# ADENOSINE 3′:5′-MONOPHOSPHATE CONTENT AND ACTIONS IN THE DIVISION CYCLE OF SYNCHRONIZED HeLa CELLS

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ABSTRACT

The involvement of adenosine 3′:5′-monophosphate (cAMP) in the regulation of the cell cycle was studied by determining intracellular fluctuations in cAMP levels in synchronized HeLa cells and by testing the effects of experimentally altered levels on cell cycle traverse. Cyclic AMP levels were lowest during mitosis and were highest during late G-1 or early S phase. These findings were supported by results obtained when cells were accumulated at these points with Colcemid or high levels of thymidine. Additional fluctuations in cAMP levels were observed during S phase.

Two specific effects of cAMP on cell cycle traverse were found. Elevation of cAMP levels in S phase or G-2 caused arrest of cells in G-2 for as long as 10 h and lengthened M. However, once cells reached metaphase, elevation of cAMP accelerated the completion of mitosis. Stimulation of mitosis was also observed after addition of $CaCl_2$. The specificity of the effects of cAMP was verified by demonstrating that: (*a*) intracellular cAMP was increased after exposure to methylisobutylxanthine (MIX) before any observed effects on cycle traverse; (*b*) submaximal concentrations of MIX potentiated the effects of isoproterenol; and (*c*) effects of MIX and isoproterenol were mimicked by 8-Br-cAMP.

MIX at high concentrations inhibited G-1 traverse, but this effect did not appear to be mediated by cAMP. Isoproterenol slightly stimulated G-1 traverse and partially prevented the MIX-induced delay. Moreover, low concentrations of 8-Br-cAMP (0.10–100 $\mu$M) stimulated G-1 traverse, whereas high concentrations (1 mM) inhibited. Both of these effects were also observed with the control, 8-Br-5′-AMP, at 10-fold lower concentrations.

The role of cyclic nucleotides as regulators of cell proliferation has been investigated intensively in recent years and has been the subject of several critical reviews (1, 14, 18, 40, 67). Despite the research effort expended in this area, substantial inconsistencies remain in the studies reported to

† Deceased 9 March 1974.

date. The results reported here provide new evidence supporting a regulatory role for cyclic AMP in cell cycle traverse.

Although there is general agreement that endogenous adenosine 3′,5′-monophosphate (cAMP) levels are at a minimum during mitosis, changes in cAMP levels during other stages of the cell cycle have been determined only in a few cell types with somewhat variable results. There are also conflicting reports on the effects of experimentally elevated cAMP levels. For example, prolongation of S phase (33, 64), G-2 (65, 68, 71), and G-1 (10, 39) have been observed in response to $N^6$,2′-*O*-dibutyryl-cAMP (dbcAMP) or other cyclic AMP-elevating agents. On the other hand, cAMP (or dbcAMP) has also been reported to stimulate cells in G-1 (30, 68) and to have no effect on S phase (24, 68) or G-2 (15). The interpretation of these results is difficult, since catabolites of cAMP and its analogues can exert potent inhibitory effects on cell growth (43, 62), since other agents such as papaverine (7), methylxanthines (60, 66), and prostaglandins (28) may have effects on systems other than those involved with cAMP metabolism, and since elevation of cAMP in some tissues may result in the production of other antagonists of cAMP (25, 35).

The purpose of this study was to determine those cell cycle loci at which cAMP might normally facilitate progress through the cell cycle and those loci at which cAMP in physiologically relevant concentrations might retard cell cycle traverse. First, cell cycle-dependent changes in endogenous cAMP levels were analyzed in order to determine when elevated or depressed cAMP levels occur during the cell cycle. Then, on the basis of these results, cAMP levels were experimentally elevated at various times either in or out of phase with natural cAMP fluctuations, and the effects of such treatment on cell cycle traverse were analyzed.

We adopted a set of criteria for demonstrating cAMP-specific effects based on those originally outlined by Robison et al. (50), that is: (*a*) to show a temporal correlation between increased cAMP levels and a given effect on cell cycle traverse; (*b*) to show a potentiation of the effect using a HeLa cell adenylate cyclase agonist (isoproterenol) and a cyclic nucleotide phosphodiesterase inhibitor (1-methyl, 3-isobutylxanthine, MIX); and (*c*) to show that the effect can be mimicked with an analogue of cAMP (8-Br-cAMP). We have previously established the optimal concentrations of isoproterenol and MIX necessary for raising intracellular cAMP in HeLa cells (70).[1] Because of the uncertainty in interpreting results obtained after prolonged exposure of cells to analogues of cAMP or to cAMP-elevating agents, we considered that an additional requirement for specificity of an agent to affect cell cycle traverse relatively quickly after exposure, e.g., 1–3 h. Preliminary reports of these studies have appeared previously (71, 72, 73).

## MATERIALS AND METHODS

### *Culture Techniques*

HeLa cells, with a cell doubling time of about 24 h, were maintained in suspension culture at 37°C in spinner modified, Eagle's minimal essential medium (MEM), supplemented with 5% calf serum, streptomycin (6,000 μg/liter), penicillin (6,000 U/liter), (all from Grand Island Biological Co., Grand Island, N. Y.), and 0.1% Pluronic F-68 (Wyandotte Chemicals Corp., Wyandotte, Mich.). The strain of HeLa cells used in these studies was obtained as a gift from Dr. P. N. Rao, University of Colorado Medical Center, Boulder, Colo. The cells were routinely assayed for mycoplasma (4) and were free of contamination.

### *Synchronization Techniques*

SYNCHRONIZATION AT THE BEGINNING OF S PHASE: Cells were routinely grown to approx. 500,000 cells/ml, sedimented in prewarmed glassware at 800 *g* for 5 min at room temperature, and then resuspended in fresh warm Eagle's MEM (spinner formulation with 5% calf serum) to approx. 75,000 cells/ml. The cells were allowed to grow for an additional 24 h before initiation of synchronization. This procedure was adopted to provide a common culture history and was found to reduce variations in basal cAMP levels. The cells were synchronized using a double thymidine block technique (5) by sequential exposure to 2 mM thymidine for 17 h, fresh thymidine-free media for 11 h, and 2 mM thymidine for an additional 13 h. The second thymidine block was reversed by resuspension of the cells in thymidine-free media at a density of approx. 160,000 cells/ml. Alternatively, the cells were synchronized by successive exposures to hydroxyurea ($5 \times 10^{-4}$ M) for 17 h, fresh media for 11 h, and amethopterin ($1 \times 10^{-6}$ M) plus adenosine ($5 \times 10^{-5}$ M) for 13 h. The amethopterin block was reversed by the addition of thymidine (20 μg/$10^6$ cells) (36).

SYNCHRONIZATION IN MITOSIS: In studies on

[1] Zeilig, C. E., R. A. Johnson, E. W. Sutherland, and D. L. Friedman. 1975. Modulation of intracellular adenosine 3′:5′-monophosphate levels in HeLa cells. Submitted for publication.

cAMP content, the double thymidine technique was used in combination with a mitotic selection method (61) to obtain synchronized G-1 cells in relatively high yield. The second thymidine block was reversed by resuspension of the cells in medium (monolayer formulation) at a density of 250,000 cells/ml. Aliquots of 200 ml of the cell suspension were seeded into 32-oz prescription bottles and maintained in monolayer culture at 37°C. After 8 h, the mitotic index (M.I.) of the population was monitored at 15-min intervals on an inverted phase-contrast microscope. When an M.I. of 15% was observed (usually about 9 h after reversal of the second thymidine block), the monolayer were shaken vigorously at room temperature to dislodge debris, mitotic, and early G-1 cells. The monolayer medium was decanted and replaced with 50 ml of prewarmed suspension media. The cells were incubated at 37°C until an M.I. of ca. 20% was observed (30–60 min). These cells were then selectively dislodged by gentle agitation at room temperature, resuspended in prewarmed media to a density of about 180,000 cells/ml, and maintained in suspension by magnetic stirring at 37°C. In studies on the effect of chemical agents on cells in M and G-1, the synchronization procedure was modified in that a single exposure to thymidine was used followed by selection of mitotic cells with shaking.

### *Determination of Cell Cycle Parameters*

Progress through the cell cycle was monitored by cell density using a model $F_N$ Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.), M.I., and labeling index (L.I.) by autoradiography. For L.I. measurements, aliquots of cells were exposed to [5-$^3$H]deoxycytidine (10.4 Ci/mmol) or [*methyl*-$^3$H]thymidine (84 Ci/mmol) (both from ICN Pharmaceuticals, Inc., Cleveland, Ohio) and processed for autoradiography using standard procedures (44). In experiments in which the effects of chemical agents were tested, cells were preincubated for 15 min in fresh media in order to remove drugs before addition of [$^3$H]thymidine for determination of the L.I. L.I. and M.I. were expressed as percent of the total population, and cell density was expressed as percent change from the initial seed density.

### *Assay of cAMP*

For the determination of cAMP content, 15- to 30-ml aliquots, each containing $2 \times 10^6$ to $6 \times 10^6$ cells, were withdrawn from the cultures and rapidly sedimented at 1,350 *g* for 3 min. The pellet was disrupted by the addition of 1.0 ml of 0.3 M perchloric acid containing 5,000–10,000 cpm of [G-$^3$H]cAMP (24 Ci/mmol, New England Nuclear Corp., Boston, Mass.) to monitor recovery of cAMP during subsequent purification. Cyclic AMP was extracted from the pellet by five cycles of rapid freezing and thawing (11) and was purified by cation exchange chromatography (Bio-Rad AG 50-X8, 100–200 mesh, $H^+$ form, Bio-Rad Laboratories, Richmond, Calif.) on $10 \times 0.7$ cm columns equilibrated and eluted with 0.1 N HCl (27). The fraction containing cAMP was lyophilized, redissolved in 0.5 ml of 50 mM acetate buffer, pH 4.0, and assayed in duplicate essentially according to the method of Gilman (27), using beef muscle protein kinase prepared according to published procedures (21, 34). Values were corrected for recovery of [$^3$H]cAMP (recoveries ranged from 70–80%).

Values from blank samples, consisting of 0.3 M $HClO_4$ also chromatographed as described above, were routinely subtracted from cell-derived samples, since such blanks were found to contain column-derived material which assayed as cAMP ($1.87 \pm 0.26$ ($n = 22$) pmol, or 70 fmol per ml of column effluent). Otten et al. (38) recently made comparable observations. After correction for blank values, the remaining assayable material derived from cells could be completely abolished by prior incubation with beef heart cyclic nucleotide phosphodiesterase. The cAMP values derived from cell extracts were additive with internal cAMP standards and were linear with cell dilution, indicating that the samples did not contain additional materials which interfered with the cAMP-binding assay. Possible contamination of cAMP from the medium in which the cells were grown was judged negligible since no cAMP was detected in a 20-ml aliquot of used growth medium. Cyclic AMP levels were expressed on the basis of cell protein which was determined by the method of Lowry et al. (29) on separate aliquots of cells that had been washed three times in 0.9% NaCl to remove extracellular protein.

### *Materials*

The following compounds were obtained from the sources indicated: caffeine, theophylline, hypoxanthine, and 1-isoproterenol·HCl, (Sigma Chemical Co., St. Louis, Mo.); $N^6$-monobutyryl-cAMP (mbcAMP), 5′-AMP $Na_2$, 5′-GMP, 8-Br-cAMP and 8-Br-cGMP, C.F. Boehringer and Sons, Mannheim, Germany; 8-Br-5′-AMP, P-L. Biochemicals, Inc., Milwaukee, Wis. MIX was a gift of G. D. Searle & Co., Chicago, Ill.

## RESULTS

### *Cyclic AMP Content in Synchronized HeLa Cells*

In order to minimize the effects of synchrony decay with time and to diminish the chance of artifact that might result from the synchronization method, two techniques (double thymidine block and mitotic selection) were used to achieve synchrony during the earlier and later stages of the cell cycle. The results of these procedures on cell cycle traverse are illustrated in Figs. 1 A and B. The rate at which the cell population as a whole entered any given phase of the cell cycle (the degree of synchrony) and the duration of each

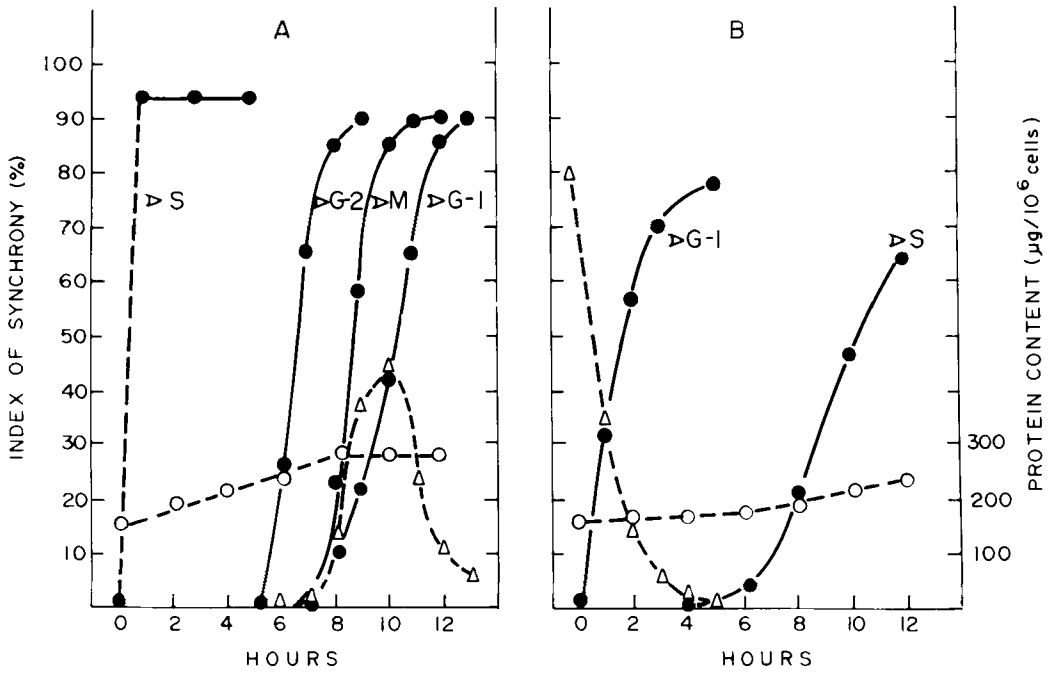


FIGURE 1 Synchronization of HeLa Cells. Suspension cultures of HeLa cells were synchronized either by a double thymidine block (*A*) or by mitotic selection (*B*). Progress through the cell cycle was determined from measurements of L.I. (percent), M.I. (percent), increase in cell density (percent), and cellular protein content. Closed symbols (●) represent the cumulative percentage of the total population entering a given phase (arrows) with time; (Δ---Δ), M. I., normalized to the premitotic cell density (19); (O---O), cellular protein content normalized to the premitotic (A) or postmitotic (B) cell density. The cumulative curve for S phase is a direct determination of the L. I. while the curve for G-2 was obtained from the maximal L.I. during S phase minus the L. I. obtained as cells exited from S phase. The cumulative mitotic curve was obtained by adding the percent increase in cell density to the normalized M. I. The cumulative G-1 curve is a direct determination of the percent increase in cell density. Values shown are the averages of data from five experiments for protein and 10 experiments for the other parameters.

phase are given in Table I. The results indicate that the synchrony decays rapidly with time, a finding which is typical of all methods for synchronizing mammalian cells (59). The rate and time at which cells flowed into S phase after mitotic selection were similar to those obtained for HeLa cells when other procedures were used (47). With the double thymidine block technique, the time required for 50% of the population to enter either G-2 or mitosis never varied more than 1 h between experiments. However, with the mitotic selection technique, the time at which 50% of the cells entered S phase varied between 9.6 and 12.5 h after selection. The 2–4 h discrepancy in the duration of the cell cycle in synchronized cells and the population doubling time in unsynchronized cells (see Table I) may be a result of unbalanced growth induced by synchronization (16) and can be accounted for primarily by a decrease in the length of G-1 resulting from the thymidine block (Kurz, J. B. and D. L. Friedman, preliminary observations).

Basal cAMP levels during S, G-2, M, and early G-1 were monitored at hourly intervals after reversal of the second thymidine block. The average values from seven experiments are shown in Fig. 2. The cAMP content before removal of the excess thymidine was not significantly different from the 1 h value (data not shown). However, by $t = 3$ h, the cAMP content had declined to a level of about 60% of the 1 h value (Fig. 2). This initial decline in cAMP content was prevented by readdition of 2 mM thymidine, suggesting that the decline in cAMP levels was not a consequence of the resuspension procedure used to reverse the thymidine block. At $t = 4$ h, an elevation of cAMP was observed. The magnitude of this peak was variable but with paired analysis was statistically significant

($P < 0.01$). In some but not all experiments, an additional smaller peak was observed at $t$ = 6 h. As the cells entered G-2 ($t$ = 7 h), cAMP levels decreased again, reaching a minimum concentration (6.97 ± 1.20 pmol/mg protein, 38 ± 5% of the 1 h value) at a time which corresponded to the mitotic peak. As the cells completed mitosis and entered G-1 ($t$ = 11–12 h), cAMP levels began to rise, reaching a plateau at $t$ = 12 h and remaining relatively constant until the end of the experiment ($t$ = 15 h).

TABLE I

*Cell Cycle Parameters in Synchronized HeLa Cells*

| Cell cycle stage | Rate of entry: Double thymidine | Rate of entry: Mitotic selection | Length of stage: Double thymidine | Length of stage: Mitotic selection |
|---|---|---|---|---|
| | % cells/h | | h | |
| S | 100* | 11.1 | 6.5 | – |
| G-2 | 34.3 | – | 2.1 | – |
| M | 33.3 | – | 1.4 | – |
| G-1 | 18.2 | 32.5‡ | – | 10.2 |
| Generation time (synchronized cells) | | | 20.2 | |
| Population doubling time (unsynchronized cells) | | | 22–24 | |

* Since the thymidine block allows cells to enter S phase (16), it was assumed that essentially all of the cells initiated DNA synthesis simultaneously.

‡ Entrance into G-1 after mitotic selection was not a sigmoidal function but appeared to be exponential. Accordingly, the rate of entry into G-1 was estimated from a logarithmic plot of the data.

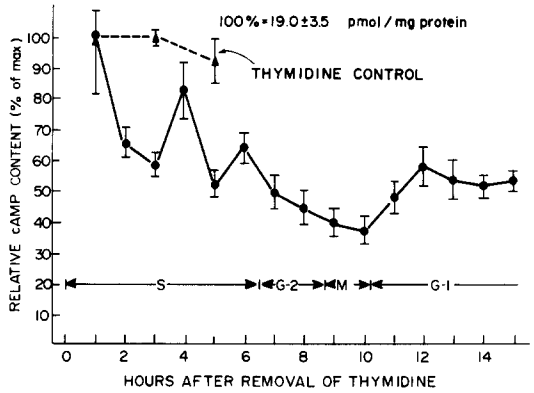


FIGURE 2 Cyclic AMP content in cells synchronized by the double thymidine block method. At 0 time, the cells were resuspended in fresh thymidine-free growth medium and allowed to proceed through the cell cycle. Thymidine (2 mM, final concentration) was immediately re-added to a portion of the cultures to prevent further DNA synthesis. Cyclic AMP content was determined at various times as indicated. (●——●), no thymidine; (▲——▲), + thymidine. Values represent the means and standard errors of three experiments with thymidine and seven without. Within each experiment, points represent average values from duplicate samples from a single culture, each sample assayed for cAMP in duplicate. The data from individual experiments were normalized to the 1 h value (100% = 19.0 ± 3.5 pmol/mg protein). The mean lengths of each cell cycle phase shown at the bottom of this and subsequent figures represent values determined as described in the text.

To determine whether any of these changes were peculiar to the method of synchronization, cells were also synchronized with hydroxyurea followed by amethopterin (data not shown). Initiation of DNA synthesis was effected by the addition of 20 μg of thymidine/$10^6$ cells at $t$ = 0 h without changing the growth medium. The results obtained in this experiment were essentially identical to those with double thymidine block.

To characterize more fully the timing and magnitude of the 4 and 6 h peaks (Fig. 2), an experiment was carried out in which cAMP determinations were made at 0.5 h intervals (Fig. 3). These results strengthen the observation that cAMP levels were elevated transiently at 4 and 6 h. In other experiments (Fig. 6), inclusion of a cyclic nucleotide phosphodiesterase inhibitor in the medium was found to potentiate greatly the magnitude of the 4 h peak but not the 6 h peak, suggesting an increased synthesis of cAMP at 4 h.

The marked decline of cAMP levels during G-2 and mitosis was investigated further to determine whether the minimum level was reached before or after the completion of cell division. Addition of Colcemid (0.064 μg/ml) to synchronized cells at $t$ = 7 h resulted in a decline of cAMP levels below that observed in the control culture (Fig. 4). As the cells accumulated in metaphase, cAMP levels continued to decline and at $t$ = 10–11 h reached a value of about 23% of the 1 h value. These results suggest that the reaccumulation of cAMP begins at a point later than metaphase.

In order to verify the results obtained using S-phase synchronization techniques and to examine alterations in cAMP levels in G-1 in greater detail, double thymidine-blocked HeLa cells were resynchronized by mitotic selection. At various intervals after collecting mitotic cells, aliquots were removed for cAMP determinations, the results of which are illustrated in Fig. 5. After a lag of approx. 1–2 h (as cells completed mitosis, cf. Fig. 1), cAMP levels increased gradually to a peak in late G-1 ($t$ = 8 h) which was approximately double the cAMP levels in mitotic cells. As a substantial proportion of the cells entered S phase (L.I. = 40%), a significant decline in cAMP content oc-

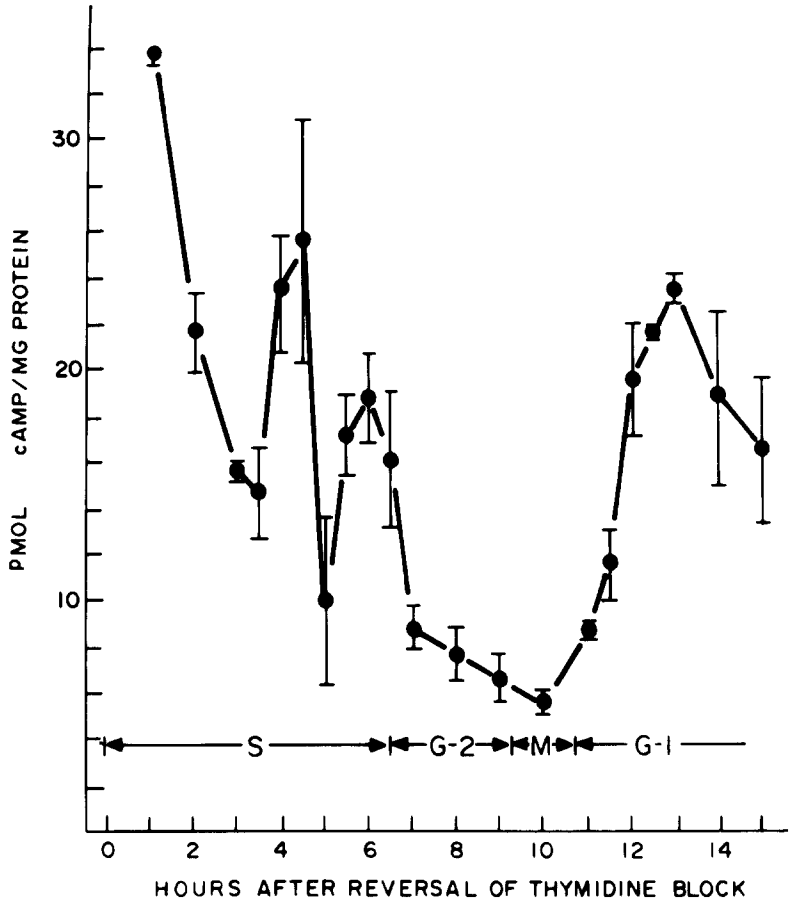


FIGURE 3 Cyclic AMP content measured at short intervals in cells synchronized by the double thymidine block method. Cyclic AMP content was determined at 0.5 h intervals, as indicated, after reversal of the second thymidine block. Results represent the means and standard errors of three cell suspensions derived at zero time from a common culture.

curred ($t$ = 10 h), followed by a second increase 2 h later.

To test the possibility that the decline in cAMP content immediately following the G-1 peak was a consequence of some of the cells having entered S phase, 2 mM thymidine was re-added to a portion of the culture at $t$ = 5 h in order to impede the progression of cells into S phase (Fig. 5). Addition of thymidine completely prevented the decline which occurred at $t$ = 10 h and resulted in a continued increase in cAMP content which appeared to reach a plateau between 8 and 12 h. These results were comparable to those shown in Fig. 2 (in that when DNA synthesis is blocked by high levels of thymidine, the S phase decline in cAMP is not observed) and suggest that the maximum levels of cAMP during the cell cycle may occur at or near the G-1/S border.

### *Effects of cAMP on Cell Cycle Traverse*

EFFECT OF MIX AND ISOPROTERENOL ON S, G-2, AND M: In studies on the effects of various agents on cell cycle traverse, an autoradiographic method for the incorporation of [$^3$H]thymidine into DNA was selected for the delineation of S phase. This method was considered preferable to liquid scintillation assays since it is less susceptible to alterations in transport capacity and precursor pool size and since it allows accurate determinations of the percentage of cells synthesizing DNA and the rate at which they enter or exit S phase.

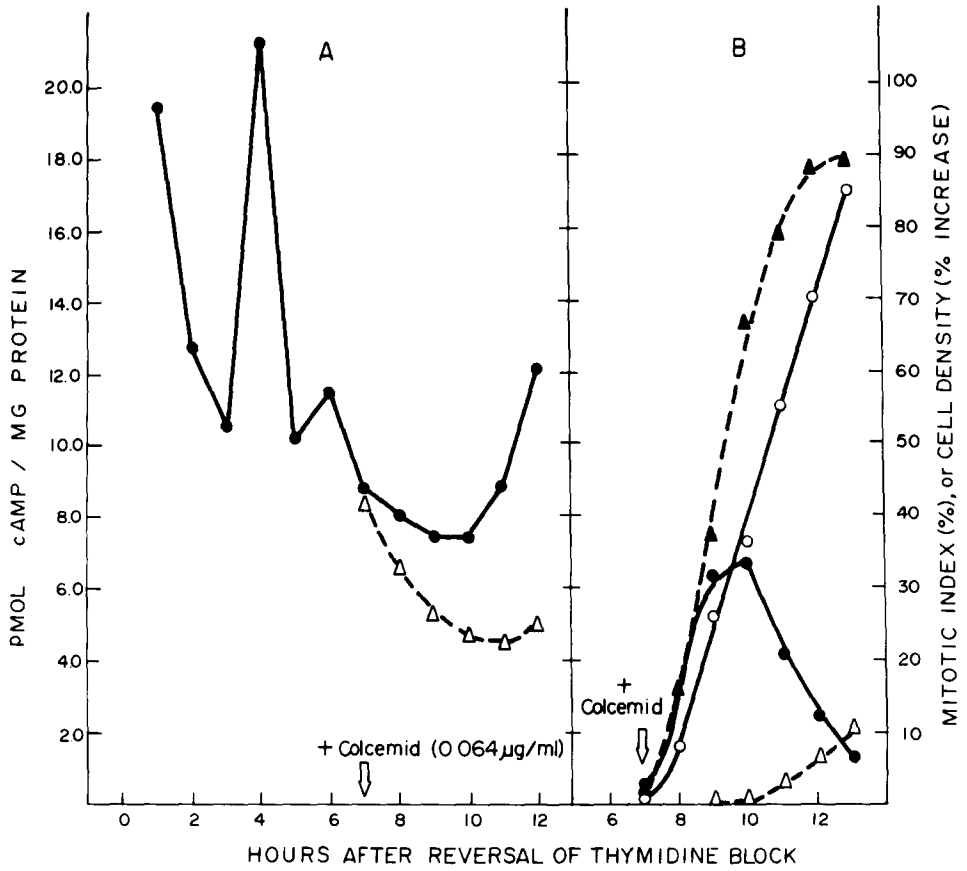


FIGURE 4 Cyclic AMP content and M.I. of Colcemid-arrested cells. 7 h after reversal of a second thymidine block, Colcemid (0.064 μg/ml, final concentration) was added to one portion of the culture. Cyclic AMP, M.I., and cell density were determined as described earlier. (A) Cyclic AMP content: (●——●), control; (△- - -△), + Colcemid. (B) M.I. (percent): (●——●), control; (▲- - -▲), + Colcemid. Cell density (percent increase): (○——○), control; (△- - -△), + Colcemid. Values represent the average of two experiments.

In preliminary experiments, the incorporation of [$^3$H]thymidine into DNA, as visualized autoradiographically, was found to be almost completely inhibited by 1 mM MIX. In contrast, accumulation of total cellular DNA in synchronized cells was essentially unaltered in the presence of MIX. This suggested that MIX was inhibiting the uptake of thymidine. An inhibition of thymidine transport has also been observed in Chinese hamster ovary cells in response to dbcAMP (24). In HeLa cells, we found that this inhibition was readily reversed within 15 min after removal of MIX. Therefore, in subsequent studies, cells were pre-incubated for 15 min in drug-free medium before [$^3$H]thymidine pulse labeling. Examination of autoradiograms of treated and untreated cells showed nearly equal grain densities, confirming that this procedure yielded valid estimates of the percentage of cells synthesizing DNA at any given time.

In order to study the effects of elevated cAMP levels on the progress of cells through S phase, cultures were exposed to 1 mM MIX immediately after reversal of a double thymidine block. A single exposure of the cells to MIX resulted in a two- to threefold increase in cAMP levels throughout S phase (Fig. 6 A) except for a transient eightfold increase in cAMP content 4 h after reversal of the S-phase block, corresponding to the time at which a rise in basal cAMP levels was observed (cf. Fig. 2). Despite the elevation of cAMP, there was no demonstrable effect of MIX either on the total percentage of pulse-labeled cells or on the

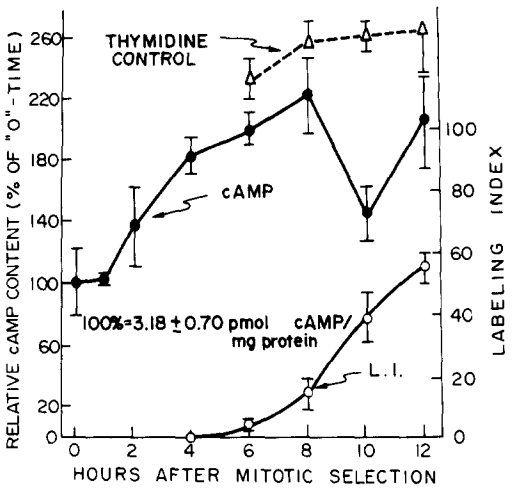


FIGURE 5 Cyclic AMP levels in HeLa cells synchronized by mitotic selection. At zero time, mitotic cells were selectively dislodged from monolayer cultures previously synchronized by a double thymidine block and placed in suspension culture. Cyclic AMP, M.I. (not shown), and L.I. were monitored at various times thereafter. At 5 h, the cultures were split and thymidine (2 mM final concentration) was added to one group of cells. Cyclic AMP content: (●——●), control; (△- - -△), + thymidine. L.I.: (○——○), control. Values are the means and standard errors of three experiments. Within each experiment, values for cAMP represent averages of duplicate samples from a single culture. Each sample was assayed in duplicate. The data from individual experiments were normalized to the zero time value (100% = 1.18 ± 0.70 pmol/mg protein).

rate at which they exited S phase (Fig. 6 B). However, a significant prolongation of G-2 was observed (Fig. 6 B). The length of G-2 (as estimated from the difference between the half-maximal points on the L.I. and M.I. curves) was increased from 2.3 to 10.7 h by MIX (Fig. 6 B). Furthermore, the rate at which the MIX-treated population flowed into mitosis was decreased, and the time spent in mitosis appeared to be increased.

When MIX was added to cells at or near the beginning of G-2 (6 h), a six- to eightfold rise in cAMP was observed (Fig. 7 A) which was associated with a lengthening of both G-2 and mitosis (Fig. 7 B). Even though cAMP levels remained substantially elevated, the cells were not irreversibly blocked in G-2 and eventually progressed to mitosis. The effect on G-2, however, was considerably smaller than that observed after addition of MIX at the beginning of S phase. In separate experiments (data not shown), the last point in the cell cycle before mitosis that MIX inhibits was determined in unsynchronized cells by the method of Petersen et al. (42). In these experiments, the rate at which Colcemid-blocked metaphase cells accumulated began to slow 30–40 min after addition of MIX to the culture. Addition of MIX to synchronized cells as late as 5 h after removal of thymidine was found to produce as effective a G-2 block as addition at the beginning of S phase. Taken together, these results suggest that MIX inhibits a cell cycle process which begins in late S phase and extends into late G-2.

Since methylxanthines can exert a variety of effects on cells perhaps unrelated to their effects on cyclic nucleotide metabolism (60, 66), the specificity of MIX was investigated by comparing its action on G-2 with that of other xanthine analogues. The agents were added at the beginning of S phase to cells synchronized by a double thymidine block. At a concentration of 1 mM, only MIX delayed cells in G-2 significantly. The lengths of G-2 were: saline, 3.0 h; theophylline, 3.3 h; caffeine, 2.8 h; hypoxanthine, 3.2 h; and MIX, 10.0 h. In previous studies with logarithmic cells (our unpublished results)[1] only MIX produced significant increases in cAMP content while other analogues had little or no effect at concentrations of 1 mM.

An additional test for the involvement of cAMP in the MIX-induced G-2 delay is the demonstration that the effect of isoproterenol, an adenylate cyclase agonist in these cells (70),[1] can be potentiated by a submaximal concentration of MIX (Fig. 8). Essentially no effect of 10 μM isoproterenol in the presence or absence of 0.5 mM MIX was observed on the progress of cells through S phase. The duration of G-2 in controls was estimated at 3.1 h, whereas with isoproterenol or MIX alone it was 3.5 or 4.3 h, respectively. In the presence of both agents, the duration of G-2 was increased to 8.8 h.

EFFECT OF CAMP ANALOGUES ON THE DURATION OF S PHASE AND G-2: To study the effects of cAMP analogues on the cell cycle, 8-Br-cAMP was chosen since it is somewhat resistant to hydrolysis by cyclic nucleotide phosphodiesterase and activates cAMP-dependent protein kinase (58). We have previously shown that the use of native cAMP is undesirable because of the cytotoxic effects of 5′-AMP, which is readily formed by the action of phosphodiesterase found in the serum used in the growth medium.[1] $N^6$,2′-*O*-dibutyryl-cAMP was also found to be undesira-

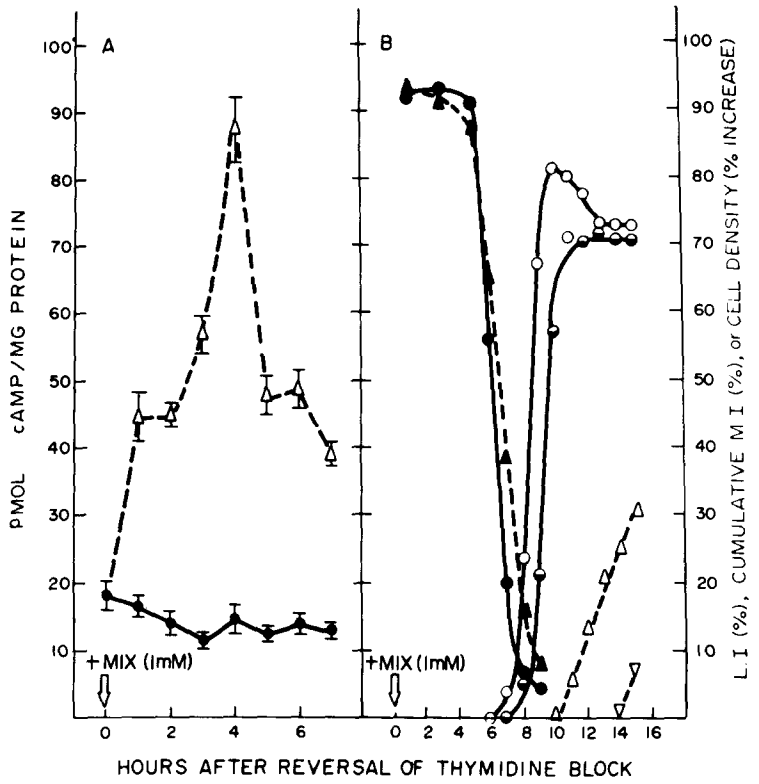


FIGURE 6 Effect of MIX on cAMP levels during S phase and on the lengths of S phase, G-2, and M. Cells were synchronized in early S phase by the double thymidine block technique. After resuspension of the cells in thymidine-free medium, MIX was added (arrow) to the indicated cultures to give a final concentration of 1 mM. (A) Cyclic AMP content: (●——●), control; and (△- - -△), MIX. Values represent the mean ± SE of three examples. (B) L.I.: (●——●), control; and ▲- - -▲), MIX Percent cumulative M.I.: (○——○), control; and (△- - -△), MIX. Percent increase in cell density: (◒——◒), control; and (▽- - -▽), MIX. Values shown are the average of two experiments. The cumulative M.I. curve is equivalent to the curve that would be obtained with a mitotic blocking agent. Each point in the curve is obtained by summing the M.I. (expressed as percent and corrected for increasing cell density) and the percent increase in cell density. To determine the M.I., cells were fixed in media containing 2.5% glutaraldehyde. Concentrated samples were gently squashed with a cover slip and viewed under phase-contrast microscopy. Single cells without a clearly defined nucleus were scored as mitotic cells. Prophase cells, which could not be clearly identified by this procedure, and newly divided doublets were scored as nonmitotic. The M.I. is expressed as the percent of mitotic cells in the total population.

ble for these studies, since it is unstable and free butyrate was cytostatic (70).

Addition of 8-Br-cAMP (1 mM) at the beginning of S phase delayed the time at which 50% of the maximum thymidine-labeled population exited S phase by approx. 40 min, which is in contrast with the lack of effect of isoproterenol and MIX. In addition, the presence of 8-Br-cAMP increased the duration of G-2 from 3.4 to 5.9 h. The magnitude of this effect was comparable to that observed with 0.5 mM MIX (Fig. 8). However, the interpretation of this observation is complicated by the finding that 8-Br-5'-AMP (1 mM) produced similar effects as 8-Br-cAMP on traverse through both S phase and G-2. The possibility that 8-Br-cAMP was acting as the cyclic nucleotide and that these two compounds have independent actions was supported by other studies with unsynchronized cells.[2] These experiments showed that the inhibition of G-2 traverse by 8-

[2] Kurz, J. B. and D. L. Friedman. Manuscript in preparation.

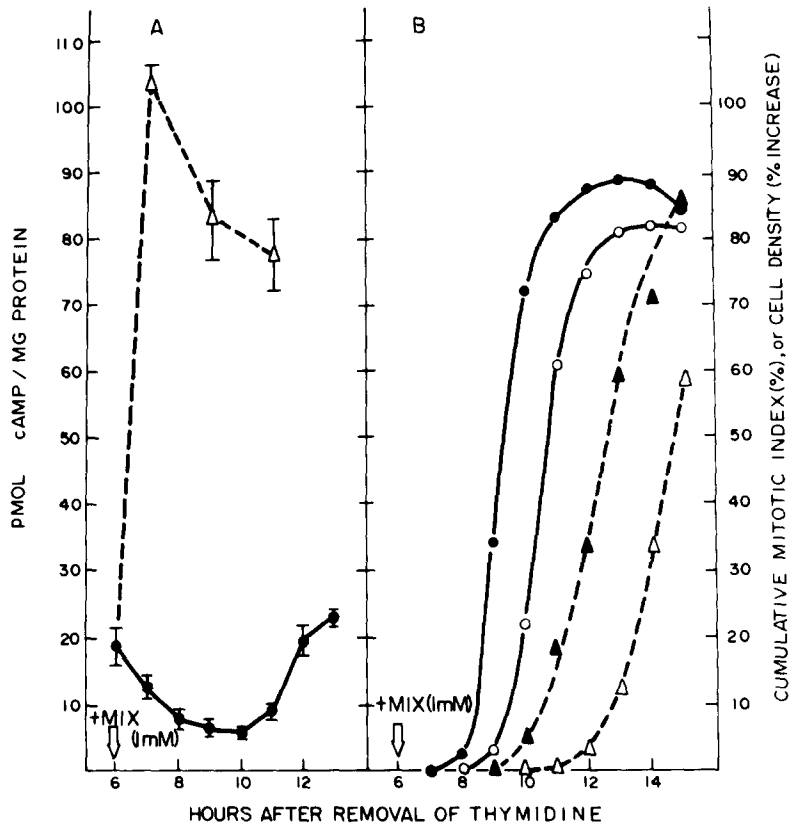


FIGURE 7 Effect of late addition of MIX on cAMP levels during G-2 and on the lengths of G-2 and M. Mix (1 mM) was added 6 h after removal of the second thymidine block (arrow). (A) Cyclic AMP content: (●——●), control; and (△- - -△), MIX. Values represent the mean and standard error of three samples. (B) Percent cumulative M.I.: (●——●), control; (▲- - -▲), MIX. Percent increase in cell density: (○——○), control; and (△- - -△), MIX. Values are an average of two experiments.

Br-cAMP, but not by 8-Br-5'-AMP, can be potentiated by MIX.

EFFECT OF VARIOUS AGENTS ADDED DURING MITOSIS UPON SUBSEQUENT TRAVERSE THROUGH MITOSIS: In contrast with the premitotic G-2 inhibitory effect of cAMP, once cells have progressed beyond the G-2 phase and have reached metaphase, elevated cAMP levels apparently can enhance the rate at which cells complete mitosis and enter G-1 (Fig. 9). When synchronized cells were isolated in metaphase by the mitotic selection technique and exposed to 1 mM MIX, the half-time for completion of mitosis was decreased from approx. 48 min to 15 min (Fig. 9 A). Coincident with the stimulatory effect of MIX, a ninefold increase in intracellular cAMP was observed 60 min after addition of the drug (Fig. 9 B).

As illustrated in Fig. 10, a synergistic interaction between 0.5 mM MIX and 1.0 $\mu$M isoproterenol was observed. This would further suggest that elevated cAMP levels may mediate the increased rate of exit from mitosis.

A variety of other agents were tested for their ability to mimic these effects, and the results of several experiments are summarized in Table II. Of the agents tested, only those capable of elevating endogenous cAMP levels or acting like intracellular cAMP were able to stimulate cell division.

In view of the requirement for $Ca^{++}$ in a variety of cellular processes (6, 48), the effect of increasing the $Ca^{++}$ concentration in the medium was compared with that of MIX (Fig. 11). Addition of 2.73 mM $CaCl_2$ to mitotic cells was found to be essentially as effective in stimulating cell division as 1 mM MIX. Simultaneous additions of both

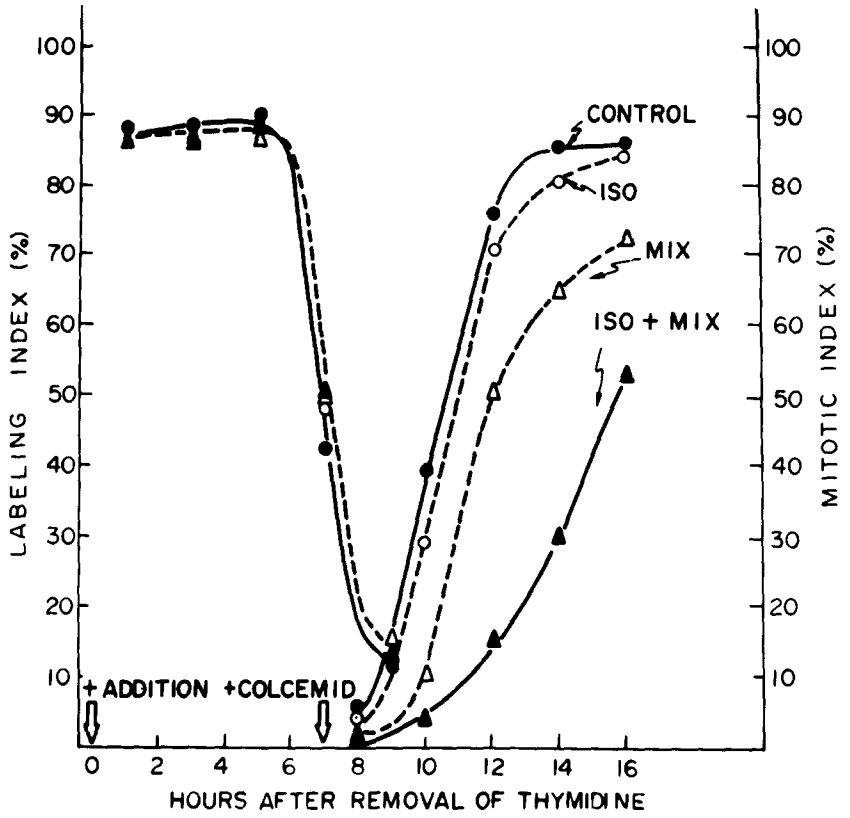


FIGURE 8 Effects of isoproterenol and MIX on the duration of S phase and G-2. The various agents were added immediately after reversal of the second thymidine block (arrow). Colcemid (0.064 μg/ml) was added at 7 h (as indicated) to accumulate cells in metaphase. L.I. (0–9 h) or M.I. (8–16 h): (●——●), control; (○- - -○), 10 μM *L*-isoproterenol·HCl; (△- - -△), 0.5 mM MIX; and (▲——▲), isoproterenol + MIX.

$Ca^{++}$ and MIX to the cultures did not result in any significant further increase in the rate at which mitosis was completed.

STUDIES ON TRAVERSE OF G-1: When MIX was added to synchronized cells immediately after mitotic selection, a concentration-dependent inhibition of the flow of cells into S phase was observed (Fig. 12 A). The effect of 1 mM MIX was associated with a relatively large initial increase in cAMP content for the first 4 h of exposure to MIX (Fig. 12 B). At later times, however, cAMP content decreased despite the continued presence of MIX. Theophylline, caffeine, and hypoxanthine (at concentrations of 1 mM) were all found to extend only slightly (<1 h) the length of G-1.

In order to ascertain whether or not the inhibitory effects of MIX could be attributed to a mechanism involving cAMP, cultures were treated at various times after mitotic selection with 10 μM isoproterenol with or without 0.5 mM MIX (Figs. 13 A, B, and C). The inhibitory effect of 0.5 mM MIX appeared to be maximal when added early in G-1, shortly after most of the cells had completed cell division (Fig. 13 B). Addition of MIX either before or after this time produced a less effective G-1 delay. Isoproterenol by itself had a slight stimulatory effect on entrance into S phase, but unexpectedly partially reversed the MIX inhibition of DNA synthesis (Fig. 13 A, B, and C).

These results prompted further experiments on the effect of isoproterenol by itself. It was found that exposure of cultures to 10 μM isoproterenol either at zero time or at 3 h shortened the length of G-1 by 0.5 h ($P < 0.01$) and 1.1 h ($P < 0.02$), respectively (not shown). However, with a range of concentrations of MIX from 2 to 200 μM and isoproterenol from 0.1 to 10 μM, it was not possi-

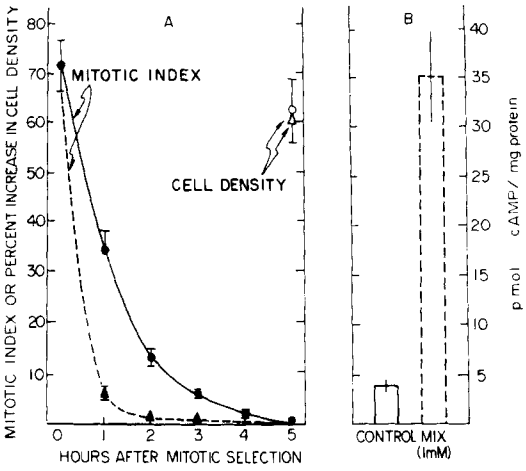


FIGURE 9 Effects of MIX on cyclic AMP levels and on completion of mitosis. MIX was added to synchronized cells, immediately after isolation in metaphase by mitotic selection, to give a final concentration of 1 mM. (A) Effect of MIX on completion of mitosis. M.I.: (●——●), control; and (▲- - -▲), + MIX. Percent increase in cell density: (○), control; and (△) + MIX. Values represent the mean ± SE from four separate experiments. Mitotic cells were determined as described in the legend of Fig. 6. (B) Effect of MIX on cAMP content of mitotic cells. cAMP levels were measured 60 min after the addition of either 0.9% saline or 1 mM MIX in 0.9% saline. Solid bar, control; broken bar, MIX. Vertical lines represent the range of cAMP levels in duplicate experiments.

ble to demonstrate a synergism between suboptimal doses of these two agents. These results suggested that either isoproterenol or MIX was acting by a mechanism which did not involve cAMP.

In an attempt to distinguish among these possibilities, the effect of 8-Br-cAMP on the length of G-1 was examined. 8-Br-cAMP (1 mM) was added for various intervals of time to synchronized cells (Fig. 14 A, B, and C). The effects of 8-Br-5'-AMP were also examined in these experiments to control for the possible hydrolysis of 8-Br-cAMP by serum phosphodiesterase.[1] Continuous exposure of the cells to 8-Br-cAMP over the interval from 0 to 12 h delayed the flow of cells into S phase by about 1 h. However, a greater inhibition was observed in response to 8-Br-5'-AMP (Fig. 14 A). The effect of 8-Br-cAMP was not seen when it was added at a later time (Fig. 14 B) nor when the cells were first treated and then resuspended in drug-free medium (Fig. 14 C). On the other hand, 8-Br-5'-AMP produced significant inhibition when added at 3 h (Fig. 14 B), and the effects were not completely reversed upon its removal (Fig. 14 C).

The results shown in Fig. 15 suggest an additional effect of 8-Br-cAMP which may be due to its hydrolysis to 8-Br-5'-AMP. Varying concentrations of 8-Br-5'-AMP or 8-Br-cAMP were added to synchronized cultures 3 h after mitotic selection, and the L.I. was measured at 10 h (Fig. 15 A). In contrast to the inhibitory effects observed with high concentrations, at low concentrations ($10^{-8}$–$10^{-7}$ M) both nucleotides produced a 50% stimulation in the labeling indices (indicative of a shortening of G-1). 8-Br-5'-AMP was approximately one order of magnitude more potent than 8-Br-cAMP. The estimated half-maximal concentrations for the rising phase of the dose response curves were approx. 6 nM for 8-Br-5'-AMP and 40 nM for 8-Br-cAMP. At concentrations greater than $10^{-4}$ M, 8-Br-5'-AMP was inhibitory. In contrast, 8-Br-cAMP was stimulatory at all concentrations tested, but the magnitude of this stimulation decreased at concentrations in excess of $10^{-4}$ M. Both the inhibitory and stimulatory effects of 8-Br-cAMP may be attributed to

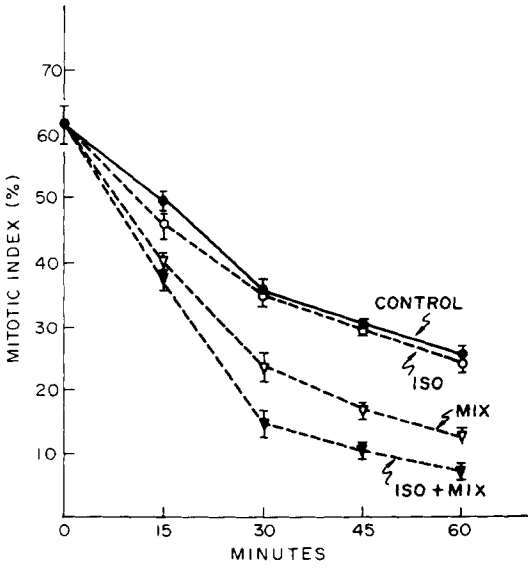


FIGURE 10 Effect of isoproterenol and MIX on mitosis. Agents were added at zero time and M.I. was followed for 60 min. (●——●), 0.9% saline; (○- - -○), isoproterenol (1 μM); (▽- - -▽), MIX 0.5 mM; and (▼- - -▼), isoproterenol + MIX. Values represent the mean ± SE from three experiments.

TABLE II

*Effect of Various Agents on Completion of Cell Division*

| Addition | Concentration | M.I. | |
|---|---|---|---|
| | | *% of control* | |
| Saline | 0.9% | 100 | (15) |
| MIX | 0.2 mM | 75 ± 4* | (4) |
| Isoproterenol | 1 μM | 88 ± 6 | (4) |
| Isoproterenol + MIX | – | 53 ± 7* | (4) |
| Carbachol | 10 μM | 94 ± 5 | (4) |
| Carbachol + MIX | – | 85 ± 5 | (4) |
| MIX | 1 mM | 40 ± 1* | (7) |
| Theophylline | " | 100 | (2) |
| Caffeine | " | 102 | (2) |
| Hypoxanthine | " | 99 ± 2 | (3) |
| 8-Br-cAMP | " | 64 ± 1* | (4) |
| mbcAMP | " | 68 ± 5* | (4) |
| 8-Br-5'-AMP | " | 102 ± 9 | (4) |
| 5'-AMP | " | 97 ± 5 | (4) |
| 8-Br-cGMP | " | 106 ± 8 | (4) |
| 5'-GMP | " | 98 ± 5 | (4) |

HeLa cells were synchronized by mitotic selection, and incubated for 45 min in the presence of various agents at the indicated final concentrations. The initial control M.I. was 62 ± 4%. Of the remaining cells, 27 ± 4% were postmitotic doublets. The results of several experiments are expressed as a percent of the control at 45 min (30 ± 4%). Numbers in parentheses represent the number of observations.

* $P < 0.01$ compared with control saline.

the presumed formation of 8-Br-5'-AMP or its catabolites.

## DISCUSSION

The absolute changes in cAMP at any given time must be viewed as an approximation, since they will be greatly affected by the degree of cell synchrony at that time. In general, if there is a sharp rise (or fall) in cAMP at a particular time in the cell cycle, the measured change would always represent an underestimate of the true value for an individual cell. Thus, when cells were accumulated either in mitosis with Colcemid or at the G-1/S border with 2 mM thymidine, the mean change in the cAMP content of the whole population would be increased as the individual cells in the population reached a uniform position in the cell cycle. If the duration of a cAMP fluctuation is brief enough (ca. 5–15 min), it is likely that such a change would escape detection entirely.

In spite of these limitations, the results reflect certain broad cell cycle changes which may be summarized as follows: (*a*) Cyclic AMP levels begin to rise early in G-1 and reach a plateau in late G-1. (*b*) Coincident with the G-1 to S transition or shortly thereafter, cAMP levels begin to decline; this decline continues for approx. 50% of the total length of S-phase. (*c*) During the latter half of S-phase, at least one and possibly two transient elevations in cAMP content occurs. (*d*) As cells traverse G-2 and M, the concentration of cAMP declines, attaining its lowest level in mitosis.

Some general aspects of the changes in cAMP content observed in this study with malignant cells have also been observed in untransformed cells (52, 53, 54, 56). However, the levels of cAMP during the first half of G-2 appear to be somewhat variable among different cell lines. In HeLa cells under the culture conditions employed in these studies and in some other cell types (10, 54), cAMP levels appear to decline throughout G-2. On the other hand, in human lymphoid (33), mouse lymphoma (Zeiling C. E., R. A. Johnson, and D. L. Friedman, unpublished observations) and rat Novikoff hepatoma cells (22), a rise of cAMP content in late S and early G-2 has been observed. Several reports confirm that cAMP lev-

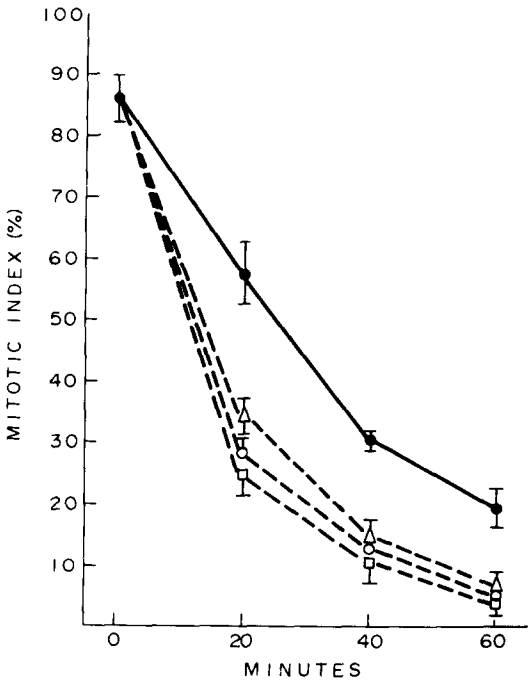


FIGURE 11 Effect of calcium and MIX on mitosis. $CaCl_2$ or MIX was added to mitotic cells at zero time in final concentrations of 2.73 mM and 1 mM, respectively. (●——●), control; (△- - -△), MIX; (○- - -○), $CaCl_2$; and (□- - -□), $CaCl_2$ + MIX. Values represent the mean ± SE from triplicate cell suspensions.

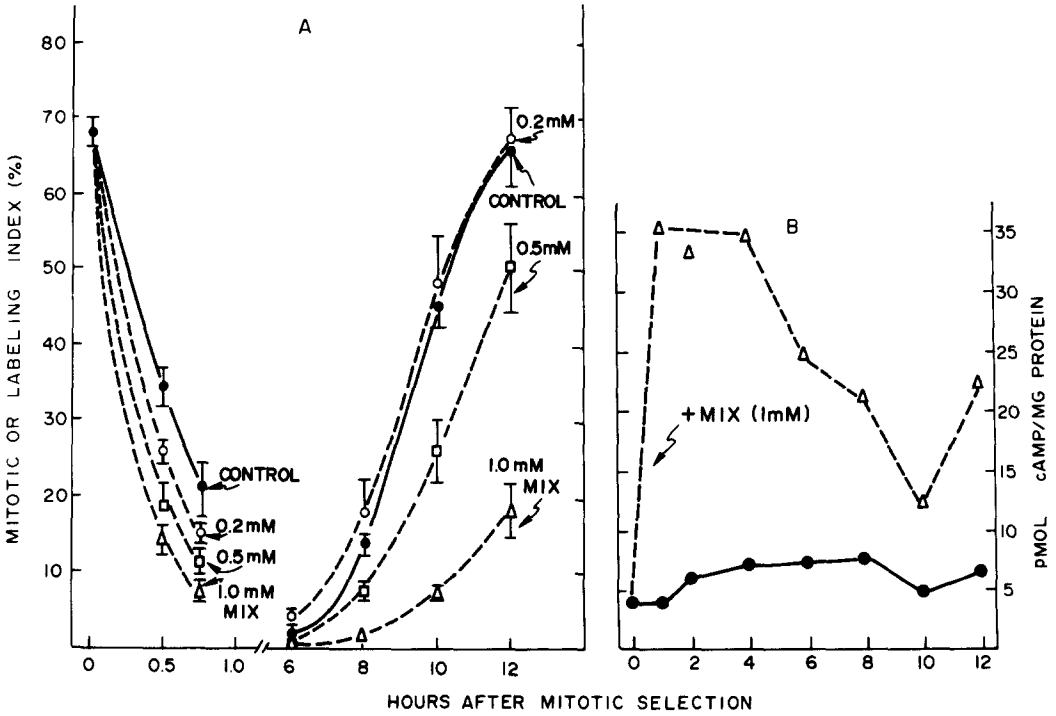


FIGURE 12 Effects of MIX on G-1. (A) The effect of varying concentrations of MIX on the duration of M and G-1. All additions were made immediately after selection of mitotic cells (zero time). M.I. (0–0.75 h) or L.I. (6–12 h): (●——●), control; (○- - -○), MIX (0.2 mM); (□- - -□), MIX (0.5 mM); and (△- - -△), MIX (1.0 mM). Values represent the mean ± SE from four experiments. (B) The effect of MIX on cAMP levels during G-1: (●——●), control; and (△- - -△), MIX (1.0 mM). Values are the average of two experiments.

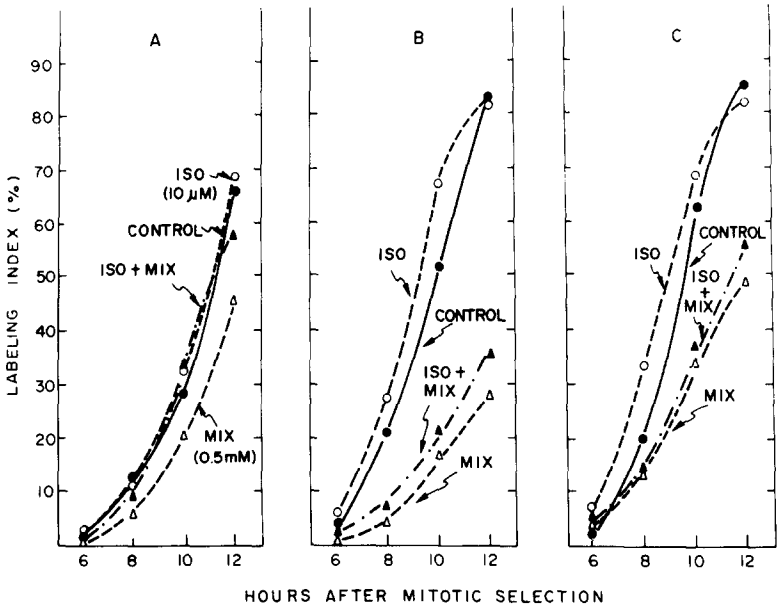


FIGURE 13 Effects of isoproterenol and MIX on the duration of G-1. L.I.: (●——●), control; (△- - -△), MIX (0.5 mM); (○- - -○), isoproterenol (10 μM); and (▲— · —▲), isoproterenol + MIX. (Values are the average of two experiments.) (A) Additions at zero time. (B) Additions at 2 h. (C) Additions at 4 h.

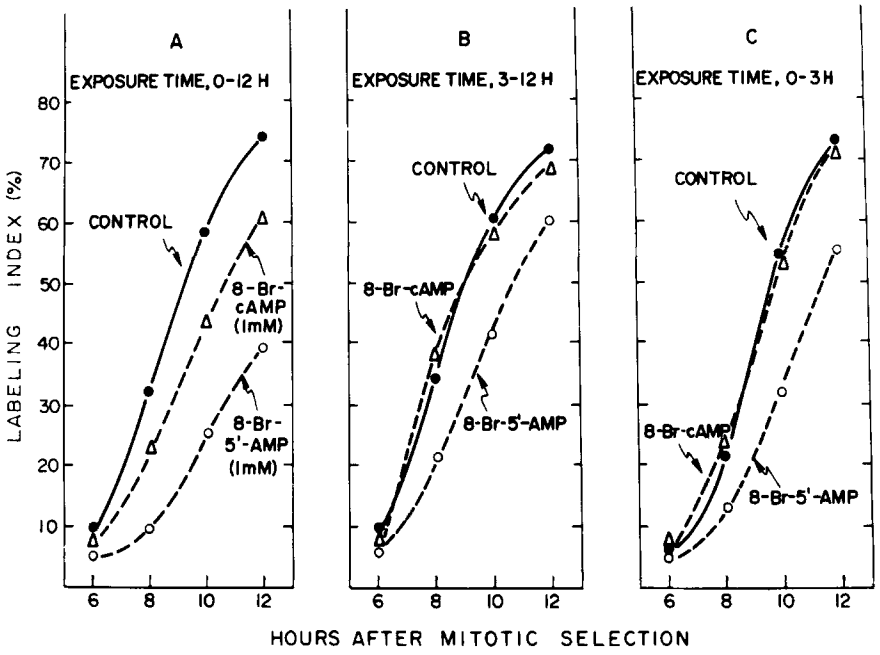


FIGURE 14 Effects of 8-Br-cAMP and 8-Br-5'-AMP on the duration of G-1. L.I.: (●——●), controls; (△- - -△), 8-Br-cAMP (1 mM); and (○- - -○), 8-Br-5'-AMP (1 mM). (Data represent the average of two experiments.) (A) Additions at zero time. (B) Additions at 3 h. (C) Addition at zero time. Agents were removed at 3 h by three sequential washes in prewarmed drug-free medium at 37°C.

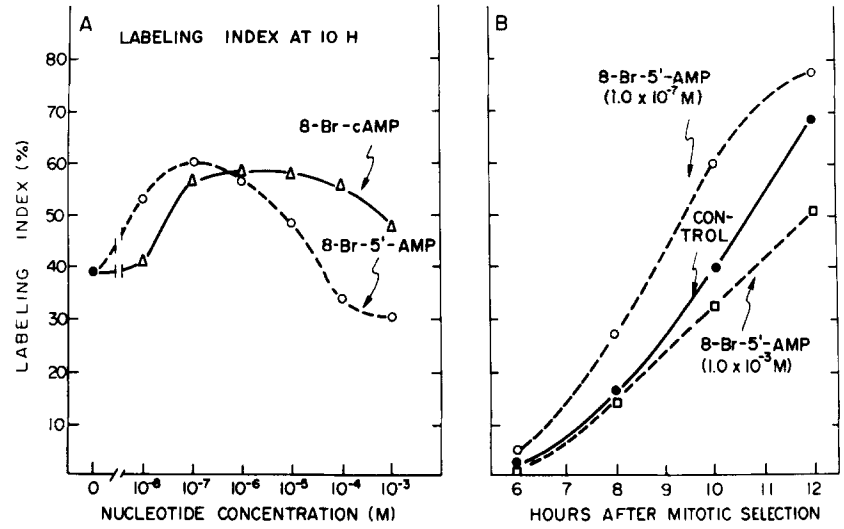


FIGURE 15 Effects of varying concentrations of 8-Br-cAMP and 8-Br-5'-AMP on the duration of G-1. All additions were made 3 h after mitotic selection. Values are the average of two experiments. (A) Effect of varying concentrations of 8-Br-cAMP and 8-Br-5'-AMP on L.I. measured at 10 h: (○- - -○), 8-Br-5'-AMP; and (△——△), 8-Br-cAMP. (B) Effect of 8-Br-5'-AMP on the length of G-1. L.I.: (●——●), control; (○- - -○), 8-Br-5'-AMP (0.1 μM); and (□- - -□), 8-Br-5'-AMP (1 mM). Similar relationships of concentration effects of 8-Br-5'-AMP and 8-Br-cAMP as indicated in panel A at 10 h were also observed at the other time-points indicated in panel B.

els in growing mammalian cells are at a minimum during mitosis (10, 32, 33, 53, 54, 56). The recent observation that cAMP levels vary cyclically in cleaving sea urchin zygotes, exhibiting minima at each cytokinesis (69), suggests that this phenomenon may be a common feature of mitosis in eucaryotic animal cells.

A number of reports confirm a peak in cAMP levels during G-1 phase. Reinitiation of growth in quiescent fibroblasts by fresh serum (10, 19, 38, 51, 53), trypsin (10, 38), or viral infection (49) is associated with a transient fall in cAMP. However, cAMP content eventually returns to a relatively high level before initiation of DNA synthesis (10, 53). Prereplicative peaks in cAMP content have also been observed in regenerating liver (31, 57, 63) and in synchronized cultures of CHO cells (52, 56) and human lymphoid cells (32, 33). The timing of these peaks in relation to DNA synthesis is quite variable in the different systems, suggesting that increased cAMP levels are not the immediate signal for initiation of S phase. However, cAMP may be intimately involved in the events leading up to DNA synthesis.

Several lines of evidence support a significant role for the prereplicative rise in cAMP (3, 12, 23, 30, 41, 52, 57, 68). However, in studies with S49 lymphoma cells, Coffino et al. showed that genetic mutants deficient in cAMP-dependent protein kinase progressed through an essentially normal cell cycle (15). This was interpreted as indicating a lack of involvement of cAMP in the regulation of the cell cycle. However, such an interpretation is premature until it can be shown that: (*a*) other cellular compensatory mechanisms do not operate to bypass a defective regulatory subunit of cAMP-dependent protein kinases, (*b*) mutant kinases (26) as measured in vitro are totally inactive in vivo, and (*c*) receptor proteins other than protein kinase are not involved in cAMP effects on the cell cycle.

An inverse correlation has been reported between growth rate and cAMP content (37). Our results suggest a plausible explanation for these observations. Since growth rate is primarily a function of the length of G-1 (45), the more rapid the growth rate the greater will be the proportion of cells in S, G-2, and M in an asynchronous culture. This would predict an inverse relationship of cAMP levels with growth rate, assuming that relatively high cAMP levels are maintained during most of G-1 and lower levels at other stages of the cell cycle.

Several reports indicate that cAMP levels are lower in malignant cells when compared with those of untransformed cells during exponential growth (13, 37, 55). It would be of interest to know whether this difference is reflected in only one part of the cell cycle or whether cAMP levels are proportionately lower in all stages. Direct comparison within the same cell line before and after transformation will be required to establish such differences.

In the studies of cell cycle traverse, we have attempted to determine the effects of cAMP levels elevated experimentally in phase or out of phase with naturally occurring cell cycle-dependent fluctuations in endogenous cAMP. Where possible, we have studied effects demonstrable within a relatively short period after cellular cAMP levels were elevated because of the uncertainty in judging physiological specificity of long-term treatments. Elevation of intracellular cAMP in cells which have initiated DNA synthesis and which normally exhibit falling cAMP levels (early S phase) had little or no effect on the subsequent timing of S phase. Our results are in agreement with those of Willingham et al. in normal fibroblasts (68) but are in contrast with studies on human lymphoid cells (33) and rat hepatoma cells (64). These discrepancies may reflect differences in cell lines or nonspecific effects of the agents used to elevate cAMP.

Our studies indicate that high levels of cAMP inhibit cell cycle traverse in G-2. A role for cAMP is supported by several observations. First, MIX raises cAMP and inhibits progression of cells through G-2. Other methylxanthines which have little effect on cAMP levels in these cells[1] did not affect passage through G-2. Secondly, the combination of isoproterenol, which activates adenylate cyclase in these cells (70),[1] and suboptimal concentrations of MIX leads to a markedly potentiated inhibition of G-2 traverse. The inhibition of G-2 traverse by high cAMP levels together with the demonstration that endogenous cAMP levels normally fall during G-2 indicates the existence of a cAMP-mediated G-2 control point in HeLa cells. The mechanism for the inhibition of G-2 traverse by cAMP is not known, but our observation of a more complete G-2 block with the addition of MIX before onset of G-2 suggests an additional level of complexity in the mechanism of this effect of MIX. Whatever the mechanisms underlying the effect of cAMP in G-2, the endogenous level of cAMP during late S phase and G-2 could

regulate the length of G-2. The possibility that such regulation occurs naturally is supported by the findings that, in a number of tissues, catecholamines or dbcAMP have been shown to slow G-2 traverse (8, 46, 65, 68), that in certain tissues a significant proportion of cells are arrested in G-2 in vivo (20), and that, in vitro, passage of cells through G-2 is serum dependent (2).

In contrast with the inhibitory effect of premitotic elevations of cAMP on G-2 and M, increased cAMP levels during mitosis were found to stimulate the completion of cell division as evidenced by the rapid fall in M.I. (Figs. 9, 10, and 11). The possibility has been considered that this effect resulted from an inhibition of G-2 cells contaminating the mitotic population rather than from a stimulation of mitosis. This possibility was ruled out by the finding that the cell number increased more rapidly in MIX-treated mitotic cells than in control cells. Furthermore, in many recent experiments the initial M.I. was as high as 95%. Even if the remaining 5% of the cells were all in G-2, their inhibition could account for only a small fraction of the mitotic effect observed after MIX.[3] Although cAMP levels were not measured, a somewhat similar effect of epinephrine on mitosis in mouse epidermis has been reported (9, 17).

If cAMP is normally involved in events leading to completion of mitosis, a rise in intracellular cAMP levels in late M would be expected. With the synchronization methods employed here, a highly transient increase in cAMP at this time would not be easily detected. Using a slightly different approach to this problem, we have recently found a transient increase in the production of cAMP at a time which does correlate with the latter half of mitosis[4] in Novikoff hepatoma cells. The ability of increased extracellular calcium levels to mimic the cAMP-induced stimulation of cell division suggests a possible mechanism for the effect of cAMP. Cyclic AMP could promote an increased concentration of free cytoplasmic calcium either by promoting cellular uptake of calcium from the medium or by releasing sequestered intracellular stores of calcium.

Interpretation of the effects of various agents on G-1 in these studies is obviously complex. Since isoproterenol failed to enhance the inhibitory effects of MIX, the ability of high levels of MIX to prolong G-1 may not be due to its inhibition of cAMP phosphodiesterase. For example, MIX could elevate cellular cGMP levels, alter transmembrane ion fluxes, etc. The results obtained with cyclic nucleotide analogues support the conclusion that cAMP is ineffective as a negative modulator of G-1 traverse in HeLa cells. Since 8-Br-5′-AMP was far more effective than 8-Br-cAMP and since it is known that both compounds are at least partially metabolized (58), it seems likely that the effects of the cyclic nucleotide could be attributed to 8-Br-5′-AMP or its metabolities, e.g., adenosine. Independent actions of these two agents cannot be excluded. The observations that isoproterenol is slightly stimulatory in G-1, that it can partially prevent the MIX-induced inhibition, and that cAMP levels are naturally elevated during this stage suggest a permissive role of cAMP in G-1 traverse. Although our data are not inconsistent with this hypothesis, more substantial evidence is required to verify such a role.

In conclusion, our studies and those in other laboratories implicate a role of cAMP in the regulation of the cell division cycle. However, none of the possible underlying mechanisms have yet been established by which it might influence a given cell cycle event. The ability to establish such roles and to evaluate causal mechanisms is severely impaired by our lack of understanding of the fundamental processes that occur in the cell division cycle. In addition, the mechanisms that underlie fluctuations in cyclic nucleotides during the cell cycle are also not established, and further work will be required to ascertain whether the appropriate cyclases or diesterases are involved. Perhaps then, more important questions could be posed regarding the mechanisms by which the activities of those enzymes may be regulated.

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