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The Effects of Arsenic, a Toxic Oil Shale Constituent, on Cell Proliferation and Histone Phosphorylation

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by

L. R. Gurley, R. A. Walters, J. H. Jett, and R. A. Tobey

ABSTRACT

Mobilization of arsenic to the environment during coal-based and oil shale-based energy production represents a potential health hazard of unknown magnitude. Because of the casual association between arsenic and cancer in man, studies were initiated to determine if subtoxic levels of arsenic have effects on cell proliferation and chromatin constituents. Cultured CHO cells were treated with both As(III) and As(V), as sodium arsenite ($NaAsO_2$) and sodium arsenate (Na_2HAsO_4), respectively, to assess their biological impact. Quantitatively, arsenite was ten times more toxic than arsenate, with 5 x 10^{-5} *M* arsenite causing irreversible inhibition of culture growth. At subtoxic concentrations, 10^{-5} *M* $NaAsO_2$ induced culture growth kinetics that were radiomimetic, that is, they caused a cell division delay period followed by a spontaneous recovery of cell division. During this division delay, histone H1 phosphorylation was reduced to 40% of control. It then recovered to control levels just before recovery of cell division. Histone H2A phosphorylation was stimulated in the middle of the division delay period, followed by a stimulation of H3 phosphorylation just before recovery of cell division. The DNA synthesis rate was also reduced during division delay, but it did not recover before resumption of cell division. The ATP pool levels were unaffected at these low arsenite concentrations. Flow microfluorometric analysis demonstrated that cell-cycle progression was inhibited throughout the entire cycle during the division delay period. Upon recovery of cell division, cells in S and G_2 regained the capacity to resume cycle traverse earlier than did cells in G_1. These results support the conclusions that interphase H1 phosphorylation is not tightly coupled to DNA synthesis, but is vitally involved in cell-cycle progression. The results indicate that sodium arsenite modulates those histone phosphorylations involved with cell proliferation.

INTRODUCTION

About 50 000 tons of arsenic (AsO_3) are produced each year as a by-product of copper smelting. This AsO_3 serves as the basic raw material for a variety of insecticides, herbicides, fungicides, algicides, sheep dips, wood preservatives, feed additives, and medicinals (Nelson 1977), which will eventually find

their way into the environment. In the past, fallout from the ore-smelting plants also introduced significant quantities of arsenic into the environment in localized areas, but this problem recently has been reduced by emission controls (Nelson 1977). Although smelting activity is an obvious source of industrially mobilized arsenic, it is not the principal source of arsenic emission in the United States. The 400 million tons per year of coal burned in power plants produce 3000 tons per year of arsenic (Nelson 1977). This emission undoubtedly will increase manyfold as the use of coal is increased as an alternate energy source for natural gas.

As the demand for petroleum production increases, the retorting of the great reserves of oil shale to produce petroleum is expected to mobilize a new source of arsenic to the environment (Fruchter et al. 1977; Poulson et al. 1975). Trace element analysis of oil shale indicates that this energy source is highly enriched in selenium, arsenic, and antimony. These elements have raw oil shale/earth crust average abundances (enrichment factors) of 86, 45, and 20, respectively (Fruchter et al. 1977). Analysis from the Laramie Energy Research Center 10-ton oil shale retort revealed the existence of significant levels of highly toxic arsine in the off-gas stream (Fruchter et al. 1977). Retort water has also been shown to be excessively high in arsenic, as has the crude oil product itself (Poulson et al. 1975). Because arsenic can be so easily mobilized to the environment during oil shale processing, there is concern about what damaging effects it might have on biological processes. Inorganic arsenicals are demonstrated to be skin carcinogens in man, and a casual association between arsenic and cancer of the respiratory, lymphatic, and hematopoietic systems is suspected (Wildenberg 1978).

It is clear that a balance of interdisciplinary studies involving many specialties, especially in the life sciences, will be necessary to answer questions concerning arsenic's biological impact (Brinckman et al. 1977). Such studies are necessary if a basis is to be established for the meaningful forecasting of arsenic-associated health problems. Likewise, they are vital for the development of environmental control strategies (Brinckman et al. 1977). As a result of these considerations, we have performed studies to determine what effects subtoxic exposure to arsenic have on cell metabolism, cell proliferation, and chromatin constituents and how these effects might contribute to the casual relationship between arsenic and cancer in man (Wildenberg 1978). We paid particular attention to the impact of arsenic on growth-related histone phosphorylation because arsenic and phosphorus are both class 5A elements exhibiting similar chemistry. Previous experiments at the Los Alamos Scientific Laboratory (LASL) have correlated histone phosphorylation with the cell division process (Gurley et al. 1977a, b, 1978b), suggesting an involvement of histone phosphorylation with cell-cycle progression (Gurley and Walters 1971; Gurley et al. 1973a,b, 1974a, b, 1975, 1978a, c: Hohmann et al. 1975, 1976). Thus, histone phosphorylation is a likely target for arsenic interference.

The results in this report show that sodium arsenite produces radiomimetic effects (Gurley and Walters 1971) on both culture growth kinetics and histone phosphorylation. These effects are manifested as a dose-dependent cell division delay followed by a spontaneous recovery of proliferation. During the division delay, histone H1 phosphorylation is reduced, but spontaneously recovers before recovery of cell division. These results support our previous suggestions, as well as the suggestions of others, that histone phosphorylation is vitally involved in cell-cycle progression. (Gurley and Walters 1971; Gurley et al 1973 a, b, 1974a, b, 1975, 1977a, b, 1978a, b, c; Hohmann et al. 1975, 1976; Ajiro et al. 1975; Balhorn et al. 1972, 1975; Bradbury et al. 1973a, b; Lake and Salzmann 1972; Lake et al. 1972; Marks et al. 1973; Ord and Stocken 1968; Stevely and Stocken 1968).

MATERIALS AND METHODS

Cell Cultures

Chinese hamster cells (line CHO) were grown in suspension culture in F-10 medium supplemented with 15% newborn calf serum, streptomycin, and penicillin (Tobey et al. 1966). Cultures were maintained free of *Mycoplasma* contamination as determined by periodic assays (Walters et al. 1974). The cell concentration of cultures was determined to a statistical precision of better than 1% by using an electronic particle counter (Tobey et al. 1967). The fraction of S-phase cells in a culture was determined by autoradiography following a 15-min pulse with [^{3}H]thymidine (Tobey and Ley 1970).

Isotope Labeling of Cells

Labeling of histones with ^3H was accomplished by exposing exponential cultures to 50 μCi of L-[^{3}H]lysine (specific activity 8 Ci/mmol) per liter of culture medium for 41 h before exposure to arsenic. The phosphorylation of histones with [^{32}P]phosphate was accomplished by pulse-labeling 500-mℓ cultures for 1 h with 10 mCi of $H_3{}^{32}PO_4$ (carrier-free) at various intervals during arsenic treatment.

Arsenic Treatment

Cultures growing exponentially with a generation time of ~17 h were treated with either sodium arsenite ($NaAsO_2$), or sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$), which had been dissolved in water and sterilized by filtration through a 0.22-μm Millipore Swinnex-13 filter. These arsenic solutions (100 times the final concentration in the cultures) were prepared fresh before addition to the cultures. The concentration used is given with each experiment.

Cell-Cycle Distribution Studies

The cell-cycle distribution of cells in a culture was determined by FMF (flow microfluorometry) analysis. Approximately 8 x 10^6 cells were removed from a culture and treated with the fluorescent DNA stain mithramycin as previously described by Crissman et al. (1977). The relative DNA content of each cell was then determined from the fluorescence of the DNA-bound dye by FMF as previously described by Kraemer et al. (1972). The fluorescence data from 20 000 to 30 000 cells were accumulated in the FMF multichannel pulse-height analyzer, producing a DNA histogram that was transferred to a magnetic disk for storage. The fractions of cells in G_1, S, and G_2 + M were derived from this DNA histogram with the program described by Dean and Jett (1974) using a PDP 11/40 computer (Digital Equipment Corporation, Maynard, Massachusetts). The data analysis procedure fits a broadened second-degree polynomial to describe S-phase cells and Gaussian distributions to describe the G_1 and G_2 + M populations (Dean and Jett 1974).

By extending the interpretation of this cell-cycle analysis result beyond that given by Dean and Jett (1974), the distribution of cells within four subcompartments of S phase was calculated as described below. The computer fit of the DNA histogram generates nine parameter values that mathematically describe a DNA distribution [(see Table I of Dean and Jett (1974)]. These values include the coefficients α, β, and γ of the polynomial P(X) used to fit the S-phase population [$P(X) = \alpha + \beta X + \gamma X^2$], and the values $\bar{X}_1$ and $\bar{X}_2$ representing the FMF modal channel numbers for G_1 and G_2 + M, respectively. The area under the curve P(X) between $\bar{X}_1$ and $\bar{X}_2$ is the number of cells in S phase as determined by the Dean and Jett method (1974). The number of cells in each quarter of S phase was obtained by integrating the polynomial P(X) over each quarter of the region bounded by $\bar{X}_1$ and $\bar{X}_2$. That is, the number of cells N_i in the i^{th} quarter of S phases is given by

$$N_i = \int_{XBC_i}^{XEC_i} P(X) \qquad (1)$$

or $N_i = \alpha (XEC_i - XBC_i) + 1/2\beta (XEC_i^2 - XBC_i^2) + 1/3\gamma (XEC_i^3 - XBC_i^3)$,

where XBC_i and XEC_i are the beginning and ending fractional FMF channel numbers of the i^{th} quarter of S phase.

Cell Survival Studies

Suspension cultures of CHO cells at ~140 000 cells/mℓ were treated with various concentrations of $NaAsO_2$. After a 24-h exposure to arsenite, 10-mℓ aliquots were removed from each culture. These aliquots were centrifuged and the cells resuspended in 10 mℓ of fresh growth medium (without arsenite). The cell concentration of this suspension was determined, dilutions were prepared, and known numbers of cells were plated into 60-mm plastic tissue culture dishes. The dishes were incubated for 7 days at 37°C, then rinsed with physiological saline, fixed with 70% ethanol, and stained with 1% crystal violet in methanol to determine colony formation (i.e., 50 or more cells). Cell survival was expressed (six replicate samples per point) as a percentage of the colonies formed in parallel untreated cultures.

DNA Synthesis Studies

The rate of DNA synthesis during arsenic treatment was measured from the incorporation of

[^{3}H]thymidine into DNA. At hourly intervals after arsenic addition, 60 mℓ were removed from the culture and incubated with 300 μC_i of [methyl-^{3}H]thymidine (227 mCi/mg) for 1 h. The cells were then divided into duplicate samples, poured over crushed frozen F-10 medium to stop incorporation of thymidine, and harvested by centrifugation. The cells were washed once with cold 0.25 *M* sucrose, extracted twice for 30 min with 10% trichloroacetic acid, and extracted twice for 15 min with cold 95% ethanol-2% potassium acetate. DNA in the cell pellet was then hydrolyzed in 1 *M* $HClO_4$ at 70°C, and subjected to the diphenylamine DNA assay as described by Friesen (1968). Incorporated [^{3}H]thymidine was measured by counting aliquots of the 1 *M* $HClO_4$ hydrolysate in liquid scintillation fluid in a Packard Tri-Carb spectrometer. The relative synthesis rate of DNA was then calculated as the cpm of [^{3}H]thymidine incorporated into DNA per unit of DNA expressed as the absorbance at 600nm produced by the diphenylamine reaction.

Histone Isolation

Histone fractions were prepared from 1.2 x 10^8 cells by the first method of Johns (1964), as previously described for cultured cells by Gurley and Hardin (1968), with two modifications: (a) 0.05 *M* sodium bisulfite was present in the 0.15 *M* NaCl solutions used to disrupt the cells and wash the chromatin and in the 5% perchloric acid used to extract H1 (Panyim and Chalkley 1969); and (b) 0.14 *M* 2-mercaptoethanol was present in the ethanol solutions used for the extraction and recovery of arginine-rich histones to prevent the dimerization of H3 (Smith et al. 1970). This method separates histones into three fractions: (1) the very lysine-rich histone H1; (2) a histone complex containing H2A, H3, and H4; and (3) the lysine-rich histone H2B. This procedure was performed to separate the H2B from the H2A and H3 before preparative electrophoresis because H2A, H2B, and H3 are not adequately separated by that procedure (Gurley and Walters 1971). The histones were dissolved in water and lyophilized to dryness before electrophoresis.

Histone Fractionation and Purification by Preparative Electrophoresis

Histone H1 and the complex of H2A, H3, and H4 from 1.2 x 10^8 cells labeled with [^{3}H]lysine and $^{32}PO_4$ were subjected separately to preparative polyacrylamide gel electrophoresis as previously described by Gurley and Walters (1971), using the urea-acetic acid method of Panyim and Chalkley (1969), adapted for use with a Canalco Prep-Disc apparatus (Canalco, Inc., Rockville, Maryland). Purified histones were removed from the bottom of the gel by a cross flow of acetic acid buffer and collected in 2-mℓ fractions for liquid scintillation counting. Individual histone fractions were located by the patterns of incorporated [^{3}H]lysine, and incorporated $^{32}PO_4$ was determined from the ^{32}P associated with each fraction. The relative phosphate incorporation into each histone was determined from the ^{32}P/^{3}H ratio in each peak. Histone H2B was not examined in this study since it is not phosphorylated in CHO cells (Gurley and Walters 1973).

Determination of Cellular ATP

Approximately 10^8 cells were removed from 500-mℓ aliquots of an arsenite-treated culture by centrifugation and transferred to a small tube with 10 mℓ of saline-GM [the balanced salt and glucose "solution-G" of Puck et al. (1958) minus $MgSO_4$ and $CaCl_2$]. The cell pellet was extracted twice with 0.5 mℓ of 6% TCA (trichloroacetic acid) for 15 min, recovering the cells each time by centrifugation. The ATP concentration in the pooled TCA extracts was measured enzymatically using the reactions described by Bücher(1947), and modified by Adams (1963). Enzymes and reagents were supplied by Sigma Chemical Co., St. Louis, Missouri. This procedure uses the reaction between 3-phosphoglycerate and ATP catalyzed by phosphoglycerate kinase to generate 1,3-diphosphoglycerate, which is then dephosphorylated by the oxidation of NADH to NAD. The amount of ATP in the TCA extract was determined from the decrease in the NADH absorbance of the solution at 340 nm (Sigma Chemical Co. 1974) using a Gilford Model 240 Spectrophotometer.

RESULTS

To determine what differences the oxidation state of arsenic might have on cell proliferation, the effect of inorganic arsenic(III) and arsenic(V) on cell culture growth was examined (Fig. 1). As(III) and As(V) were administered to cells as sodium arsenite

($NaAsO_2$) and sodium arsenate (Na_2HAsO_4), respectively. Quantitatively, arsenite was much more toxic than arsenate: 5×10^{-5} *M* $NaAsO_2$ caused immediate irreversible inhibition of culture growth (Fig. 1e), whereas 5×10^{-4} *M* Na_2HAsO_4 was necessary for the same result (Fig. 1). At lower arsenic concentrations, cells exhibited qualitatively different growth kinetic responses to these two arsenic salts. At 10^{-4} *M* arsenate, culture growth rate was simply reduced (Fig. 1j). However, at 10^{-5} *M* arsenite, growth kinetics were radiomimetic in character, exhibiting first a cell division delay period, and then a spontaneous recovery of cell division (Fig. 1d).

The arsenite-induced division delay period was examined in detail at a variety of $NaAsO_2$ concentrations (Fig. 2). It was found that cell division stopped after 1 h of exposure to arsenite and the duration of the division delay period was

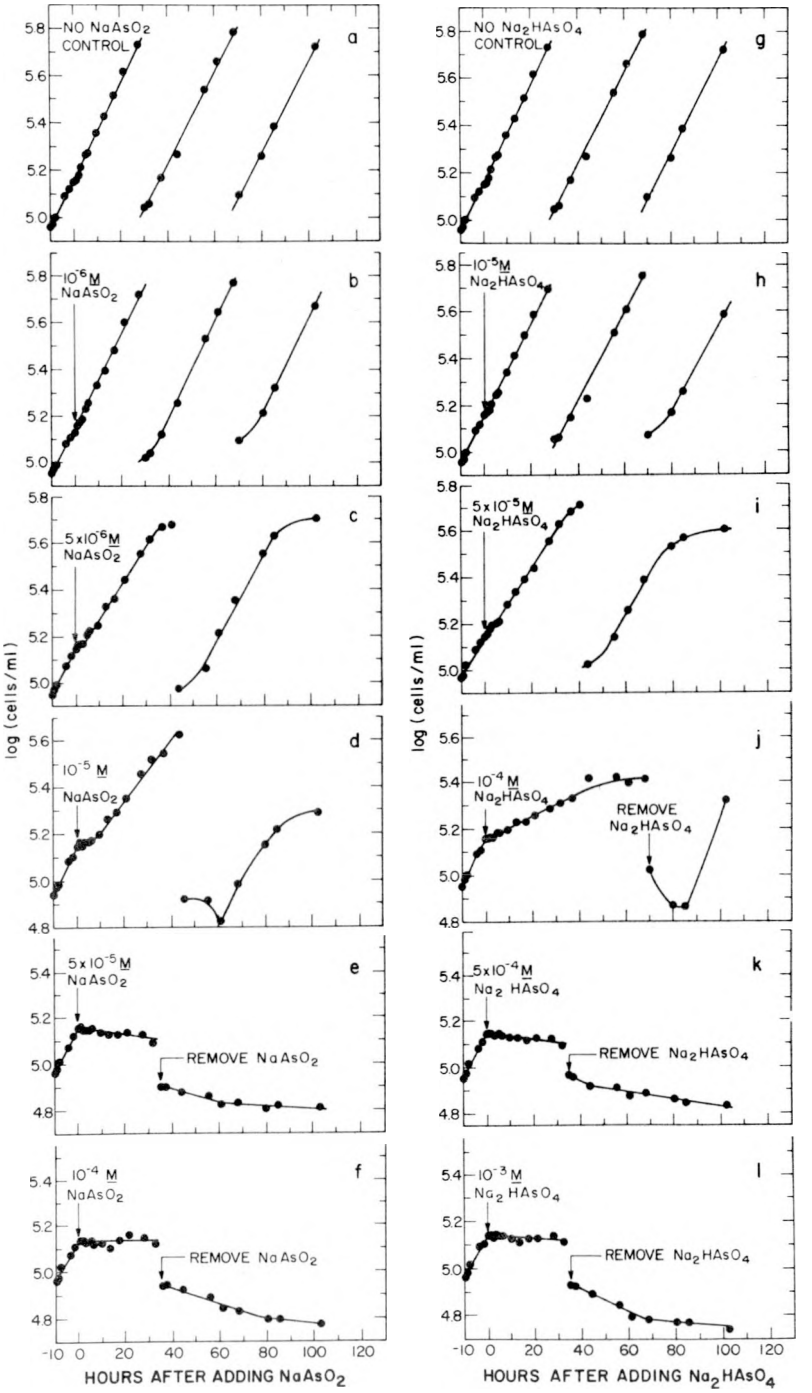

Fig. 1.
Effects of arsenic on CHO culture growth: a-f, various concentrations of As(III) as sodium arsenite ($NaAsO_2$); g-l, various concentrations of As(V) as sodium arsenate (Na_2HAsO_4). Cultures were diluted periodically with arsenic-containing medium except where otherwise indicated in e, f, j, k, and l.

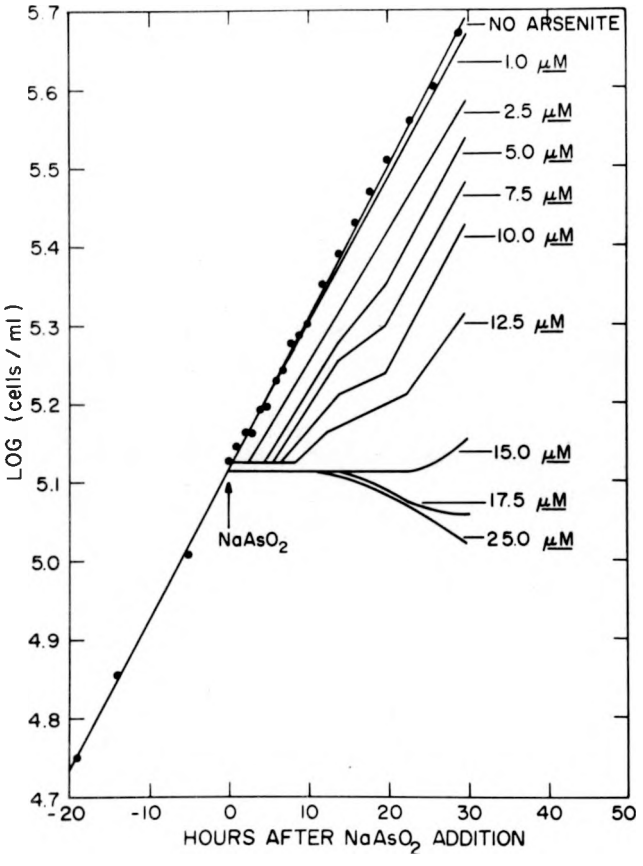

Fig. 2.
Dependence of the cell division delay period on sodium arsenite ($NaAsO_2$) concentration.

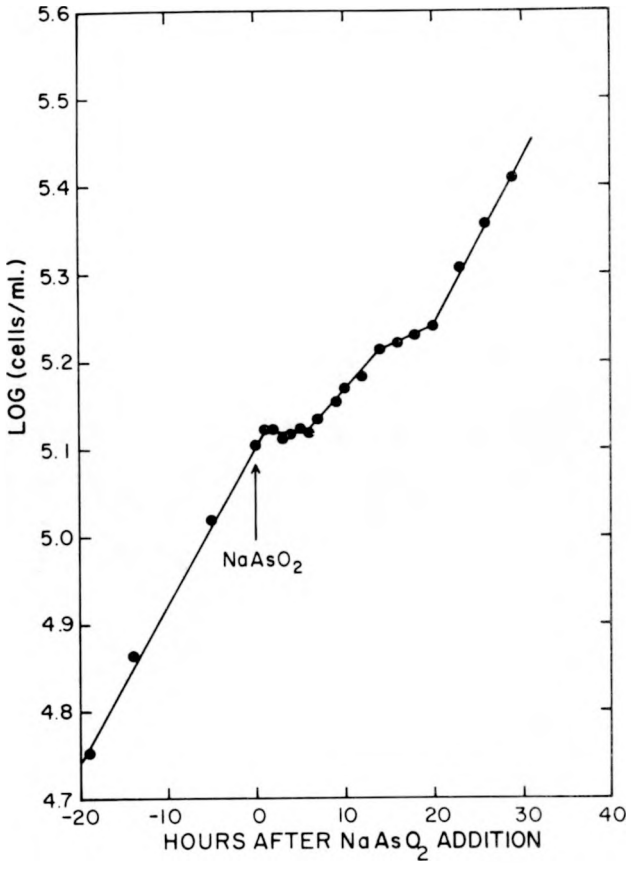

Fig. 3.
Detailed analysis of CHO division delay and recovery in the presence of continuous exposure to 10^{-5} M sodium arsenite ($NaAsO_2$).

concentration-dependent, ranging from 2 h at 2.5 x 10^{-6} *M* $NaAsO_2$ to 24 h at 1.5 x 10^{-5} *M* $NaAsO_2$. At concentrations of 1.5 x 10^{-5} *M* or greater, cell division was inhibited immediately upon addition of arsenite. At 1 x 10^{-5} *M* $NaAsO_2$, cell division stopped after 1 h of exposure and remained inhibited through the sixth hour of exposure (Fig. 3). The recovery of cell proliferation after 6 h occurred spontaneously without removal of the $NaAsO_2$. Such culture growth behavior is similar to that observed when CHO cells are treated with low doses of x-irradiation (Walters and Petersen 1968a). This radiomimetic response suggests that CHO cells can (to some extent) repair the arsenite damage to the cell proliferation process, or detoxify the arsenite itself. A secondary reduction in cell division rate was observed commencing at 14 h after arsenite addition (Fig. 3). Normal exponential growth resumed at 20 h. The reasons for this perturbation in the culture growth will be presented later in this report.

In previous studies with x-irradiated cells, we observed that histone H1 phosphorylation was inhibited during the division delay period, but recovered before recovery of cell division (Gurley and Walters 1971). To determine if histone phosphorylation behaves in a similar manner during the arsenite-induced division delay, [^{3}H]lysine-prelabeled exponential CHO cells were treated with 10^{-5} *M* $NaAsO_2$. At hourly intervals during division delay, cells were pulse-labeled for 1 h with $H_3{}^{32}PO_4$. The ^{32}P-labeled histones were isolated and fractionated by preparative electrophoresis as previously described (Gurley and Walters 1971). The electropherograms are not shown since they are similar to those presented in previous work (Gurley and Walters 1971; Gurley et al. 1973a, b, 1974a, b). The relative phosphate incorporation into the four phosphorylated histones during cell division arrest is presented in Fig. 4 as the ^{32}P/^{3}H ratios of the

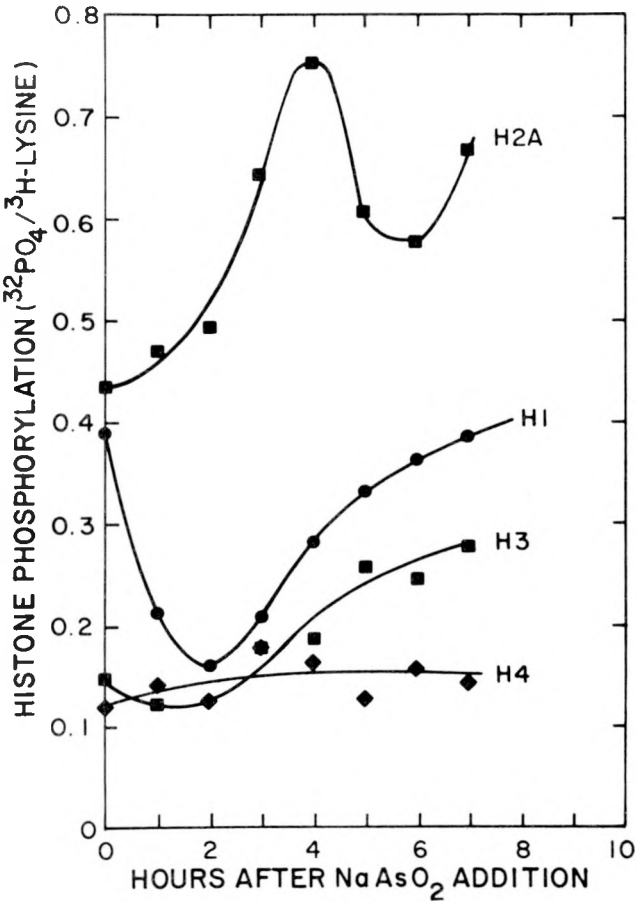

Fig. 4.
Effect of 10^{-5} M sodium arsenite ($NaAsO_2$) on histone phosphorylation during arsenite-induced cell division delay.

electrophoretic peaks. The phosphorylation of H1 was inhibited immediately by the arsenite treatment reaching a minimum value 2 h after exposure. Thereafter, the H1 phosphorylation recovered, reaching the control level by 7 h (Fig. 4), the time of resumption of cell division (Fig. 3). Thus, the response of H1 phosphorylation to arsenite treatment is also radiomimetic (Gurley and Walters 1971). The response of histone H2A phosphorylation to arsenite treatment was stimulatory, exhibiting a peak at 4 h after exposure. Histone H3 phosphorylation remained at control levels until the latter half of the division delay period; then it increased. There was essentially no change in the limited phosphoyrlation of histone H4 during cell division arrest.

The diverging responses of H1 and H2A phosphorylation to arsenite treatment indicate it is unlikely that the perturbations of histone phosphorylation result from changes in cellular ATP pools. However, because arsenite is a known respiratory inhibitor (Mahler and Cordes 1971), the ATP pool during arsenite treatment was measured (Fig. 5). No significant changes in cellular ATP levels were observed following 10^{-5} *M* $NaAsO_2$ addition, which could account for the large fluctuations in H1 and H2A phosphorylations observed in Fig. 4. The small increase in ATP levels between 1 and 2 h after arsenite addition (Fig. 5) may reflect a sparing of ATP as a result of a decrease in culture activity reflecting the cessation of cell division that occurs after the first hour of arsenite exposure (Fig. 3).

Following x-irradiation, CHO cells continue to traverse interphase until they reach a point late in G_2 (Walters and Peterson 1968a), approximately 0.9 h before cell division (Walters and Peterson 1968b). At this point (just before prophase) cell-cycle traverse is arrested causing division delay. As a

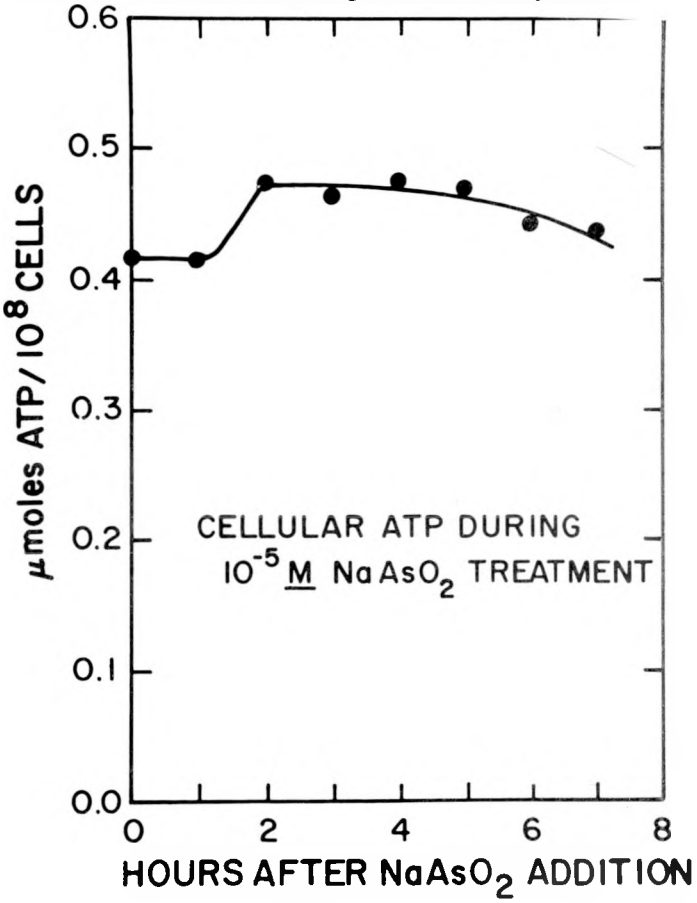

Fig. 5.
Effect of 10^{-5} M sodium arsenite ($NaAsO_2$) on cellular ATP pools during arsenite-induced cell division delay.

result, irradiated cultures accumulate large populations of cells in G_2 during division delay, which has been demonstrated by FMF analysis (Enger et al. 1976). To determine if arsenite caused cell-cycle arrest at a specific point in the cell cycle similar to x-irradiation, an FMF analysis was performed on CHO cultures during arsenite-induced division delay (Fig. 6). From these data the cell-cycle distribution in arsenite-treated cultures was determined (Fig. 7). In contrast to irradiated cells, 10^{-5} *M* arsenite appears to arrest cells in all phases of the cell cycle. Very little enrichment of cells in G_2 was observed during division delay (Figs. 6 and 7), indicating that arsenite is not radiomimetic with respect to this parameter.

[^{3}H]Thymidine autoradiography of 10^{-5} *M*-arsenite-treated cells confirmed that there was no significant enrichment in the fraction of S-phase cells in the culture during the 6-h division delay (Fig. 8A). This is in contrast to x-ray treated cultures that are enriched in S-phase cells during division delay (Enger et al. 1976) as a result of continued cell-cycle traverse of G_1 cells into S phase. The observations that (1) S-phase cells continue to incorporate [^{3}H]thymidine during arsenite-induced division delay (Fig. 8A), but that (2) the sizes of the G_1 and S compartments remain unchanged at a time when cell division is not occurring (Fig. 7), suggest that the rate of DNA synthesis must be reduced greatly by arsenite, but not completely stopped. The reduction in rate of DNA synthesis was confirmed by measuring the rate of incorporation of [^{3}H]thymidine into DNA following 10^{-5} *M* $NaAsO_2$ treatment (Fig. 8B). It was observed that DNA synthesis was reduced to 39% of the control rate by arsenite treatment. Unlike histone H1 phosphorylation, which recovered before resumption of cell proliferation (Fig. 4), DNA synthesis remained

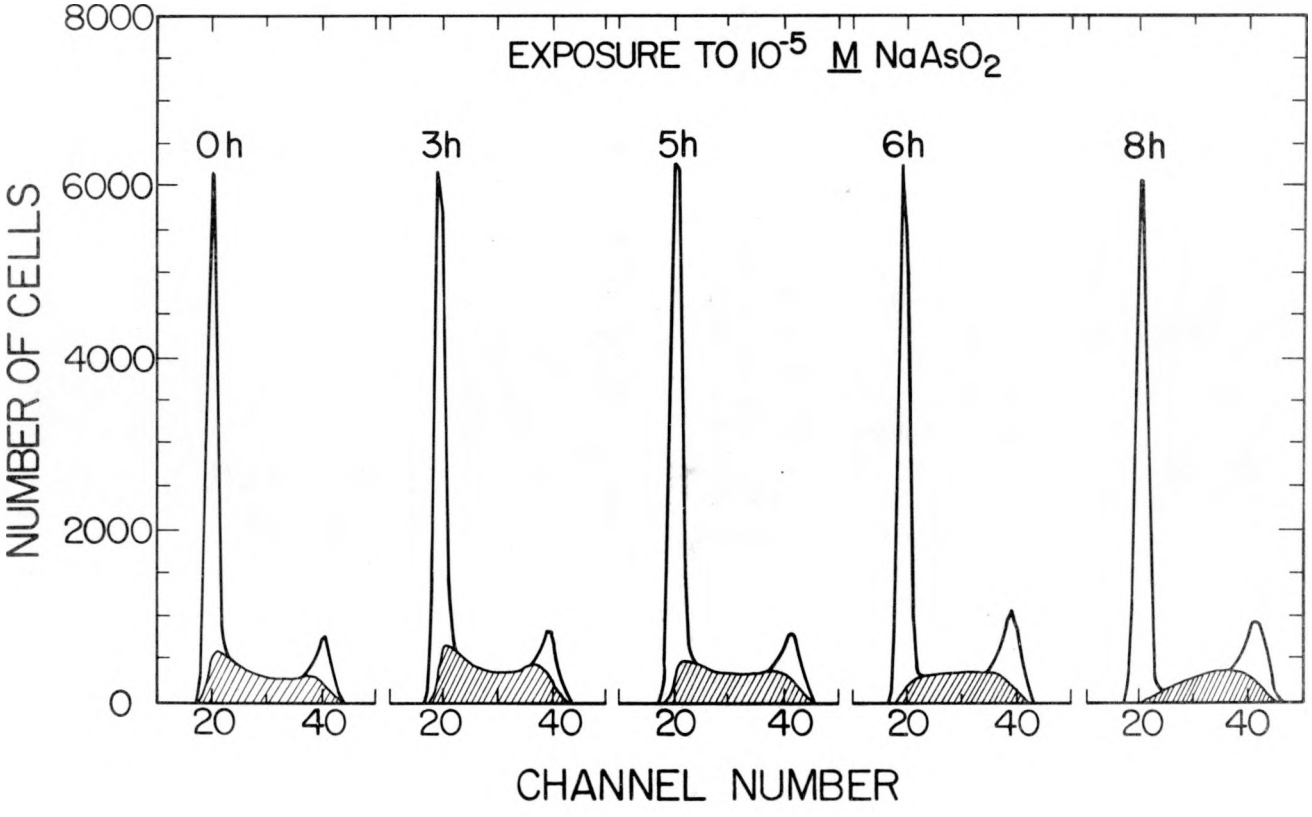

Fig. 6.
FMF analysis of CHO cultures during cell division delay induced by 10^{-5} M sodium arsenite ($NaAsO_2$). Exposure to arsenite is continuous for the times indicated. Cell division resumed by 8 h. The shaded area represents the population of cells in S phase as determined by computer analysis described by Dean and Jett (1974).

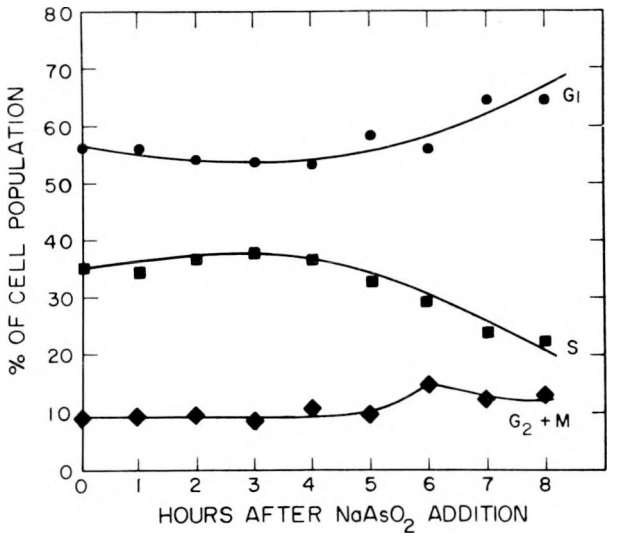

Fig. 7.
Cell-cycle distribution of CHO cells during the cell division delay period induced by 10^{-5} M sodium arsenite ($NaAsO_2$). The percent of cells in each cell cycle phase was computed from DNA histograms (Fig. 6) as described by Dean and Jett (1974).

reduced throughout the division delay period (Fig. 8B). In this respect, the effect of arsenite on DNA synthesis is not radiomimetic. The [^{3}H]thymidine incorporation into DNA in x-irradiated cells is observed to recover to control rates before resumption of cell proliferation (Gurley and Walters 1971).

From the FMF analysis of arsenite-treated cultures it appeared that there was a redistribution of cells in S phase after 6 h of treatment with arsenite (Fig. 6). To examine this possibility in greater detail, S phase was divided into quarters and the number of cells in each quarter was quantified (Fig. 9). It was found that the number of cells in each subcompartment of S phase remained constant throughout the first 4-5 h of division delay, but thereafter, the number of cells in the first quarter of S was reduced to nearly zero, whereas the number in the other quarters remained constant up to 8 h after 10^{-5} *M* $NaAsO_2$ treatment (Fig. 9). It is concluded that, upon resumption of cell proliferation, those cells in S and G_2 resume cell-cycle traverse, but cells in G_1 delay entry into S phase for a while longer, resulting in an emptying of early S (Fig. 9).

These FMF data explain the secondary reduction in cell division observed between 14 and 20 h after

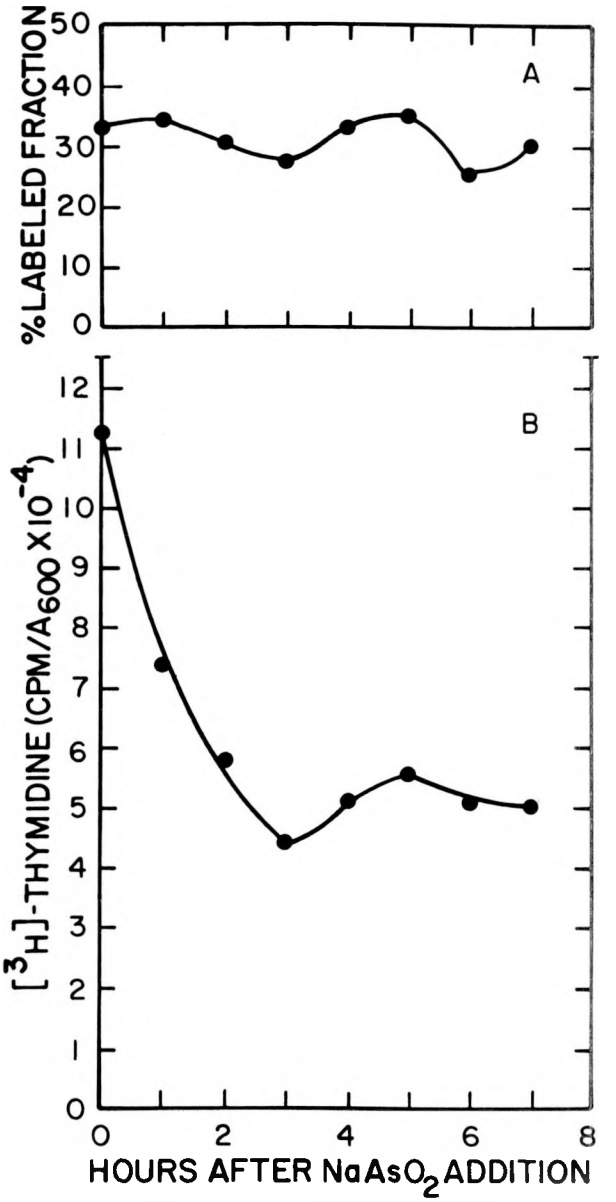

Fig. 8.
DNA synthesis during cell division delay induced by 10^{-5} M sodium arsenite ($NaAsO_2$). (A) Autoradiography of exponential CHO cells pulse-labeled for 15 min with [^{3}H]thymidine every hour after addition of arsenite. (B) DNA synthesis rate measured as the incorporation of [^{3}H]thymidine into DNA isolated after a 1-h exposure of cells to [^{3}H]thymidine.

10^{-5} *M* arsenite treatment (Fig. 3). After 6 h of exposure to arsenite, the S and G_2 compartments resume cell-cycle traverse, resulting in proliferation from 6 to 14 h. However, because the entry of G_1 cells into S phase remained inhibited for a time after 6 h

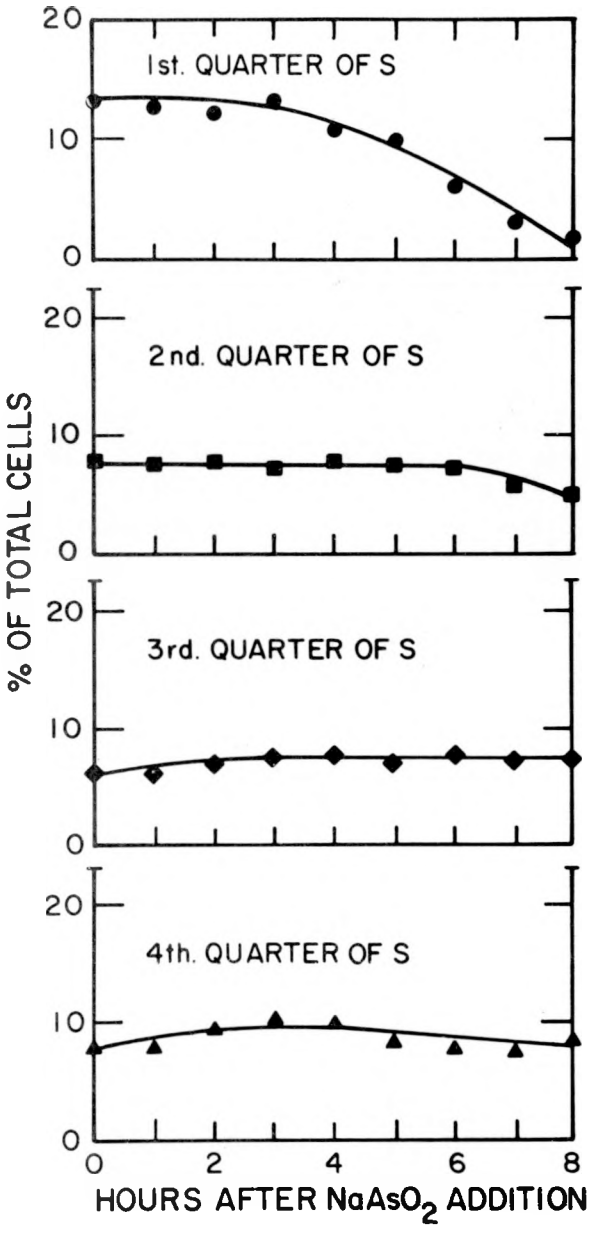

Fig. 9.
Distribution of cells within four subcompartments of S phase during the cell division delay period induced by 10^{-5} M sodium arsenite ($NaAsO_2$). The number of cells in each quarter of S is calculated as a percentage of the total cells in all phases of the cell cycle.

(Fig. 9), there was a significant reduction in the rate of cell division commencing at 14 h (Fig. 3), a time when the cells originally arrested in S and G_2 have already passed through mitosis. Since normal exponential growth resumes after 20 h of exposure (Fig. 3), it is concluded that the inhibition of entry of G_1 cells into S phase is not permanent, but rather, G_1 cells resume cell-cycle traverse about 6 h after the resumption of traverse by those cells arrested in S and G_2.

The spontaneous recovery of cell proliferation from arsenite-induced cell division arrest (Fig. 3) indicated that the cells could temporarily adjust their metabolism to accommodate 10^{-5} *M* arsenite. To determine whether any long-term effects on proliferation had occurred, cells treated with various concentrations of $NaAsO_2$ for 24 h were removed from the arsenite-containing medium and plated in dishes to determine their survival (Fig. 10). It was found that 89% of the cells survived following treatment for 24 h with 10^{-5} *M* arsenite. Some information concerning the nonsurviving 11% can be deduced from the data gathered in this study. FMF data (Fig. 7) indicated that ~44% of the untreated culture existed in S, G_2, and M before arsenite treatment. However, only ~33% of the treated cells divided between the times of resumption of S and G_2 traverse at 6 h and the resumption of normal exponential growth at 20 h (Fig. 3). The fraction that failed to divide (~11%) is equivalent to the fraction

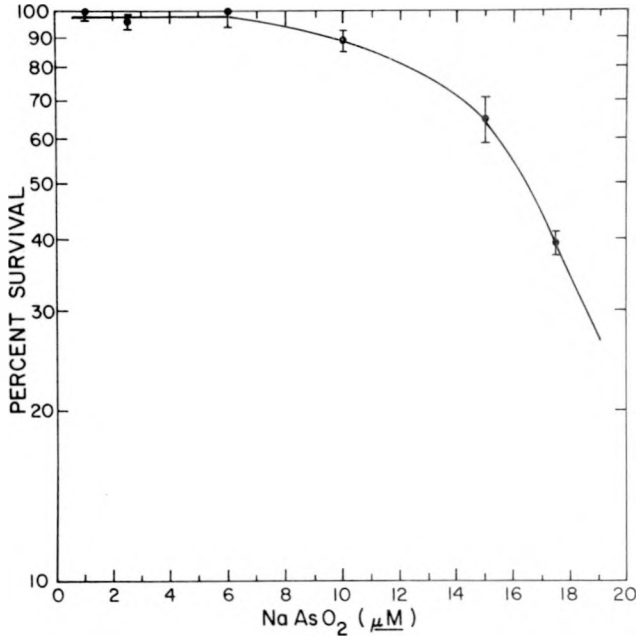

Fig. 10.
Survival of CHO cells after treatment with various concentrations of sodium arsenite ($NaAsO_2$) for 24 h.

of cells that failed to survive, suggesting that the bulk of the arsenite-killed cells were those cells in S and G_2 at the time the arsenite was added.

DISCUSSION

Arsenic is a labile element that can undergo valence state changes over a range of redox conditions likely to be found in soils and biological systems (Bohn 1976). Thus, arsenic mobilized by industrial processes is found both as As(III) and As(V) in the environment. For example, in aerated soils, As(V) predominates as arsenate, whereas in flooded soils, in the hypolimnion region of stratified lakes and seas, As(III) exists as arsenite in significant quantities (Ferguson and Gavis 1972; Walsh et al. 1977; Woolson 1977). Obviously, studying the effects of As(V) on cell systems is important because much environmental arsenic is eventually oxidized to that state. However, the effects of As(III) may be even more important for initial studies for the following reasons. (1) We found As(III) to be ten times more toxic than As(V) in CHO cells, an observation consistent with other biological systems. (2) The subtoxic effects of arsenite on cell proliferation were spontaneously reversible, which may make As(III) more dangerous than As(V) because any genetic damage that may have occurred as a result of exposure to arsenite could be transmitted to future generations. (3) The cell division delay and recovery induced by arsenite treatment was similar to that which we had previously encountered in x-irradiation studies (Gurley and Walters 1971; Walters and Petersen 1968a, b), thus offering some direction in our search for metabolic perturbations. (4) Most airborne arsenic particulates consist of inorganic As(III) compounds (Barman 1977). (5) Arsenic enters the food chain predominantly through the methylating activity of anerobic bacteria (McBride and Wolfe 1971) operating in sediments where As(III) concentration is highest. (6) Arsenate is reduced to arsenite by bacteria before the methylations are performed (Wood 1974); thus, arsenite may be the natural metabolically active form of inorganic arsenic. For these reasons we have restricted this initial investigation to the effects of sodium arsenite.

Sodium arsenite produced a culture growth kinetic response in CHO cells not typical for most toxic substances. Rather, the cell division delay and subsequent recovery of proliferation following arsenite treatment was similar to the response of cells to x-irradiation (Walters and Petersen 1968a, b). This prompted us to investigate histone phosphorylation during arsenite-induced division delay to see if H1 phosphorylation also behaved in a radiomimetic manner (Gurley and Walters 1971). We found that it did, with the phosphorylation of H1 being reduced immediately following exposure to arsenite, and later recovering to control levels spontaneously before cell proliferation was reinitiated. This reduction of H1 phosphorylation associated with cell division arrest and recovery of H1 phosphorylation just before resumption of cell division supports previous suggestions (Ord and Stocken 1968; Stevely and Stocken 1968; Gurley and Walters 1971; Balhorn et al. 1972) that a functional relationship exists between H1 phosphorylation and the cell's ability to proliferate. It is important that we now have a chemical probe for producing this phenomenon. With x ray we had no means of tracing the course of action of that physical probe. By using arsenic isotopes, we should now be able to trace the perturbing agent's activity and provide some insight into the specific sites and processes that are affected.

Previous observations at LASL have correlated histone phosphorylations with condensed states of chromatin (Gurley et al. 1974b, 1977a, b, 1978a, b, c; Hildebrand et al. 1977, 1978; Hohmann et al. 1976). Interphase H1 phosphorylation was correlated specifically with chromatin reorganization (Hildebrand et al. 1977, 1978), presumed to be associated with the interphase chromatin condensing cycle (Gurley et al. 1977a, b, 1978b) suggested by Mazia (1963). No chromatin structural measurements were made in this initial arsenic study; however, some interesting structural observations were reported recently by Vossen et al. (1977) that are relevant to our observations. Using *Drosophila* salivary glands, Vossen et al. (1977) found that (a) 7.5×10^{-5} *M* sodium arsenite-induced chromosome puff formation in 2 h and that then (b) these puffs spontaneously regressed 4-8 h later while still in the presence of the arsenite. The time course of this chromosome structural response to arsenite is essentially the same as that which we observed for the reduction and recovery of H1 phosphorylation. Assuming that H1 phosphorylation responds to arsenite the same way in both salivary glands and CHO cells, we would correlate the reduction of H1

phosphorylation with the more extended chromosome puffed structures, and the rephosphorylation of H1 with the regression of the puffs to the condensed chromosome state. Thus, the arsenite observations of Vossen et al. (1977) and those of this report are consistent with our general experience (for review, see Gurley et al. 1978b) that dephosphorylated H1 is associated with a more extended chromatin structural state, whereas phosphorylated H1 is associated with a more condensed chromatin structure. These observations suggest that arsenite may be a good agent to use to manipulate chromatin structural changes in the laboratory. If chromatin structural changes in response to arsenite can be measured in CHO cells, it will be possible to investigate directly the association of histone phosphorylation with chromatin condensation.

Following the arsenite-induced inhibition of H1 phosphorylation and the onset of division delay, histone H2A phosphorylation increased, reaching a peak of activity 4 h after exposure. This stimulation was not correlated with a change in DNA synthesis rate, nor with any change in cellular ATP concentration. There was a small increase in the number of cells in the third and fourth quarters of S phase; and DNA synthesis, although greatly reduced at this time, was not completely stopped. We have previously measured elevated H2A phosphorylation in synchronized CHO cultures rich in G_2 and late S-phase cells (Gurley et al. 1973b). Thus, it is suspected that the increase in H2A phosphorylation in the middle of the division delay period results from the slow movement of cells through late S and G_2 in the absence of cell division. We have recently correlated H2A phosphorylation with the amount of heterochromatin in a cell (Gurley et al. 1978c). Since heterochromatin is late-replicating, the increase in H2A phosphorylation may signal the return of heterochromatin synthesis as the first step in resumption of cell-cycle traverse.

Following the increase in H2A phosphorylation, there was an increase in H3 phosphorylation. We have previously shown that phosphorylated H3 does not normally appear in CHO until prophase (Gurley et al. 1978a). The appearance of elevated H3 phosphorylation at the end of the division delay period (5-7 h after arsenite addition) suggests that cells are slowly entering the early stages of mitosis at this time.

Although the inhibition of DNA synthesis, H1 phosphorylation, and cell division are all radiomimetic in response to arsenite treatment, upon recovery, only H1 phosphorylation and cell division are radiomimetic; the recovery of DNA synthesis is not. H1 phosphorylation recovers before cell proliferation begins, whereas the DNA synthesis rate remains reduced. This observation is consistent with our earlier arguments that H1 phosphorylation is not necessarily coupled to DNA synthesis (Gurley et al. 1974a, b); rather, it appears to be coupled more directly to cell-cycle traverse.

Another nonradiomimetic response to arsenite is the inhibition of the entry of G_1 cells into S phase. It appears that arsenite inhibits a G_1 process obligatory for cell-cycle traverse. The recovery of H1 phosphorylation to normal levels is complete by 7 h after adding arsenite, whereas at least another 6 h is required before G_1 cells recover the ability to resume cell-cycle traverse. This suggests that the inhibition of entry into S phase is not the result of an effect on H1 phosphorylation. Similarly, the DNA synthesis rate, while reduced at the time of resumption of cell division, continued at a sufficient rate to nearly empty the first quarter of S phase. Therefore, it is also unlikely that DNA synthesis is the obligatory process inhibited. Thus, these results eliminate two possible processes frequently suspected of controlling entry into S phase as the mechanism involved in the arsenite-induced G_1 traverse delay. The underlying mechanisms involved in controlling the initiation of S phase warrant further investigation, and it appears that arsenite can serve as a probe for studying these processes.

In conclusion, the results of this study indicate that sodium arsenite perturbs histone phosphorylation. Accordingly, consideration should be given to the possibility that the carcinogenic properties attributed to arsenite may involve its effects on the histone phosphorylation processes that are coupled to cell proliferation.

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