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Cell-cycle-related changes of 3′:5′-cyclic GMP levels in Novikoff hepatoma cells

(3′:5′-cyclic AMP/cell synchrony/mitosis)

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ABSTRACT **Intracellular and extracellular levels of 3′:5′-cyclic GMP and 3′:5′-cyclic AMP were studied in synchronized Novikoff rat hepatoma cells. Intracellular levels of cyclic GMP increased spontaneously from 2-fold (without colcemid) to 10-fold (with colcemid), in proportion to the number of cells in mitosis. As cells entered mitosis, cellular cyclic AMP declined simultaneously with the rise in cyclic GMP. These reciprocal changes in cyclic nucleotide levels were reversed as cells passed out of metaphase and through anaphase. Maximum cyclic AMP and minimum cyclic GMP concentrations occurred during G-1. Less marked reciprocal fluctuations in both cyclic nucleotides were also found in S-phase and early G-2, where the ratio of cyclic AMP to cyclic GMP concentrations first fell and then increased. These changes in cyclic nucleotide ratios were closely correlated with major cell-cycle transitions at the boundaries between G-1/S-phase, S-phase/G-2, G-2/prophase, and metaphase/anaphase.**

Most, but not all, of the extracellular cyclic nucleotides were extruded when cells traversed mitosis. Colcemid or vinblastine completely prevented the appearance of extracellular cyclic AMP but augmented the appearance of extracellular cyclic GMP in parallel with the accumulation of mitotic cells. These results reflected changes in intracellular cyclic nucleotides and indicated that increased intracellular turnover of cyclic GMP and cyclic AMP occurred before and after metaphase, respectively.

Elevated cyclic GMP levels during mitosis and S-phase are consistent with potential modulatory roles for this cyclic nucleotide in proliferation.

Cyclic AMP (adenosine 3′:5′-cyclic monophosphate) (1–3) and cyclic GMP (guanosine 3′:5′-cyclic monophosphate) (4) have been implicated as regulators of cell proliferation. Cyclic AMP varies during the cell cycle (5–7), and some evidence suggests that these fluctuations reflect discrete periods in the cell cycle during which elevated cyclic AMP may enhance or retard cell-cycle progression (6, 8). A transient increase in cyclic GMP content has been reported to occur in early G-1 of partially synchronized mouse fibroblasts (9), but to date little is known about cyclic GMP levels during other phases of the cell cycle. We report here that, in a fast growing rat hepatoma cell line, cyclic GMP levels vary independently and reciprocally with cyclic AMP levels during the cell cycle and that cyclic GMP levels increase up to 10-fold at the onset of mitosis. These results are consistent with possible regulatory roles for both cyclic AMP and cyclic GMP at specific stages of the cell cycle, most notably during mitosis.

Abbreviations: cyclic GMP, guanosine 3′:5′-cyclic monophosphate; cyclic AMP, adenosine 3′:5′-cyclic monophosphate; HU, hydroxyurea; I.C., intracellular; E.C., extracellular.

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METHODS

Culture Conditions and Synchronization Procedures. Suspension cultures of Novikoff hepatoma cells (N1S1-67) were maintained in Swim's medium 67 supplemented with 5% serum and 5% pancreatic autolysate, as described (10). Cells were synchronized at the G-1/S border by a double hydroxyurea (HU) block or at metaphase by a single HU block followed by a reversible colcemid block (10). With both synchrony methods, cells were resuspended at cell densities ranging from 0.5 to 1.3 $\times 10^6$ cells per ml in drug-free basal medium 42B‡ at 37° to reinitiate synchronous growth. All experiments were performed in a 37° warm room.

Cell number and volume were measured on a Coulter counter, model Zb, equipped with a volume channelizer and plotter. The flow of synchronized cells through the cell cycle was determined from analysis of cell volume, cell density, mitotic index, and, in some cases, relative DNA content on a modified Biophysics model 4801 Cytofluorograph equipped with a model 2100 distribution analyzer (13). The percentage of mitotic cells was determined using phase contrast microscopy by counting 300 to 600 cells, either before or after fixation in 2.5% glutaraldehyde in Hanks' balanced salt solution, pH 7.4.

Modal cell volume and relative modal DNA content per cell were determined from histograms of cell number against volume or relative DNA content. Relative DNA content represents the ratio of channel positions to the channel position of cells with a G-1 content of DNA. The proportion of cells in G-1, S-phase, and G-2 + M phases was estimated manually with the aid of a planimeter from DNA histograms of synchronized cells (14). All results are the mean of duplicate samples from parallel cultures, unless otherwise stated, and are representative experiments.

Extraction and Measurement of Cyclic Nucleotides. Ten- to 14-milliliter aliquots of cells were withdrawn under sterile conditions from culture flasks, and cells were separated from medium by centrifugation for 45 sec at full speed in a desk-top IEC clinical centrifuge. The medium was decanted into test-tubes containing perchloric acid at a final concentration of 0.3

‡ Basal medium 42B was identical to medium 67 except that it contained 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) and lacked pancreatic autolysate and serum. Pancreatic autolysate and serum that had not been heat-inactivated (GIBCO) were added to medium 42B to a final concentration of 5% each and the complete medium was incubated at 37° for 6–8 hr to allow the cyclic nucleotide phosphodiesterase present in serum (11, 12) to hydrolyze any measurable cyclic AMP or cyclic GMP. The phosphodiesterase activity was then destroyed by heating the complete growth medium at 56° for 45 min. After this treatment, phosphodiesterase activity was undetectable either before or after a 14-hr incubation in the presence of cells.

M. The walls of the centrifuge tubes were swabbed to remove residual medium and 1 ml of 0.3 M perchloric acid was added to the cell pellet with vigorous mixing. Samples were stored at −20°. Approximately 80 fmol of cyclic [8-^{3}H]GMP (specific activity 13 cpm/fmol) (Schwarz/Mann) and 36 fmol of cyclic [8-^{3}H]AMP (specific activity 28 cpm/fmol) (Schwarz/Mann) were added to each sample to monitor losses during subsequent purification steps. Cell pellets were subjected to five cycles of freezing and thawing in a dry-ice/ethanol bath (15). Samples were deproteinized by centrifugation and the resultant supernatant fractions and appropriate blanks were applied to 2–3 cm × 0.7 cm dry neutral alumina oxide columns. The columns were washed with 10 ml of H_2O and the cyclic nucleotides were eluted with 10 ml of 200 mM ammonium formate, pH 6.0. The eluate was allowed to drip onto 2 × 0.7 cm Bio-Rad AG-1-X8 anion exchange columns (formate form) that had been washed with H_2O. These columns were washed with 10 ml of H_2O and the major fraction containing cyclic AMP was eluted with 10 ml of 1 M formic acid. A second 10-ml wash of 1 M formic acid was required to prevent residual cyclic AMP from coeluting with cyclic GMP. Cyclic GMP was eluted with 10 ml of 6 M formic acid. The fractions containing cyclic nucleotides were evaporated at 40° on a Buchler Evapo-Mix and reconstituted in 500 μl of either H_2O or 25 mM Tris·Cl, pH 7.5, containing 1 mM $MgCl_2$. Recoveries were monitored by determining the radioactivity in 100-μl aliquots of each reconstituted sample in 3a70B scintillation fluid (Research Products International) on a Beckman scintillation spectrometer. Recoveries were about 45% for cyclic GMP and 35% for cyclic AMP. Cyclic GMP and cyclic AMP were acetylated and assayed at appropriate dilutions, in duplicate, by a modification[§] of the method of Steiner *et al.* (17). 2′-*O*-Succinyl cyclic GMP-, or cyclic AMP-, tyrosine methyl ester (Sigma) was iodinated by the procedure of Cailla *et al.* (18). The antisera to cyclic GMP were prepared from goats in this laboratory and the antiserum to cyclic AMP was a gift from Dr. A. L. Steiner. Cyclic GMP values were corrected for added cyclic [^{3}H]GMP and for blanks not eliminated by phosphodiesterase. Such blanks (0–3 fmol/100 μl), when present, were uniform between samples and equal to column blanks. The authenticity of material assayed as cyclic GMP or cyclic AMP was confirmed by treatment of samples with beef heart phosphodiesterase (Boehringer-Mannheim) and parallel-line analysis (19). Variation between duplicate samples was less than 20% for cyclic GMP and less than 10% for cyclic AMP. Cyclic nucleotide levels were expressed relative to cell number, as recommended by Mitchison (20). Relative cell volumes are also given so that concentration of cyclic nucleotides can be estimated.

RESULTS

Synchronization of cells with HU, followed by reversible colcemid arrest, yielded a mitotic population in excess of 80% that exhibited a relatively high degree of synchrony during traverse of G-1 and S-phase (Fig. 1*A–C*).

Resuspension of cells in colcemid-free medium led to an 80% fall in intracellular (I.C.) cyclic GMP and a 2-fold rise in I.C. cyclic AMP as cells exited mitosis and entered G-1 (Fig. 1*D* and *E*). The drop in I.C. cyclic GMP can be accounted for by a coincident and almost equal increase in the appearance of extracellular (E.C.) cyclic GMP after removal of colcemid. These results suggest that cellular cyclic GMP concentrations can be diminished by an efflux mechanism as well as by intracellular hydrolysis. On the other hand, E.C. cyclic AMP began to increase only after a lag period of 30 min. As cells traversed G-1, cyclic AMP levels remained elevated and cyclic GMP levels remained low relative to mitotic cells (zero time). On a cell volume basis, the maximum concentration of cyclic AMP and the minimum concentration of cyclic GMP occurred during G-1. As cells entered S phase, cyclic AMP/cell decreased and cyclic GMP/cell remained relatively constant at a time when cell volume was rapidly increasing (t = 2–4 hr). During mid- to late S-phase, a 90% increase in cellular cyclic GMP and a 50% increase in cyclic AMP were observed. These increases in cyclic nucleotides were not simultaneous, and the rise in cyclic GMP appeared to precede the rise in cyclic AMP by about 1–2 hr. Net accumulation in the medium of both cyclic nucleotides was observed during S-phase, with cyclic GMP exhibiting an increased rate and cyclic AMP a decreased rate of accumulation relative to the rates in G-1. Beyond 9 hr, synchrony decay and redistribution of cells between G-2 + M and G-1 (see Fig. 1*C*) obscured any distinct changes in I.C. cyclic nucleotides. Examination of E.C. cyclic nucleotides, however, indicated an increased rate of accumulation of both cyclic GMP and cyclic AMP as the cell population as a whole flowed through mitosis. When completion of mitosis was blocked by further addition of colcemid at 8 hr, E.C. accumulation of cyclic AMP was completely blocked while that of cyclic GMP was markedly accentuated compared to the unblocked control cultures. These results suggest that the increased appearance of cyclic GMP and cyclic AMP in the medium occurred before and after the colcemid block point, respectively. In the presence of colcemid,

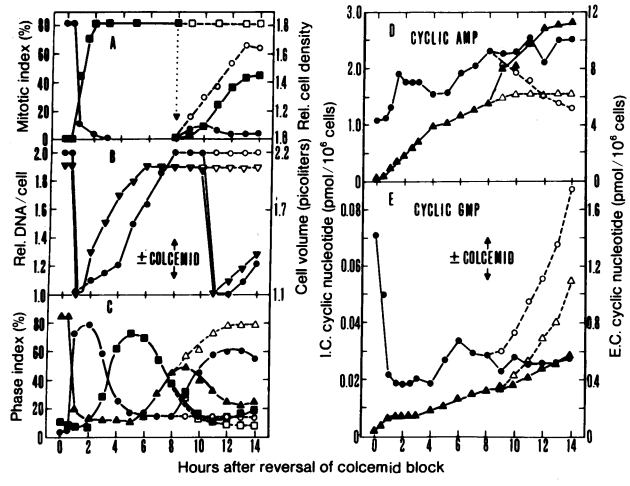

FIG. 1. Cyclic nucleotide levels in hepatoma cells synchronized by HU and colcemid. *A*. (●—●) Mitotic index; (○ - - - ○) mitotic index in the presence of 0.25 μM colcemid. In this and subsequent panels, open symbols and broken lines represent cultures to which colcemid was again added 8 hr after reversal of the initial colcemid block. (■, □) Relative cell density. At 8 hr the relative cell density was arbitrarily set at 1.0 (dotted line) for graphical purposes. *B*. (●, ○) Relative DNA content per cell (see *Methods*); (▼, ▽) cell volume. *C*. (▲, △) G-2 + M cells; (●, ○) G-1 cells; (■, □) S-phase cells. *D*. (●, ○) Intracellular (I.C.) cyclic AMP; (▲, △) extracellular (E.C.) cyclic AMP. *E*. (●, ○) Intracellular cyclic GMP; (▲, △) extracellular cyclic GMP. Cyclic nucleotide levels are expressed relative to the final cell density after completion of mitosis (i.e., cell density at 2 hr).

[§] The acetylation method was developed through the combined efforts of M. K. Haddox, D. B. Glass, J. H. Stephenson, C. E. Zeilig, and N. D. Goldberg at the University of Minnesota and E. A. Ham at the Merck Institute for Research. A similar assay method was developed independently by Harper and Brooker (16). Binding of antigen to antibody was stimulated 2- to 3-fold by cations. Therefore, the cyclic AMP and cyclic GMP radioimmunoassays were conducted in the presence of 1 mM $MgCl_2$ and 20 mM $CaCl_2$, respectively.

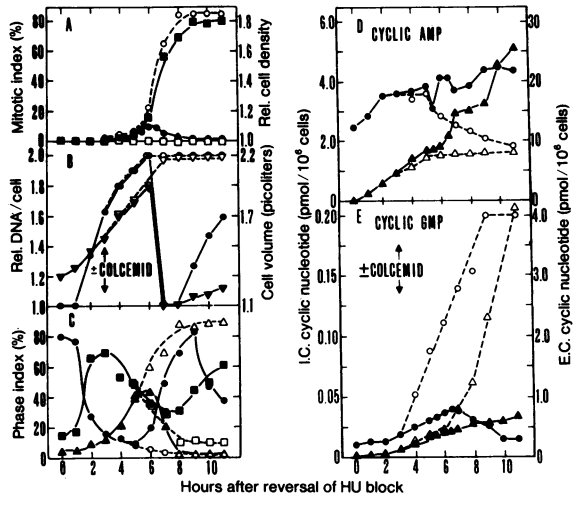

FIG. 2. Cyclic nucleotide levels in hepatoma cells synchronized by double HU block. See Fig. 1 for key to symbols. Cyclic nucleotide levels are expressed relative to the initial cell density (i.e., cell density at 0 hr).

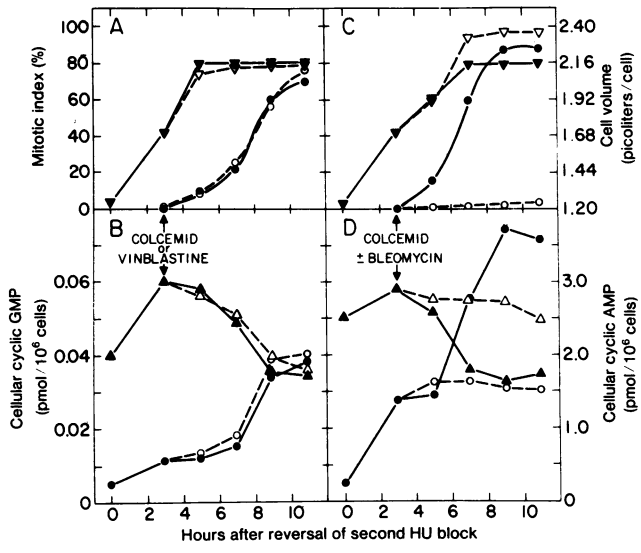

FIG. 3. Effect of colcemid, vinblastine, and bleomycin on cyclic nucleotide levels in hepatoma cells synchronized by double HU block. *A*. At 3 hr 0.25 μM colcemid (closed symbols) or 0.5 μM vinblastine sulfate (open symbols) was added to the culture medium. (▼, ▽) Cell volume; (●, ○) mitotic index. *B*. (▲, △) Cellular cyclic AMP; (●, ○) cellular cyclic GMP. *C*. At 3 hr 0.25 μM colcemid (closed symbols) or 0.25 μM colcemid plus 200 μg of bleomycin sulfate/ml (open symbols) was added to the culture medium. (▼, ▽) Cell volume; (●, ○) mitotic index. *D*. (▲, △) Cellular cyclic AMP; (●, ○) cellular cyclic GMP.

cyclic AMP/cell fell by about 50% while cyclic GMP/cell increased 3-fold, essentially coincident with the flow of cells into mitosis.

A double HU block procedure was used to confirm the general nature of cell-cycle-dependent fluctuations in cyclic nucleotides and also to observe cyclic AMP and cyclic GMP levels in more highly synchronized cells that were allowed to pass through mitosis in the absence of any colcemid treatment (Fig. 2). Comparison of the mitotic index (Fig. 2*A*) and I.C. levels of cyclic GMP (Fig. 2*E*) indicates that these cells exhibited a natural increase in cyclic GMP content in the absence of colcemid. The magnitude of this increase correlated closely with the proportion of cells in mitosis. If the rise in I.C. cyclic GMP from 0.020 pmol/10^6 cells at 3 hr to 0.038 pmol/10^6 cells at 6 hr (Fig. 2*E*) occurred only in the 10% of the population that were in mitosis at 6 hr (Fig. 2*A*) and not in pre- or post-mitotic cells, then the actual cellular level of cyclic GMP during mitosis in this experiment can be calculated to be as much as 0.200 pmol/10^6 cells. This estimate represents an increase in cyclic GMP content of approximately 10-fold over that of S or G-1 levels and is in good agreement with the actual measurements of cyclic GMP content in cells accumulated in mitosis with colcemid (Fig. 2*E*). As seen in Figs. 1 and 2, accumulation of E.C. cyclic GMP was consistent with I.C. cyclic GMP changes. This is true in the case of cyclic AMP as well because the increased E.C. accumulation observed as cells passed through mitosis was entirely blocked when cells were arrested in metaphase by the addition of colcemid. The mechanism by which cyclic AMP levels are lowered in colcemid-arrested cells cannot be accounted for by efflux into the medium, and therefore, probably results from intracellular degradation.

The possibility that colcemid itself raised cyclic GMP levels independently of its antimitotic action seems unlikely because treatment of synchronized cells with bleomycin, which arrests cells in early G-2 (21), prevented the flow of cells into mitosis as well as the fall in cellular cyclic AMP and the rise in cellular cyclic GMP, even in the presence of colcemid (Fig. 3*C* and *D*). Moreover, vinblastine, a compound structurally distinct from colcemid, produced nearly identical changes in mitotic index and in cyclic AMP and cyclic GMP levels (Fig. 3*A* and *B*).

These results also indicate that the trigger for the rise in I.C. cyclic GMP and the fall in cyclic AMP levels occurs at a point in the cell cycle later than the early G-2 bleomycin block-point. A more precise estimate of this point can be made on the basis of the data presented in Fig. 4. These data were drawn from the results of several experiments and represent the percentage change in cyclic nucleotide levels and mitotic index (referred to values at 3 hr) with time after release from a double HU block. It can be seen that cyclic GMP levels rise and cyclic AMP levels fall essentially simultaneously and within 10 min of the time at which cells enter mitosis, very close to the G-2/prophase interface.

A more detailed investigation than that described in Fig. 1 was undertaken to study changes in I.C. cyclic nucleotides in later phases of mitosis after reversal of a colcemid block (Fig.

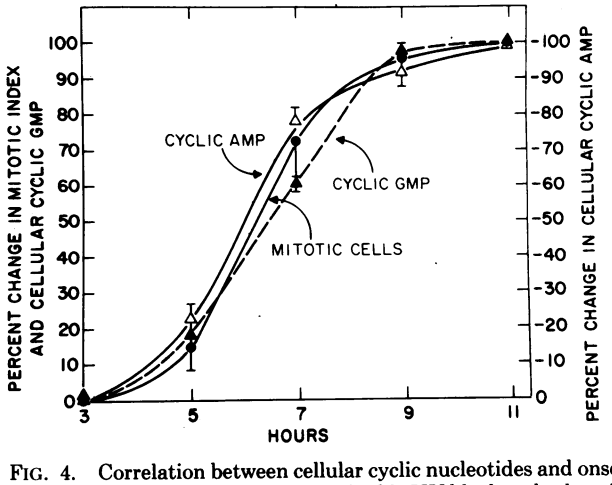

FIG. 4. Correlation between cellular cyclic nucleotides and onset of mitosis. Cells were synchronized by double HU block and colcemid (0.25 μM) was added 3 hr after reversal of the second block (see *text*). Data represent the means and standard errors from four experiments. Note that the scale on the left ordinate represents increasingly greater and on the right ordinate increasingly smaller net values.

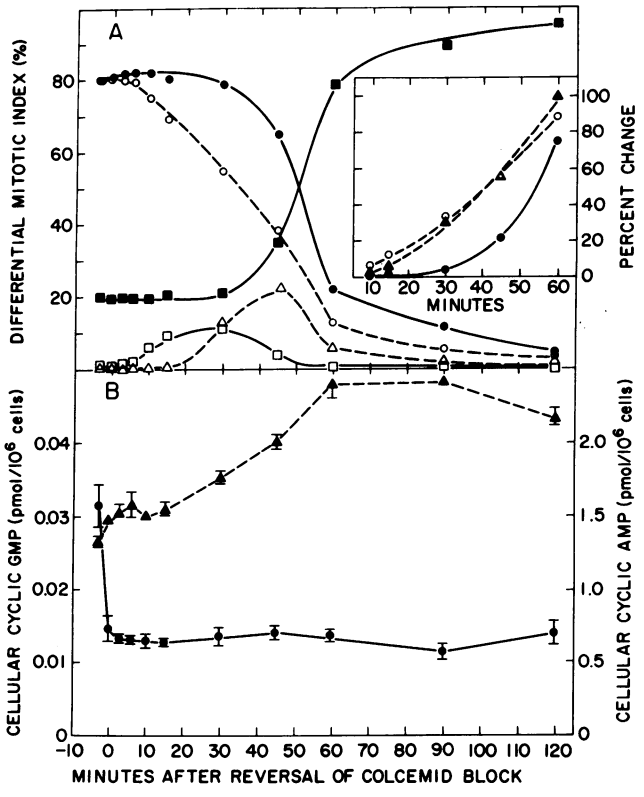

FIG. 5. Cyclic nucleotide levels during mitosis. Cells were synchronized with HU and colcemid. At zero time cells were resuspended in colcemid-free medium. *A*. (●—●) Total mitotic cells; (○ - - - ○) metaphase cells; (□—□) anaphase cells; (△ - - - △) telophase cells; (■—■) interphase cells. *B*. (●—●) Cellular cyclic GMP; (▲ - - - ▲) cellular cyclic AMP. Cyclic nucleotide levels were expressed relative to initial cell density at zero time before any cells had divided and are the means and standard errors of triplicate samples from one incubation flask. *A, insert*. (●—●) Percent change in total mitotic index; (○ - - - ○) percent change in metaphase index; (▲ - - - ▲) percent change in cellular cyclic AMP.

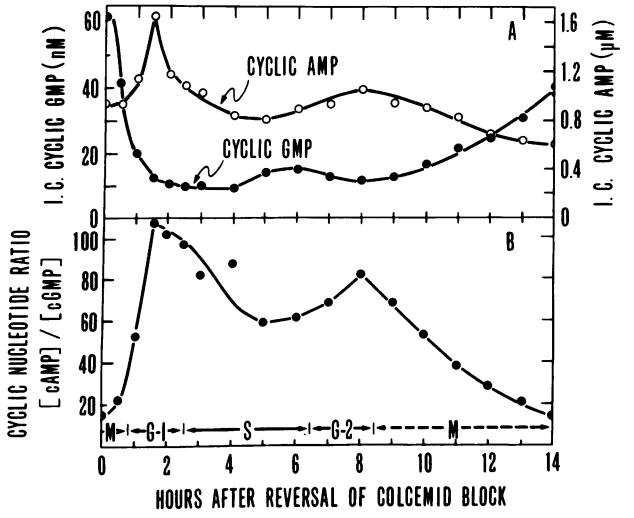

FIG. 6. *A*. Concentration of intracellular cyclic GMP and cyclic AMP during the cell cycle. Cyclic nucleotide concentrations (mol/liter of cell volume) were calculated on the basis of cyclic nucleotide levels/10^6 cells (Fig. 1*D* and *E*) and median cell volume (Fig. 1*B*). Eight hours after reversal of the first colcemid block, colcemid (0.25 μM) was again added to the cultures to reaccumulate cells in metaphase. *B*. Ratio of intracellular cyclic nucleotide concentrations during the cell cycle. Data were calculated on the basis of panel *A* and Fig. 1*D* and *E*. Cell cycle phase diagram is based on Fig. 1*C*.

5). In these experiments care was taken so as not to subject cells to a temperature change during centrifugation and resuspension in colcemid-free medium. Within the 3-min period required to change medium, cyclic GMP levels dropped to ½ to ⅓ those of colcemid-blocked cells and remained low throughout the rest of mitosis and G-1. On the other hand, after a 10-min lag, cyclic AMP levels began to increase (Fig. 5*B*) as cells exited metaphase (Fig. 5*A*). The insert in Fig. 5*A* shows that the increase in cyclic AMP clearly precedes the end of mitosis (i.e., the end of telophase) and correlates closely with the exit of cells from metaphase and entrance into anaphase. The increase in cyclic AMP that begins at the end of metaphase is not solely confined to anaphase but extends throughout anaphase and into G-1 (Fig. 5*B*).

DISCUSSION

The problem of detecting and quantifying transient changes in cyclic nucleotides in synchronized cells has been discussed previously (6). This problem is particularly apparent during mitosis (Fig. 1*D* and *E*), where changes in cyclic nucleotides can be completely obscured by poor synchrony (Fig. 1*C*). Only when the cells are accumulated at a uniform cell cycle position (as with the use of colcemid) can the full extent of alterations in cyclic nucleotide levels be assessed. This argument is also applicable during S-phase, where a relatively moderate increase in cyclic GMP was observed. Monitoring E.C. cyclic nucleotides was useful in these studies for localizing points in the cell cycle that exhibited cyclic nucleotide elevations too transient to detect by I.C. measurements (Fig. 1*D* and *E*). In a variety of other systems (22–24) the appearance of E.C. cyclic nucleotides has also proven to be a sensitive indicator of I.C. metabolism and has been directly correlated with hormonal stimulation of cyclic nucleotide synthesis (22–24).

Seifert and Rudland (9) have reported a transient increase in cyclic GMP levels in early G-1 in BALB 3T3 fibroblasts. In some earlier studies (25) we also observed a brief increase in cyclic GMP levels during G-1. These results could not be reproduced in the present studies, and the conditions responsible for a rise of cyclic GMP during G-1 remain unknown. It seems clear, however, that a G-1 rise in cyclic GMP may not be required for the progression of Novikoff hepatoma or Balb 3T3 cells through the cell cycle (26) (Trowbridge, Zeilig, Goldberg, and Sheppard, unpublished data).

When the I.C. cyclic nucleotide levels shown in Fig. 1*D* and *E* are corrected for volume changes during the cell cycle, increased cyclic GMP levels appear to be associated primarily with mitosis (Fig. 6*A*). The significance of the much smaller increase in cyclic GMP (about 50%) during S-phase is difficult to assess at the present time. However, the overall pattern of changes in the concentrations of cyclic AMP and cyclic GMP tends to be reciprocal or independent throughout the cell cycle (Fig. 6*A*). If cyclic nucleotide concentrations are expressed as the ratio of cyclic AMP to cyclic GMP, a close correlation exists between changes in this ratio and progression of cells through various phases of the cell cycle (Fig. 6*B*). Thus, transition from metaphase to G-1 (0–1.5 hr) is characterized by an increase in this ratio. Entrance of the cells into S-phase (2–5 hr) occurs in parallel with a fall in this ratio. The ratio is reversed as cells enter G-2 (6–8 hr) and, finally, sometime between the end of G-2 and the beginning of metaphase, returns to the original low value observed at zero time.

The question of whether these changes in cyclic nucleotides

play any causal or modulatory role in the progression of cells through various phases of the cell cycle remains to be answered. A functionally active form of cytoplasmic cyclic AMP-dependent protein kinase is apparently not essential for cell division in mutant mouse lymphoma cells (27). Moreover, the existence of several cell lines that exhibit subnormal cyclic AMP (28) or cyclic GMP (29) levels argues against an obligatory role for cyclic nucleotides in the cell cycle. On the other hand, there is some evidence to support a modulatory relationship between an increased ratio of cyclic AMP to cyclic GMP and completion of metaphase. Elevation of cyclic AMP levels by a variety of techniques accelerates normal completion of mitosis in HeLa cells isolated in metaphase by mitotic selection (6). These results, together with the finding that the end of metaphase occurs coincident with a rise in cyclic AMP content in Novikoff hepatoma cells, suggest that an increase in the ratio of cyclic AMP to cyclic GMP is involved in the normal physiologic mechanism that promotes onset of anaphase. If spontaneous increases in cyclic nucleotide levels at various points in the cell cycle are indicative of positive regulatory function, then cyclic GMP may be involved in the modulation of some early events in mitosis and possibly in S-phase.

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