, in preparation) are zinc metalloenzymes and RNA isolated from various cells, including *E. gracilis*, contain zinc, as well as other metals, probably required for structure stabilization (Wacker and Vallee, 1959; Prask and Plocke, 1971). Zinc has a functional role in *E. coli* DNA dependent DNA polymerase, RNA dependent DNA polymerase from various virus sources, as well as of DNA itself (Lieberman et al., 1963; Shin and Eichhorn, 1968a,b; Sandstead and Rinalde, 1969; Slater et al., 1971; Rubin, 1972; Williams and Loeb, 1973; Auld et al., 1974a,b, 1975) and is, therefore, essential for DNA synthesis and function. Zinc may also be involved in the function of microtubules, which form the mitotic apparatus during G-2/M, since the metal has been found in mitotic spindles of sea urchin eggs (Fujii, 1954).

The histogram of DNA in (−Zn, +Cd) cells shows a pattern of cells with broad distribution of DNA. There are cells in G-1, S, G-2 and, importantly, cells whose DNA content is three or four times greater than that of cells in G-1. These cells are multinucleated and do not divide (Falchuk et al., 1975b). Normally, the DNA content does not increase beyond that of cells in G-2, nor do cells become multinucleated, even if cytoplasmic cleavage does not occur. Hence, our findings suggest derangements in the regulatory mechanisms of both premitotic and mitotic events of the cell cycle of (−Zn, +Cd) cells (Figure 7). Interestingly, derangements leading to similar structural and quantitative alterations in DNA content have been noted in cells infected with some viruses (syncytia formation), in various neoplastic tissues, e.g., giant cell tumors, and in leukemic cells following treatment with a variety of antineoplastic agents (Krishan, 1972; Desai et al., 1974).

The detailed analysis of the cell cycle in nonsynchronized, synchronized, zinc deficient, or cadmium intoxicated cells illustrates the potential of the cytofluorometric approach. Moreover, the capacity to quantitate by specific and sensitive means the components of cell organelles as a function of time is not restricted to processes involving DNA and cell division. Any cell constituent which can be caused to fluoresce without staining, e.g., chlorophyll, or specifically labeled with fluorescent dyes, can be investigated. This will permit design of experiments relating to problems of cell biology and biochemistry which have hitherto been inaccessible to dynamic approaches.

References

Auld, D. S., Livingston, D., Kawaguchi, H., and Vallee, B. L. (1974a), *Biochem. Biophys. Res. Commun. 57,* 967.

Auld, D. S., Livingston, D., Kawaguchi, H., and Vallee, B. L. (1974b), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2091.

Auld, D. S., Livingston, D., Kawaguchi, H., and Vallee, B. L. (1975), *Biochem. Biophys. Res. Commun. 62,* 296.

Avanzi, S., Brunori, A., and D'Amato, F. (1969), *Dev. Biol. 20,* 368.

Brunori, A., Avanzi, S., and D'Amato, F. (1966), *Mutat. Res. 3,* 305.
Caspersson, T. (1936), *Skand. Arch. Phys., Suppl. 8,* 73.
Caspersson, T. (1950), Cell Growth and Cell Function, New York, N.Y., Norton.
Cook, J. R. (1961a), *Plant Cell Physiol. 2,* 199.
Cook, J. R. (1961b), *Biol. Bull. 121,* 277.
Cook, J. R., and James, T. W. (1960), *Exp. Cell Res. 21,* 583.
Crissman, H. A., and Steinkamp, J. A. (1973), *J. Cell Biol. 59,* 766.
Davies, H. G., and Walker, P. M. B. (1953), *Prog. Biophys. 3,* 195.
Dean, P. N., and Jebb, J. H. (1974), *J. Cell Biol. 60,* 523.
DeBruyn, P. P. A., Farr, R. S., Banks, H., and Northland, F. W. (1953), *Exp. Cell Res. 4,* 174.
DeBruyn, P. P. A., Robertson, R. C., and Farr, R. S. (1950), *Anat. Rec. 108,* 279.
Desai, L. S., Krishan, A., and Foley, G. E. (1974), *Cancer 34,* 1873.
Dhére, C. (1906), *C. R. Seances Soc. Biol. Ses. Fil. 60,* 34.
Edmunds, L. N., Jr. (1964), *Science 145,* 266.
Ellinger, P. (1940), *Biol. Rev. Cambridge Philos. Soc. 15,* 323.
Epifanova, O. I., and Terskikh, V. V. (1969), *Cell Tissue Kinet. 2,* 75.
Falchuk, K. H., Fawcett, D., and Vallee, B. L. (1975a), *J. Cell Sci. 17,* 57.
Falchuk, K. H., Fawcett, D. W. Jr., and Vallee, B. L. (1975b), *J. Ultrastruct. Cytol.,* in press.
Freed, J. J. (1969), *Phys. Tech. Biol. Res., 2nd Ed. IIIC,* 95.
Fujii, T. (1954), *Nature (London) 174,* 1108.
Gelfant, S. (1966), *Methods Cell Physiol. 2,* 359.
Heyroth, F. F., and Loofbourow, R. C. (1934), *J. Am. Chem. Soc. 56,* 1728.
Howard, A., and Pelc, S. R. (1953), Symposium on Chromosome Breakage (Supplement to Heredity Vol. 6), Springfield, Ill., Charles C Thomas.
Kasten, F. H. (1959), *Histochemie 1,* 466.
Kraemer, P. M., Deaven, L. L., Crissman, H. A., and Van Dilla, M. A. (1972), *Ann. N.Y. Acad. Sci. 2,* 47.
Krishan, A. (1972), *J. Cell Biol. 54,* 567.
Krishan, A. (1975), *J. Cell Biol.* (in press).
Krishan, A., and Paika, K. (1974), Proceedings of the American Society of Cell Biology, San Diego Meeting.
Leuchtenberger, C., Klein, G., and Klein, E. (1952), *Can. Res. 12,* 480.
Lieberman, I., Abrams, R., and Ove, P. (1963), *J. Biol. Chem. 238,* 2, 141.
Loofbourow, J. R. (1940), *Rev. Mod. Phys. 12,* 320.
Loofbourow, J. R. (1950), *J. Opt. Soc. Am. 40,* 137.
Prask, J. A., and Plocke, D. J. (1971), *Plant Physiol. 48,* 150.
Rubin, H. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 712.
Sandstead, H. H., and Rinalde, R. R. (1969), *J. Cell Physiol. 73,* 81.
Scrutton, M. C., Wu, C. W., and Goldthwait, D. A. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2497.
Shin, Y. A., and Eichhorn, G. L. (1968a), *Biochemistry 7,* 3003.
Shin, Y. A., and Eichhorn, G. L. (1968b), *Biochemistry 7,* 1026.
Slater, J. P., Mildvan, A. S., and Loeb, L. A. (1971), *Biochem. Biophys. Res. Commun. 44,* 37.
Strugger, S. (1949), Fluoreszenz mikroskopie und mikrobiologie, Hanover, Scheper.
Tobey, R. A., and Crissman, H. A. (1972), *Can. Res. 32,* 2726.
Tobey, R. A., Crissman, H. A., and Kraemer, P. M. (1972), *J. Cell Biol. 54,* 638.
Van Dilla, M. A., Trujillo, T. T., Mullavey, P. F., and Coulter, J. R. (1969), *Science 163,* 1213.
van't Hof, J. (1974), Cell Cycle Controls, New York, N.Y., Academic Press, p 77.
Vendrely, C. (1971), The Cell Cycle and Cancer, New York, N.Y., Marcel Dekker, p 227.
Wacker, W. E. C. (1962), *Biochemistry 1,* 859.
Wacker, W. E. C., and Vallee, B. L. (1959), *J. Biol. Chem. 234,* 3257.
Walker, P. M. B., and Yates, H. B. (1952), *Proc. R. Soc. London, Ser. B 140,* 274.
West, S. S., (1969), *Phys. Tech. Biol. Res., 2nd Ed. IIIC,* 253.
Williams, R. D., and Loeb, L. A. (1973), *J. Cell Biol. 58,* 594.
Yataganas, X., and Clarkston, B. D. (1974), *J. Histochem. Cytochem. 22,* 2795.
Yataganas, X., Stife, A., Perez, A., and Crissman, B. B. (1974), *Can. Res. 34,* 2795.