EFFECTS OF CAFFEINE ON RADIATION-INDUCED PHENOMENA ASSOCIATED WITH CELL-CYCLE TRAVERSE OF MAMMALIAN CELLS

RONALD A. WALTERS, LAWRENCE R. GURLEY, *and* ROBERT A. TOBEY

From the Cellular and Molecular Radiobiology Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87544

ABSTRACT Caffeine induced a state of G_1 arrest when added to an exponentially growing culture of Chinese hamster cells (line CHO). In addition to its effect on cell-cycle traverse, caffeine ameliorated a number of the responses of cells to ionizing radiation. The duration of the division delay period following X-irradiation of caffeine-treated cells was reduced, and the magnitude of reduction was dependent on caffeine concentration. Cells irradiated during the DNA synthetic phase in the presence of caffeine were delayed less in their exit from *S*, measured autoradiographically, and the radiation-induced reduction of radioactive thymidine incorporation into DNA was lessened. Cells synchronized by isoleucine deprivation, while being generally less sensitive to the effects of ionizing radiation than mitotically synchronized cells, were equally responsive to the effects of caffeine. The X-ray-induced reduction of phosphorylation of lysine-rich histone F1 was less in caffeine-treated cells than in untreated cells. Finally, survival after irradiation was only slightly reduced in caffeine-treated cells. A possible role of cyclic AMP in cell-cycle traverse of irradiated cells is discussed.

INTRODUCTION

Exposure of mammalian cells to ionizing radiation results in an inhibition of division (Walters and Petersen, 1968 *a*) and an accumulation of cells in the G_2 phase of the cell cycle (Walters and Tobey, 1970; Shepherd et al., 1972). Although it is known that the ability of Chinese hamster cells (line CHO) to recover spontaneously the capacity to divide after irradiation requires protein synthesis (Walters and Petersen, 1968 *b*), little is known of the biochemical nature of the lesion(s) responsible for division delay. In contrast to lengthening of the division delay period by treatment with inhibitors of protein synthesis, elevated concentrations of calcium and magnesium salts as well as hormone treatment reduce the division delay period of mammalian cells (Whitfield and Rixon, 1962; Whitfield et al., 1964; Perris et al., 1967; Whitfield et al., 1969). These effects, which have been attributed to the state

of chromosome condensation, are very interesting in light of recent evidence linking calcium metabolism with various hormone responses and interactions involving cyclic 3′,5′-adenosine monophosphate (cAMP) (for a review see Whitfield et al., 1973). It is thought that cAMP may play an important regulatory role in cell proliferation and, depending upon the system used, may be either mitogenic by stimulating mammalian cells to enter the DNA synthetic phase (Whitfield et al., 1973) or inhibitory as in contact inhibition of cell growth (Burger et al., 1972; Otten et al., 1972). Since cyclic AMP has been implicated in some radiation responses (Langendorff and Langendorff, 1971; Scaife, 1971; Prasad, 1972), we thought it of use to investigate the effects of various agents that may influence cyclic AMP in irradiated cells. Caffeine inhibits cyclic AMP phosphodiesterase; thus, we have investigated its effects on irradiated cells, although we are well aware of the fact that it may affect cell parameters other than cAMP. The data to be presented here indicate that caffeine can indeed reduce the magnitude of a number of radiation effects, primarily those associated with traverse of the cell cycle. The results suggest that cAMP may play a role in cell-cycle traverse of irradiated cells.

MATERIALS AND METHODS

Cell Culture and Synchronization

Chinese hamster cells (line CHO) were maintained as suspension cultures in F-10 medium supplemented with 10% calf and 5% fetal calf sera, penicillin, and streptomycin. Cells were free of mycoplasma contamination as determined by periodic assays using the method of Hayflick (1965) and improved by House and Waddell (1967). Cells were synchronized by either selectively detaching mitotic cells from monolayer cultures (Tobey et al., 1967) or by growth for 30 h in isoleucine-deficient medium, followed by resuspension in fresh medium supplemented with twice the normal concentration of isoleucine and glutamine (Tobey and Ley, 1970). In some cases, cells synchronized by isoleucine deprivation were resynchronized in late G_1 by maintaining them in hydroxyurea (10^{-3} M) for 10 h after restoration of isoleucine (Tobey and Crissman, 1972). The cell doubling time of exponentially growing cultures ranged from 16 to 18 h. Cell concentrations were determined by an electronic particle counter. Cells were X-irradiated in suspension as previously described (Walters and Petersen, 1968 *a*).

DNA Analysis

The fraction of cells in S was determined by two methods: (1) cells were pulse-labeled for 15 or 30 min with 1 μCi/ml [methyl-^{3}H]thymidine (6 Ci/mmol, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.), and autoradiographs were prepared as previously described (Walters and Petersen, 1968 *b*); and (2) DNA distribution patterns of cells in G_1, S, and $G_2 + M$ were determined by means of the Los Alamos flow microfluorometer after fixing monodispersed cells with formalin, followed by Feulgen staining with the fluorescent dye acriflavine as previously described (Kraemer et al., 1972). This method determines the relative DNA mass of individual cells by measuring the fluorescence of DNA-bound acriflavine.

Radioactive precursor incorporation into DNA was determined in the following manner. Cells were prelabeled with ~0.0075 μCi/ml [methyl-^{14}C]thymidine (55.3 mCi/mmol, Schwarz/Mann) followed by a chase period in label-free medium. Cells were then pulse-

labeled with 2.0 μCi/ml [methyl-^{3}H]thymidine (6 Ci/mmol, Schwarz/Mann) for 1 h after the desired treatment. DNA was isolated by the following protocol. Aliquots of the cell culture were cooled after labeling by pouring over frozen F-10 medium, and cells were collected by low-speed centrifugation (3,000 rpm); the pellet was resuspended in 10% trichloroacetic acid (TCA) at 3°C. After standing for 0.5 h, the precipitate was collected, washed with 10% TCA, then resuspended in 95% ethanol-2% potassium acetate. After 1 h, the precipitate was collected, washed with the ethanol-acetate solution, and dried. The pellet was resuspended in 1 ml solution (0.05 M cacodylic acid [pH 7.0], 5 mM Mg^{++}, 1 mM Ca^{++}, 100 μg pancreatic DNase [DNase I, EC 3.1.4.5, DPFF, Worthington Biochemical Corp., Freehold, N. J.]) and incubated for 3 h at 37°C. The reaction mixture was cooled to 4°C and made to 0.2 M in perchloric acid (PCA). After 0.5 h, the precipitate (containing RNA and protein) was collected by centrifugation. The supernate (containing DNA hydrolysate) was removed and combined with a wash of the pellet. Aliquots of the supernate were removed for spectrophotometric determination of absorbancy at 260 nm and for determination of radioactivity by counting 0.5-ml aliquots in a Packard Tri-Carb spectrometer (Packard Instrument Co., Downers Grove, Ill.) using Aquasol liquid scintillation fluid (New England Nuclear Corp., Boston, Mass.). Recovery of the DNA was 80–90%. ^{3}H and ^{14}C were counted simultaneously in two channels by pulse-height analysis. The rate of radioactive incorporation was determined by the ^{3}H/^{14}C ratio or by specific activity (cpm/A_{260}). Variation of the ^{3}H/^{14}C ratio between duplicate samples was $\leq$2%, and variation in specific activity between duplicate samples was $\leq$5%.

Preparation and Purification of Histones

Histones were prelabeled by growing cells in medium containing 50 μCi/liter [^{3}H]lysine (20 mCi/mg, New England Nuclear Corp.) for 50 h. After the desired treatment, cells were pulse-labeled 1 h with 20 μCi/ml [^{32}P]H_3PO_4 (carrier-free, New England Nuclear Corp.). Histone fractions were prepared from the chromatin of 3 $\times$ 10^8 cells by the first method of Johns (1964) as previously described (Gurley and Hardin, 1968). Histone f1 was extracted from chromatin with 5% perchloric acid and recovered by precipitation with 20% trichloroacetic acid. The arginine-rich histone complex containing f2a1, f2a2, and f3 was then extracted with ethanol solutions containing 0.14 M mercaptoethanol to prevent dimerization of f1 (Smith et al., 1970) and recovered by acetone precipitation. This complex was not subfractionated. Histone f2b was extracted with 0.25 N HCl and recovered by acetone precipitation. The three preparations were dissolved in water, and f1 and f2b were mixed. The two solutions, one containing lysine-rich histone (f1 and f2b) and the other containing the arginine-rich histones (f2a1, f2a2, and f3), were frozen and lyophilized to dryness. These two preparations were analyzed by preparative polyacrylamide gel electrophoresis as previously described (Gurley and Walters, 1971). During electrophoresis, individual histone fractions were eluted from the bottom of the gel by a cross flow of buffer which was collected every 2 min (2 ml/fraction). The fractions were counted in a liquid scintillation counter as described above. Since the amount of [^{3}H]lysine is proportional to the amount of protein in the peak (Gurley and Walters, 1971), the relative rate of phosphorylation in each histone fraction was determined by the ^{32}P/^{3}H ratio.

RESULTS

As will be shown later, caffeine at a 2mM concentration was most compatible for studying radiation effects with the least cytotoxic side effects. For this reason, most of the studies reported below employed 2 mM caffeine concentrations. Unless other-

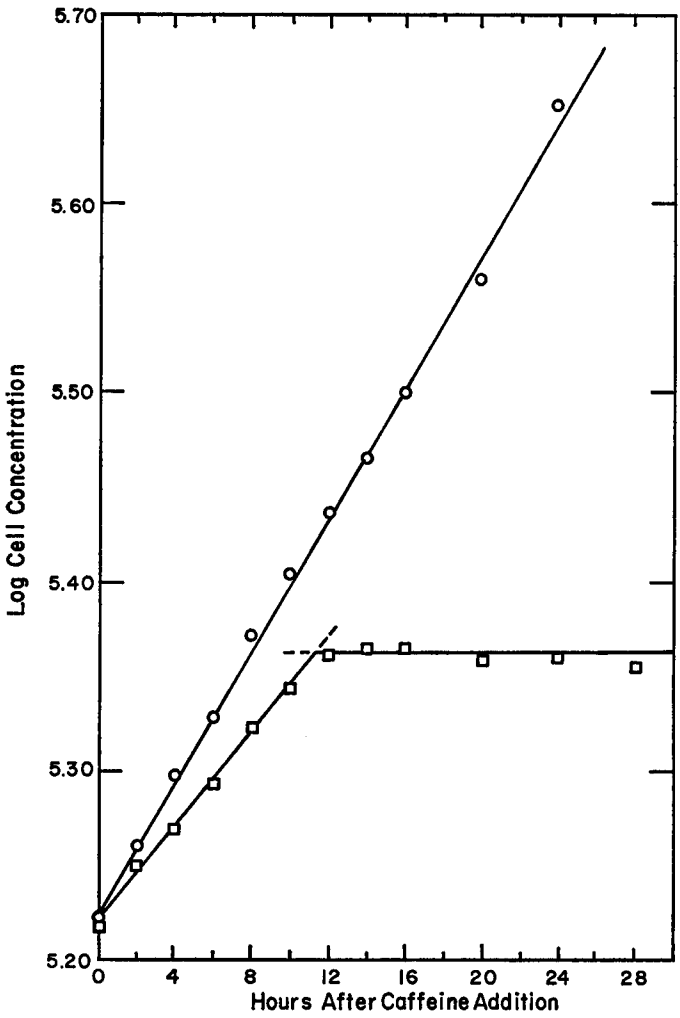

FIGURE 1 Growth rate of cells in the presence ot 2 mM caffeine. Caffeine was added at $t = 0$ to an exponentially growing culture of Chinese hamster cells. Growth was monitored with an electronic particle counter: ○, untreated controls; and □, 2 mM caffeine.

wise stated, caffeine was present continuously during the experiments. Fig. 1 shows the effect of caffeine (2 mM) on the growth rate of exponentially growing cells. The growth rate was determined in all experiments reported here by cell counting with an electronic particle counter and expressing the results as the logarithm of cell concentration. It can be seen that, in this case, caffeine reduced the growth rate, thus increasing the apparent doubling time of the culture from ~18 h to ~24 h immediately after addition. It should also be noted that between 11 and 12 h after addition of the drug cell division stopped. In view of the postulated relationship between levels of cyclic AMP and contact inhibition of cell growth (Burger et al.,

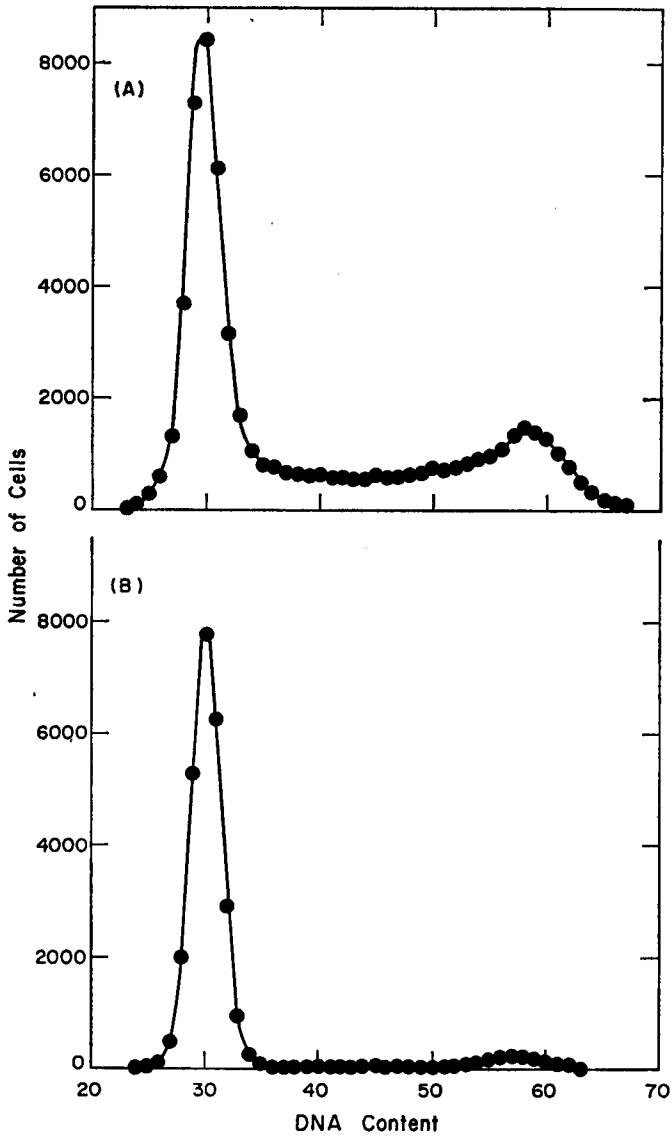

FIGURE 2 DNA distribution patterns of cells as measured by analysis with the flow microfluorometric technique. G_1 cells are represented by scale value of 30, $G_2 + M$ cells by scale value of 58: (*A*) untreated, exponentially growing cells; and (*B*) cells treated for 24 h with 2 mM caffeine. (Cells were growing exponentially prior to caffeine treatment.)

1972; Otten et al., 1972), it was of interest to determine whether caffeine treatment induced a generalized inhibition of cell growth or if, as suggested by the data in Fig. 1, there occurred a selective block of cell-cycle traverse. An inhibition of growth at 11–12 h after treatment suggested the possibility of a G_1 block with an emptying of

the S and G_2 phases of the cell cycle. To test this possibility, an exponentially growing culture was treated with caffeine (2 mM). At 24 h after treatment, samples were taken for analysis of DNA distribution patterns employing the Los Alamos flow microfluorometer (FMF) and for autoradiographic analysis of cells incorporating [^{3}H]thymidine (1 μCi/ml) during a 30 min pulse. DNA distributions obtained are shown for untreated controls (Fig. 2 *A*) and for caffeine-treated cells (Fig. 2 *B*). Under cultivation conditions employed during the course of these experiments, G_1 was $\sim$9 h, S was $\sim$6 h, and $G_2 + M$ was $\sim$3.5 h. Thus, the majority of cells in an exponentially growing culture are in G_1. The major peak in the FMF pattern (scale value of 30, Fig. 2 *A*) represented cells with a G_1 DNA content, while cells with a $G_2 + M$ DNA content exhibited a peak (scale value of 58, Fig. 2 *A*) with approximately twice the mode of G_1 cells. Cells distributed throughout S phase, containing varying amounts of DNA, were distributed between the peaks representing G_1 and $G_2 + M$ DNA contents. However, it will be noted that the culture treated with 2 mM caffeine exhibited an altered DNA distribution pattern (Fig. 2 *B*). The culture was nearly devoid of S cells ($\leq$2%); cells with a $G_2 + M$ DNA content comprised only 5% of the population and cells with a G_1 DNA content comprised $\sim$93% of the population. The distribution of cells in the untreated control was approximately 54% G_1, 35% S, and 11% $G_2 + M$. Autoradiographic analysis of cells treated with caffeine for 24 h, then pulse-labeled with [^{3}H]thymidine for 30 min, indicated that $\sim$2% of the cells were in S phase—in agreement with FMF analysis. When caffeine (2 mM) was added in G_1 (e.g., 2 h after synchronization) to cells synchronized by growth in isoleucine-deficient medium (data not shown), only 10% of the cells traversed the cell cycle and divided as compared with $\sim$80% for control cells during the same time period. Thus, caffeine appears to induce a state of G_1 arrest. It should be noted here that the effects of caffeine on cell-cycle traverse can be variable. Although caffeine never failed to induce G_1 arrest within 24 h after treatment in an exponentially growing culture, the effects on growth rate varied from a 10 to 30% inhibition, and the time required for cessation of division varied from 12 to 20 h after caffeine addition. We have no explanation for the variability of the effect of caffeine on cell growth. This variability may account for some of the variability of the results reported below and summarized in Table II.

The effect of caffeine added 15 min prior to X-irradiation on the division delay period of an exponentially growing culture irradiated with 400 rads is shown in Fig. 3. Division delay was estimated by displacement of the growth curves from the appropriate control. It is apparent that caffeine altered the division delay patterns significantly. Caffeine in 2 mM concentrations reduced division delay from 4.8 h in the untreated sample to 2.1 h (Fig. 3 *A*). In addition, inhibition of division apparentyl was not complete in the caffeine-treated culture as indicated by the continuous, though small, increase in cell number prior to resumption of division at or near the control rate. The data shown in Fig. 3 *B* indicate that higher concentrations of caffeine (4 or 6 mM), although progressively reducing the growth rate of control

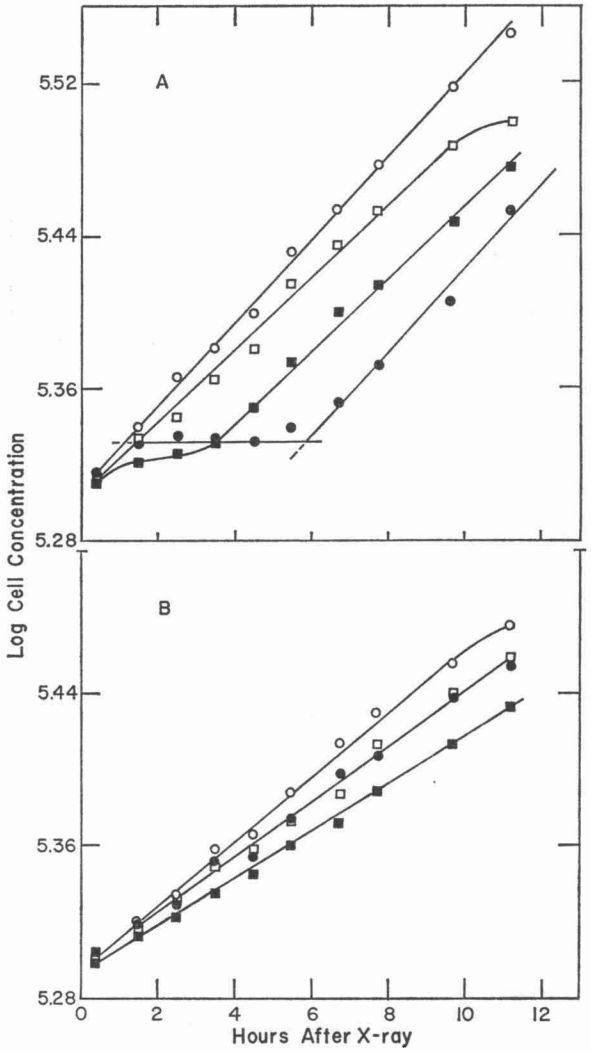

FIGURE 3 Effect of caffeine on the division delay period of cells irradiated with 400 rads X-irradiation during exponential growth. Cells were treated with caffeine 15 min prior to irradiation: (*A*) control, no caffeine (○), irradiated, no caffeine (●), control, 2 mM caffeine (□), irradiated, 2 mM caffeine (■); and (*B*) control, 4 mM caffeine (○), irradiated, 4 mM caffeine (●), control, 6 mM caffeine (□), irradiated, 6 mM caffeine (■).

cultures, were even more effective in reducing the effect of 400 rads on cell growth. As shown, division was never completely inhibited in the irradiated cultures, although the growth rate was reduced. Since 0.1 and 0.5 mM caffeine reduced the division delay period after 400 rads by ~10 and 25% (data not shown), the effect of caffeine apparently is concentration dependent. In addition, 2 mM caffeine was effective in reducing division delay after doses up to 800 rads or when added immediately after irradiation (data not shown).

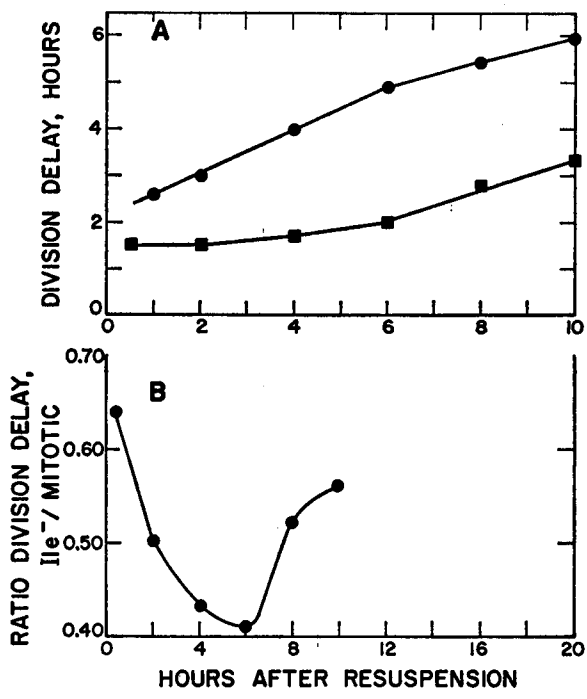

FIGURE 4 Division delay of cells synchronized by mitotic selection or growth in isoleucine-deficient medium irradiated at various times throughout the cell cycle with 700 rads: (*A*) cells synchronized by mitotic selection (●), cells synchronized by growth in isoleucine-deficient medium (■); and (*B*) ratio of division delay (Ile^-/mitotic) of cells irradiated at corresponding times in the cell cycle after synchronization by either mitotic selection or growth in isoleucine-deficient medium.

In preliminary experiments, we had noted that cells synchronized by growth for 30 h in isoleucine-deficient medium responded differently to X-irradiation than cells synchronized by mitotic selection, even though both methods of synchronization yielded cell populations with very similar cell-cycle kinetics. Cells traversed G_1, entered *S*, and divided at equivalent times after synchronization by either technique. The duration of each phase of the cell cycle was not significantly different from that reported above for exponentially growing cells (Tobey, 1973). The data in Fig. 4 *A* show the division delay of cells irradiated with 700 rads at specified intervals after synchronization by either the isoleucine-deficiency technique or mitotic selection in which cells were taken from the same culture prior to synchronization. It can be seen that, as mitotically synchronized cells traversed the cell cycle, the division delay period increased in a biphasic manner with the maximum rate of increase between 1 and 6 h after synchronization (e.g., as cells traversed G_1 and entered *S* [Walters and Tobey, 1970]), after which time the rate of increase was reduced (e.g. as cells traversed *S* and entered G_2). However, cells synchronized by growth in isoleucline-

deficient medium exhibited a consistently smaller division delay than mitotically synchronized cells, and the pattern of cell-cycle sensitivity was somewhat different. This is shown more clearly in Fig. 4 *B* where the ratio of the division delay of cells obtained by isoleucine-deficiency synchronization to that of cells obtained by mitotic selection is determined. The maximum divergence occurs at 6 h after synchronization when cells were entering *S*.

We have reported previously that, when mitotically synchronized cells were irradiated with low doses ($\leq$200 rads), the duration of the division delay period was constant as cells were irradiated throughout the cell cycle (Walters and Petersen, 1968 *a*); however, it is obvious from Fig. 4 *A* that this is not the case for higher doses of radiation. For this reason, we reexamined the effect of both high and low doses of radiation on division of mitotically synchronized cells (Fig. 5 *A*). It is apparent that cells irradiated with 200 rads at times throughout the cell cycle exhibited a uniform delay in division, in agreement with previously reported data (Walters and Petersen, 1968 *a*), while cells irradiated with 1,000 rads showed a continuously increasing division delay (although at a nonuniform rate). Since a dichotomy of results exists, mitotically synchronized cells were irradiated 1 h after synchronization (early G_1) with increasing doses, and the results are shown in Fig. 5 *B*. It can be seen that the apparent division delay (in hours per rad) was not constant. At doses $>$ 400 rads, the delay per rad falls only very slowly, indicating the expected linear dose response (Yu and Sinclair, 1967). However, the delay per rad for 200 rads is increased. Thus, a low dose given early in the cell cycle obscures the cycle dependence of division delay. Although the effect is real, it may be the result of experimental conditions since the cycle-dependent sensitivity for higher doses shown here is similar to that reported by others (Leeper et al., 1972).

Since the division delay period is reduced in either cells synchronized by the isoleucine-deficiency technique or in cells treated with caffeine, the effect of caffeine on the division delay and the DNA synthetic period of irradiated cells synchronized by the isoleucine-deficiency technique was examined to determine if the two effects might possibly be associated with a common mechanism. From the data presented above, it is clear that caffeine induces a state of G_1 arrest and inhibits cell-cycle traverse when added during G_1 after synchronization by the isoleucine-deficiency technique. To prevent this effect from interfering with the analysis, synchronized cells were resynchronized in late G_1 by maintaining cells in hydroxyurea (10^{-3} M) for 10 h after synchronization. Cells were irradiated with 400, 800, and 1,200 rads 2 h after removal of hydroxyurea and entry into *S*. In caffeine-treated cultures, the drug (2 mM) was added 15 min prior to irradiation. The effect of X-irradiation on division is shown in Fig. 6. In those cultures not treated with caffeine, irradiation with 400, 800, and 1,200 rads produced division delays (measured by the difference between the time at which irradiated cultures began dividing and the time at which the appropriate control cultures began dividing) of 1.9, 3.8, and 5.8 h, respectively.

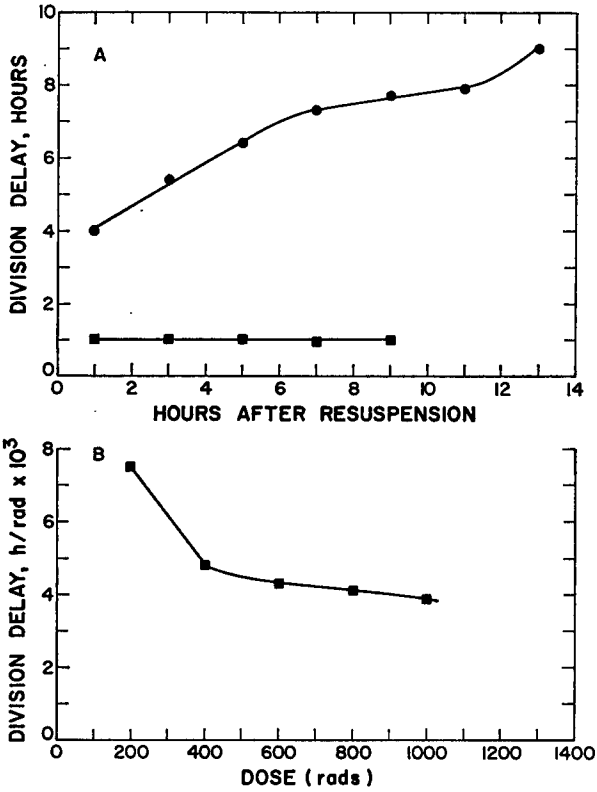

FIGURE 5 Division delay of cells synchronized by mitotic selection: (*A*) division delay of cells irradiated with 200 rads (■) or 1,000 rads (●) at various times throughout the cell cycle; and (*B*) division delay (hours per rad) of cells irradiated with increasing doses at 1 h after resuspension (early G_1).

In addition, the division rate was slowed progressively, and fewer cells completed the first postirradiation division with increasingly larger doses. The division delay for caffeine-treated cells was 1.1, 2.1, and 3.2 h after 400, 800, and 1,200 rads, respectively. It should also be noted that, in contrast to untreated cells, the division rate of irradiated caffeine-treated cells was not significantly changed with respect to the appropriate control after increasingly larger exposurses to X-irradiation, although the number of cells completing the first postirradiation division was again reduced. Thus, caffeine is nearly as effective in reducing division delay after irradiation in cells synchronized by growth in isoleucine-deficient medium as in cells irradiated during exponential growth (ie., reduction of division delay to about one-fourth that in irradiated, drug-free, nonsynchronized cells). This suggests that the mechanism(s) responsible for reduced division delay in cells synchronized by the isoleucine-deficiency technique may not be the same as that of caffeine-treated cells.

During the time that samples were taken for determination of cell concentration

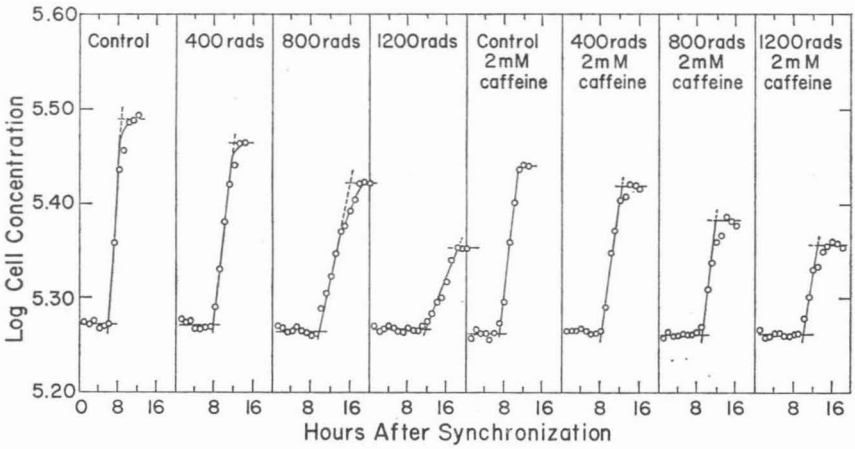

FIGURE 6 Effect of 2 mM caffeine on division delay of *S* cells synchronized by the isoleucine-deficiency technique, then resynchronized in late G_1 by treating with hydroxyurea. Cells were irradiated with 400, 800, and 1,200 rads 2 h after resynchronization. In the appropriate cultures, caffeine was added 15 min prior to irradiation. The cultures are identified in the figure.

(Fig. 6), aliquots of the same cultures were removed and pulse-labeled for 15 min with [^{3}H]thymidine. The fraction of cells in *S* was determined by autoradiography, and the results are shown in Fig. 7. As expected, cells rapidly entered *S* after removal of hydroxyurea. The radiation-induced lengthening of the *S* period (*S* retention) is defined as the additional time required by irradiated cells for one-half the cells to exit from *S* as compared with the appropriate control. The data indicate that *S* retention of untreated cells was 0.7, 1.3, and 2.5 h after irradiation with 400, 800, and 1,200 rads, respectively (Fig. 7 *A*). However, *S* retention of caffeine-treated cells was less; no significant effect could be measured after 400 rads, while retention was 0.5 and 1.1 h for 800- and 1,200-rad exposures, respectively (Fig. 7 *B*). It should also be noted here that *S* retention of irradiated, untreated cells was less than that observed previously in *S* cells after synchronization by either mitotic selection or thymidine blockade; irradiation of cells in *S* with 500 rads after mitotic synchronization produced an *S* retention of $\sim$2 h, while irradiation of *S* cells with 400 rads after release from thymidine blockade produced an *S* retention of 1.4 h (Walters and Tobey, 1970). Thus, not only division delay but *S* retention is less in cells synchronized by the isoleucine-deficiency technique than in mitotically synchronized cells. While it is possible that *S* retention after irradiation of cells resynchronized with hydroxyurea could be a result of drug effects, the observation that mitotically synchronized and thymidine synchronized cells showed similar *S* retention after irradiation makes this less likely (Walters and Tobey, 1970). Although the mechanisms are different, both excess thymidine and hydroxyurea inhibit DNA synthesis by interfering with reductase action; thymidine inhibits the conversion of cytidine diphosphate to deoxycytidine diphosphate (Moore and Hurlbert, 1966), while

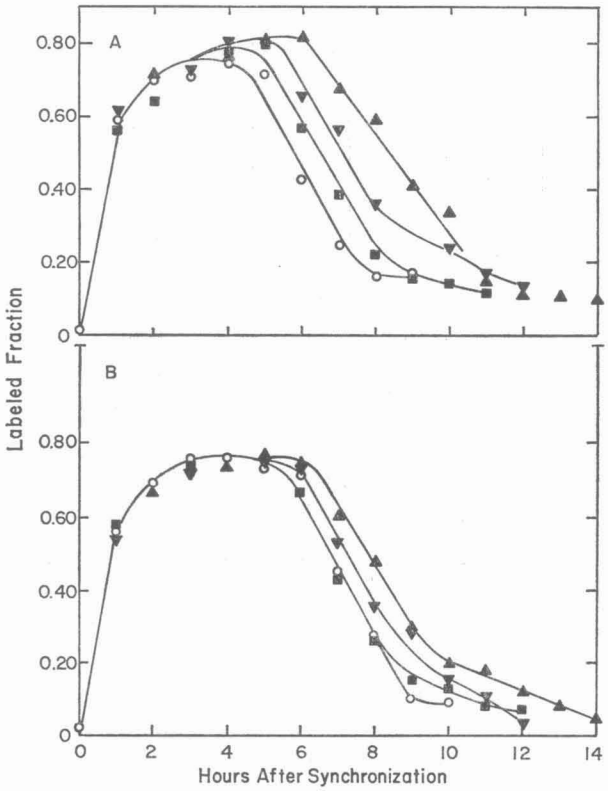

FIGURE 7 Effect of 2 mM caffeine on the radiation-induced retention of cells in *S*. Cells were synchronized by growth in isoleucine-deficient medium, then resynchronized in late G_1 by treating with hydroxyurea. Cells were irradiated 2 h after resynchronization. Caffeine was added 15 min prior to irradiation. Cells in the DNA synthetic period were assayed by autoradiographic analysis after a 15 min pulse with [^{3}H]thymidine: (*A*) cells not treated with caffeine, control (○), 400 rads (■), 800 rads (▼), 1,200 rads (▲); and (*B*) cells treated with 2 mM caffeine, control (○), 400 rads (■), 800 rads (▼), 1,200 rads (▲).

hydroxyurea completely inhibits the synthesis of only deoxyadenosine triphosphate in vivo in line CHO cells (Walters et al., 1973). Neither compound prevents RNA or protein synthesis.

The effects of caffeine on unirradiated control cells synchronized by the isoleucine-deficiency technique, then resynchronized with hydroxyurea, are consistent with the data presented in Figs. 1 and 2, showing that caffeine added to exponentially growing cells resulted in a reduced growth rate, continued division of *S* and G_2 cells, and eventual G_1 arrest. Although adding caffeine 2 h after synchronization (e.g., in G_1) allowed very few cells to traverse the cell cycle and divide ($\leq 10\%$), caffeine added 2 h after hydroxyurea removal allowed cells to complete *S* with a 1 h delay (Fig. 7) and to initiate division with a 1 h delay, although at a reduced rate (Fig. 6).

Since caffeine allows control cells to complete *S* and reduces *S* retention after

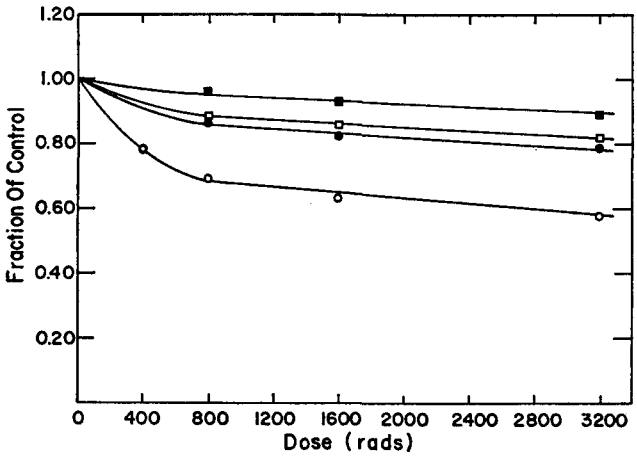

FIGURE 8 Effect of 2 mM caffeine on [^{3}H]thymidine incorporation into DNA of cells irradiated with increasingly larger doses. Cells were treated with caffeine 15 min prior to irradiation and were pulse-labeled for 1 h with [^{3}H]thymidine immediately after irradiation: (○) irradiated during exponential growth, no caffeine; (●) irradiated during exponential growth, 2 mM caffeine; (□) irradiated 2 h after hydroxyurea resynchronization of cells initially synchronized by growth in isoleucine-deficient medium, no caffeine; and (■) irradiated 2 h after hydroxyurea resynchronization of cells initially synchronized by growth in isoleucine-deficient medium, 2 mM caffeine.

irradiation, the effect of radiation on incorporation of [^{3}H]thymidine into DNA was examined. Exponentially growing cells were prelabeled with [^{14}C]thymidine (0.0075 μCi/ml) for 12 h, followed by an 18 h chase. Cells to be synchronized were prelabeled with [^{14}C]thymidine (0.0075 μCi/ml) for 18 h prior to resuspension of cells in isoleucine-deficient medium to effect synchronization. Cells were pulse-labeled for 1 h immediately after irradiation with 2.0 μCi/ml [^{3}H]thymidine. The rate of [^{3}H]-thymidine incorporation was determined from the ratio ^{3}H/^{14}C in DNA isolated from duplicate samples (3–5 $\times$ 10^6 cells/sample). The data in Fig. 8 show the dose-response curves of [^{3}H]thymidine incorporation into DNA of cells irradiated (1) during exponential growth or (2) 2 h after hydroxyurea (10^{-3} M) resynchronization of cells initially synchronized by the isoleucine-deficiency technique. Cells were treated with caffeine (2 mM) 15 min prior to irradiation. It is clear that, in both exponentially growing and synchronized cells, caffeine ameliorated the X-ray-induced reduction of [^{3}H]thymidine incorporation into DNA. This is consistent with the reduced *S* retention of caffeine-treated cells shown in Fig. 7. Furthermore, in those cultures not treated with caffeine, cells synchronized by the isoleucine-deficiency technique were less sensitive to X-ray-induced inhibition of [^{3}H]thymidine incorporation than were cells irradiated during exponential growth. Finally, caffeine treatment alone reduced [^{3}H]thymidine incorporation 20–25% in control cultures, in accord with the 1 h delay in exit from *S* seen in Fig. 7 (Cleaver, 1969). It should be noted that the reduction of [^{3}H]thymidine incorporation after caffeine treatment

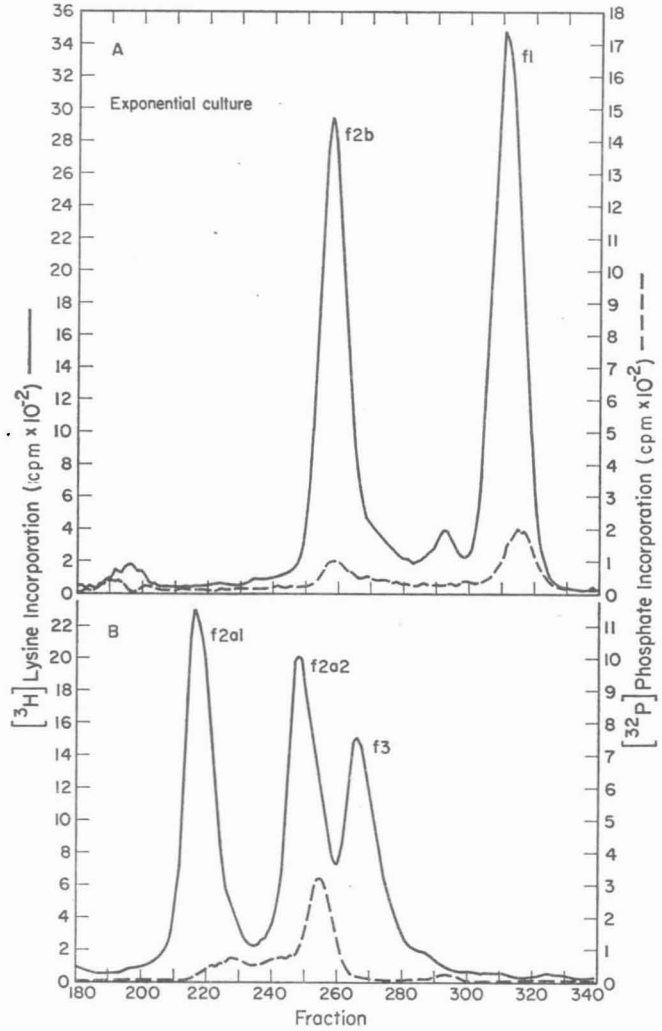

FIGURE 9 Preparative electrophoresis of histones isolated from an exponentially growing culture. Long-term [^{3}H]lysine incorporation is shown by the solid lines, and [^{32}P]phosphate incorporation after a 1-h pulse is shown by the dashed lines: (*A*) lysine-rich histone fractions f1 and f2b; and (*B*) arginine-rich histone fractions f2a1, f2a2, and f3.

of unirradiated cells has been normalized out of the data presented in Fig. 8. In addition, possible effects of caffeine on thymidine nucleotide acid-soluble pools have not been examined.

We have previously shown that, of the two major histones phosphorylated in interphase, the phosphorylation of lysine-rich histone f1 was radiosensitive while the phosphorylation of histone f2a2 was not (Gurley and Walters, 1971). Since histone phosphorylation has been implicated in the traverse of the cell cycle in

TABLE I

EFFECT OF 800 RADS X-IRRADIATION ON HISTONE PHOSPHORYLATION IN EXPONENTIAL CHINESE HAMSTER CELLS TREATED WITH CAFFEINE

Histone fraction	Caffeine	$^{32}PO_4$/[^{3}H]lysine		Effect of X-irradiation (percent of control)
		Control	X-ray	
	mM			
f1	0	0.0650	0.0356	54.8
	2	0.0583	0.0492	84.4
f2a2	0	0.1533	0.1717	112.0
	2	0.1350	0.1318	97.6

TABLE II

EFFECT OF 2 m*M* CAFFEINE ON RADIATION-INDUCED RESPONSES OF CHINESE HAMSTER CELLS ASSOCIATED WITH CELL-CYCLE TRAVERSE

Parameter	Source	Exposure	Effect of caffeine (caffeine-treated/untreated)
		rads	
Division delay	Exponential culture	400	$\frac{2.1‡}{4.8} = 0.44$
Division delay	Ile$^-$ synchronization* HU resynchronization	400–1,200	$\frac{0.0028§}{0.0048} = 0.58$
S retention	Ile$^-$ synchronization* HU resynchronization	400–1,200	$\frac{0.0008§}{0.0017} = 0.47$
Percent inhibition of [^{3}H] TdR incorporation*	Exponential culture	1,600	$\frac{14‖}{37} = 0.38$
Percent inhibition of [^{3}H] TdR incorporation*	Ile$^-$ synchronization* HU resynchronization	1,600	$\frac{7‖}{17} = 0.41$
Percent inhibition of f1 phosphorylation	Exponential culture	800	$\frac{16‖}{45} = 0.36$

* Abbreviations used: Ile$^-$, synchronization by the isoleucine-deficiency technique; HU hydroxyurea; [^{3}H]TdR, [methyl-^{3}H]thymidine.
‡ In hours.
§ In hours per rad.
‖ Percent.

preparation for division (Gurley et al., 1973 *a*, *b*; Tobey et al., 1973), the effect of caffeine on the X-ray-induced inhibition of f1 phosphorylation was examined in exponentially growing cultures. Histones were prelabeled by growing cells for 50 h in medium containing 50 μCi/liter [^{3}H]lysine, caffeine (2 mM) was added 15 min prior to irradiation with 800 rads, then cells were pulse-labeled immediately after

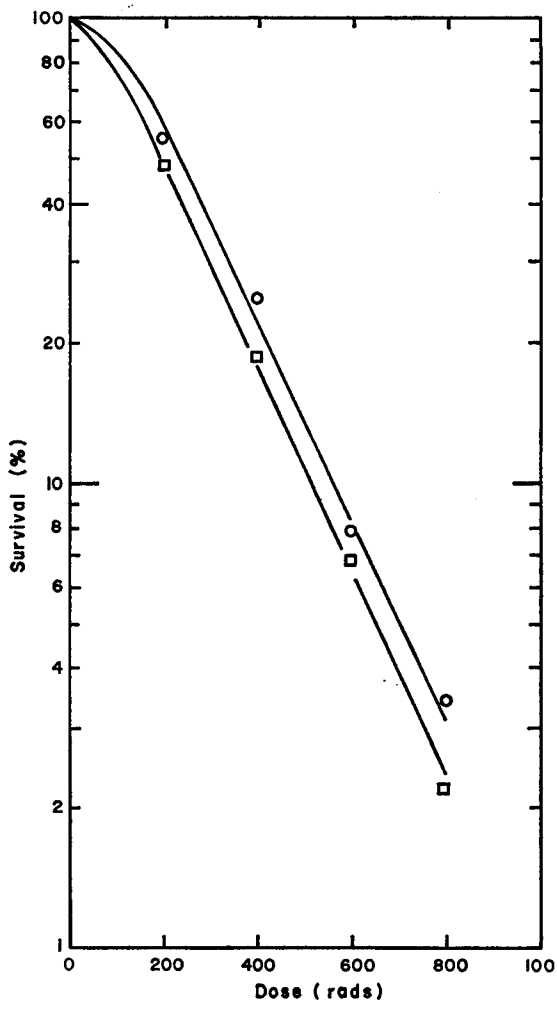

FIGURE 10 Survival, assayed by colony formation 6 days after plating, of cells irradiated during exponential growth: (○) no caffeine; and (□) 2 mM caffeine added 15 min prior to irradiation and removed 12 h after irradiation.

irradiation for 1 h with 20 μCi/ml [^{32}P]H_3PO_4. Two histone mixtures for each sample, prepared as described in Materials and Methods, were subjected to preparative polyacrylamide gel electrophoresis, and Fig. 9 shows a representative sample of the results obtained. The relative rate of phosphorylation of histones f1 and f2a2 was determined by the ^{32}P/^{3}H ratio in these fractions. The results are summarized in Table I. It is clear that treatment with caffeine reduced the X-ray-induced inhibition of f1 phosphorylation. As before, phosphorylation of histone f2a2 was not significantly affected by irradiation.

A summary of the effect of 2 mM caffeine on the radiation responses associated

with cell-cycle traverse reported above is given in Table II. It can be seen that the degree of amelioration by caffeine treatment is similar for all the parameters tested, perhaps surprisingly so in view of the number and diversity of parameters measured. That such amelioration does not translate into increased cell survival after irradiation is shown in Fig. 10. Exponentially growing cells were irradiated, and survival was assayed 6 days after plating the cells on plastic Petri dishes (Walters et al., 1970). Cells were treated 15 min prior to irradiation with 2 mM caffeine. Caffeine was removed and fresh medium added 12 h after irradiation. Caffeine treatment alone did not reduce plating efficiencies (~86% in controls), although irradiated cells were somewhat more sensitive after caffeine treatment. It appears that the small increased sensitivity was primarily due to reduction of the shoulder of the survival curve (Fig. 10).

DISCUSSION

The data presented above indicate that caffeine treatment of cells either just prior to or immediately after X-irradiation (in the case of division delay) was effective in reducing the radiation response of a number of different parameters including division delay of S and G_2 cells (Figs. 3 and 6), S retention (Fig. 7), [^{3}H]thymidine incorporation into DNA (Fig. 8), and phosphorylation of histone f1 (Table I). Furthermore, the magnitude of reduction was similar for the responses tested (Table II), although there is indeed some variability in the results reported. It is also interesting that caffeine amelioration was noted only in those parameters usually associated with cell-cycle traverse and preparation for division and not in responses leading to ultimate death of the cell as measured by survival (Fig. 10). The results suggest that radiation possibly may affect a mechanism involved in a very basic manner in cell-cycle traverse. This would imply that, of those parameters measured here, all would share, possibly through a regulatory mode, a common response mediated via a radiosensitive process. The question is posed then of what process might serve such a cellular function. Although we recognize that other interpretations of the data are certainly possible, we feel that cyclic AMP metabolism should be regarded as one of the alternatives when considering mechanisms of the role of caffeine in radiation-induced phenomena.

A number of observations are compatible with a role for cyclic AMP in caffeine amelioration of selected radiation responses. Cyclic AMP is thought to play a regulatory function in cell-cycle traverse and proliferation, and caffeine, an inhibitor of cyclic AMP phosphodiesterase, can potentiate cAMP-related phenomena (Whitfield et al., 1970 *a*, *b*; MacManus et al., 1971). It has been suggested that cyclic AMP is involved in the mitogenic action of Ca^{++} and various hormones in thymocytes (Whitfield et al., 1973), and interestingly many of the same agents are capable of reducing radiation-induced mitotic delay (Whitfield and Rixon, 1962; Whitfield et al., 1964; Perris et al., 1967; Whitfield et al., 1969). Although the mitogenic action

of Ca^{++} in thymocytes is thought to be related to a stimulation of entry of cells into the DNA synthetic phase, this apparently is not the case here. In fact, caffeine is inhibitory to the $G_1 \rightarrow S$ transition and leads to G_1 arrest (Figs. 1 and 2) when added in sufficient concentrations to exponentially growing cells. However, this is not incompatible for a possible role of cAMP since (1) dibutyryl-cyclic AMP and theophylline, another inhibitor of cAMP phosphodiesterase, can both induce G_1 arrest (Froehlich and Rachmeler, 1972; Rozengurt and Pardee, 1972), and (2) high cAMP levels apparently are associated with contact inhibition of cell growth (Burger et al., 1972; Otten et al., 1972). Thus, the role of cAMP in cell proliferation appears to be complex, but this might be expected in view of the ability of cAMP to activate protein kinases of mammalian cells, enzymes that phosphorylate a number of different proteins (Langan, 1969; Rasmussen, 1970; Reiman et al., 1971). Phosphokinase activation is also consistent with the caffeine amelioration of X-ray-induced inhibition of histone f1 phosphorylation reported here, since a cAMP-dependent phosphokinase specific for histone f1 substrate has been reported in Chinese hamster cells (Lake, 1973). However, it should also be noted that unirradiated cells treated with caffeine exhibit some inhibition of f1 phosphorylation (Table I), although this may be a result of the slowing of the cell growth rate upon addition of caffeine (Fig. 1).

The observation that cells synchronized by the isoleucine-deficiency technique were less sensitive to division delay, *S* retention, and [^{3}H]thymidine incorporation after irradiation is interesting, although we have no explanation for the results. Cells arrested in G_1 by growth in isoleucine-deficient medium are in a unique biochemical state in which DNA replication cannot be initiated but where synthesis of all major species of RNA and protein synthesis continue, although the rates of synthesis of individual RNA species are differentially reduced when compared with exponentially growing cells (Enger and Tobey, 1972). It is possible that cells in isoleucine-deficient medium can either accumulate a product whose synthesis is both radiosensitive and required for cell-cycle traverse or accumulate products that ameliorate radiation-induced responses. In any event, there does not appear to be a direct relationship between the reduced radiation effect achieved by isoleucine-deficient synchronization and caffeine treatment (Table II).

Again it should be stated that the above interpretations regarding a possible role of cAMP in radiation-induced responses are tentative. Preliminary experiments with other agents known to influence cAMP metabolism, including $N^6,0^{2'}$-dibutyryl cyclic 3′,5′-adenosine monophosphate and theophylline, have indicated that caffeine is much more effective in reducing radiation responses, although prostaglandin E_1 appears to reduce somewhat the division delay of cells irradiated during exponential growth (unpublished data). However, so little is known of the relative in vivo effectiveness of individual agents that, until more information becomes available, little more can be said of such comparisons. Further, caffeine effects other than inhibition of cAMP phosphodiesterase may be involved, since caffeine

can affect enzymes, binds to denatured DNA (Domon and Rauth, 1969; Ts'o and Lu, 1964), and may induce a state in which DNA is synthesized in smaller units (Lehmann, 1972). However, it would seem that such effects might result in caffeine potentiation of radiation damage (Cleaver and Thomas, 1969) as seen in survival of ultraviolet-irradiated (Rauth, 1967) or X-irradiated mammalian cells (Fig. 10). It is also possible that caffeine amelioration of certain radiation responses could result from the general slowing of growth rate of caffeine-treated cellls (Fig. 1), although division delay, for example, tends to be longer for cells with increased doubling times (Dewey et al., 1964). However, caffeine amelioration of radiation responses associated with cell-cycle traverse provides a tool for studying the mechanisms of radiation damage.

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