Concentration-Dependent Effects of Sodium Butyrate in Chinese Hamster Cells: Cell-Cycle Progression, Inner-Histone Acetylation, Histone H1 Dephosphorylation, and Induction of an H1-like Protein†

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ABSTRACT: This paper presents the first unified quantitative study of the effects of butyrate concentration upon (1) cell-cycle progression, (2) modification of all inner histones, (3) dephosphorylation of histone H1, and (4) enhancement of an H1-like protein (BEP) in CHO cells. Flow cytometric analysis shows that exposure to butyrate enriches CHO cultures in G_1 cells and, at sufficient butyrate concentration, leads to G_1 arrest. Additionally, butyrate alters the rate of cell-cycle progression through G_2/M and through S. Two-dimensional polyacrylamide electrophoresis and radiolabeling in butyrate-treated cultures indicate the presence of at least one site of internal acetylation in histone H2A, four sites of internal acetylation in histone H2B, five sites of internal acetylation in histone H3, and four sites of internal acetylation in histone H4. Histone H2A is also appreciably phosphorylated, so that it is acetylated and phosphorylated at a total of up to three sites. The distribution of modified species for all the inner (core) histones has been quantified from two-dimensional gels by using three different methods of analysis: (1) direct densitometry of excised portions of the gel, (2) scintillation spectrometry of ^{3}H-labeled histones, and (3) microdensitometry of photographic negatives. At 15 mM butyrate, 26% of H2B is acetylated at three to four sites, 37% of H3 is acetylated at three to five sites, and 50% of H4 is acetylated at three to four sites. Histone H1 is dephosphorylated as a function of butyrate concentration, and the dephosphorylation can be correlated with an increased proportion of G_1 cells in culture. There is also a significant increase in the cellular content of two other proteins when cells are exposed to butyrate. The increase in one of these, BEP, has been quantified as a function of butyrate concentration after 24 h of exposure to butyrate. BEP appears to be related to histone $H1_0$ [Panyim, S., & Chalkley, R. (1969) *Biochem. Biophys. Res. Commun. 37*, 1042] and to an induced protein IP_{25} [Keppel, F., Allet, B., & Eisen, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 653]. The other protein (UP), which has a molecular weight of ~15 000, has not been identified. Butyrate induces a twofold increase in the cellular content of UP and a change in the distribution of UP molecular species.

Sodium butyrate induces profound effects upon cell growth and cell morphology (Wright, 1973; Ginsburg et al., 1973; Fishman et al., 1974; Hennebery et al., 1975; Ghosh et al., 1975; Leder & Leder, 1975; Prasad & Sinha, 1976; Altenburg et al., 1976; Hagopian et al., 1977; Tralka et al., 1979) and upon the rate of DNA synthesis (Hagopian et al., 1977). Butyrate also induces change in several molecular species. Among such changes are (1) hyperacetylation of the inner histones at the ϵ-amino group of specific internal lysines (Riggs et al., 1977; Candido et al., 1978; Sealy & Chalkley, 1978a; Vidali et al., 1978), (2) increased production of a nonhistone protein, IP_{25} (Candido et al., 1978), (3) increased quantities of histone H1 (Riggs et al., 1977), and (4) induction or modulation of several other enzymes and proteins, some of which have been associated with cellular differentiation (Griffin et al., 1974; Leder & Leder, 1975; Prasad & Sinha, 1976; Ghosh & Cox, 1976; Lieblich et al., 1977; Rastl & Swetly, 1978; Reeves & Cserjesi, 1979; Rubenstein et al., 1979; Tralka et al., 1979).

It has been shown that butyrate inhibits histone deacetylase which results in the accumulation of multiacetylated forms of the inner histones in chromatin (Boffa et al., 1978; Candido et al., 1978). The highly acetylated chromatin from butyrate-treated cells is more susceptible to digestion by DNase I (Vidali et al., 1978; Sealy & Chalkley, 1978b; Simpson, 1978; Mathis et al., 1978), and there are changes in the accessibility of particular DNA sites within the nucleosome core particle to attack by DNase I (Simpson, 1978).

Despite these discoveries, questions remain about the effects of butyrate upon cell-cycle progression, the extent of modification of the inner histones, changes in the distribution of the modified species of histones H2A, H2B, and H3, and the possible effects of butyrate treatment on H1 phosphorylation. First, Rastl & Swetly (1978) have shown that erythroleukemia cells become arrested in G_1 when they are exposed to 1.0–1.5 mM butyrate for 31 h. On the other hand, in most studies of chromatin structure, cultures have been treated with 5–10 mM butyrate for 6–24 h. Since different cell lines have different doubling times and perhaps different sensitivities to butyrate, the concentration of butyrate may alter the period required for G_1 arrest, or some concentrations of butyrate may induce indiscriminate cell-cycle arrest. If this is the case, then those molecular properties which are cell-cycle dependent will vary with butyrate concentration and the length of butyrate exposure. Therefore, the effects of butyrate concentration upon cell-cycle progression must be defined for a specific cell line to interpret accurately molecular changes in that cell. Second, while it is well established that butyrate induces hyperacetylation of inner histones by inhibiting histone deacetylation (Boffa et al., 1978; Candido et al., 1978; Reeves & Candido, 1978; Sealy & Chalkley, 1978a; Vidali et al., 1978), the extent of internal acetylation of only histone H4 has been measured in butyrate-treated cells. The extent of modification and the distribution of modified species of histones H2A, H2B, and H3 have not been reported, because electrophoresis of histones in a single dimension does not adequately resolve modified

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species between histone classes [e.g., see Perry et al. (1979)]. Consequently, there is considerable overlap between histones H2A, H2B, and H3. Third, previous studies [see Gurley et al. (1978a,b) for a review] have shown that the phosphorylation of histone H1 is highly cell-cycle dependent. If butyrate affects cell-cycle progression, does it also affect H1 phosphorylation which in turn could affect chromatin structure and function?

In order to answer the above questions, we have examined some of the effects of sodium butyrate in Chinese hamster (line CHO) cells. Our objectives in the study were (1) to determine the effects of sodium butyrate concentration upon CHO cell growth and the cell-cycle distribution of CHO cells, (2) to determine the extent and origin of modification of the inner histones, (3) to determine if butyrate affected the extent of phosphorylation of histone H1, and (4) to quantify changes in the distribution of molecular species as functions of butyrate concentration. To accomplish those objectives, we have used flow cytometry (FCM),[1] isotopic labeling, one-dimensional polyacrylamide electrophoresis, two-dimensional polyacrylamide electrophoresis, and three procedures for quantifying histones in two-dimensional gels.

During this investigation we also observed significant quantitative changes in two other proteins which migrate in the histone region of our gel systems. One of the proteins is extracted with H1 and has a molecular weight of about 2.2×10^4. The increase in this butyrate-enhanced protein (BEP) has been quantified as a function of butyrate concentration. The other unidentified protein (UP) has a molecular weight of ~15000. Butyrate induces a twofold increase in the cellular content of UP, and it alters the distribution of UP molecular species.

Experimental Procedures

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 assay (Walters et al., 1974). Cell concentrations in the cultures were determined with an electronic particle counter (Tobey et al., 1967).

Sodium Butyrate Treatment. A 1.0 M solution of sodium butyrate was prepared by adjusting *n*-butyric acid (Sigma Chemical Co.) in physiological saline to pH 7.3 with NaOH. The sodium butyrate solution was then sterilized by filtration through a 0.2-μm filter. For growth studies, 1-L suspension cultures of CHO cells at 125000 cells/mL in exponential growth were treated with various amounts of this butyrate solution to make the cultures 0–15 mM with respect to sodium butyrate. Since the pH of the F-10 medium is about 7.0, the ratio of butyrate to butyric acid should be $\sim 1.3 \times 10^2$:1. At various times after butyrate addition, 2.0- and 40-mL samples were withdrawn from the cultures for cell concentration analysis and FCM analysis, respectively. After 24 h in sodium butyrate, some cultures were released from butyrate treatment by resuspending the cells in 1 L of fresh F-10 medium containing no butyrate.

Cell cultures used to determine the effects of butyrate upon histone modification were treated in a similar fashion. Sodium butyrate was added to exponentially growing cultures (cell concentration of 1.4×10^5 cells mL^{-1}). The volume of these cultures varied with butyrate concentration from 500 to 850 mL, so that 1.6×10^8 cells could be harvested after 24 h in butyrate.

Cell-Cycle Distribution Studies. The cell-cycle distribution of cells in a culture was determined by FCM (Van Dilla et al., 1969). Approximately 5×10^6 cells were removed from a culture and treated with the fluorescent DNA stain mithramycin as previously described (Crissman et al., 1977). The relative DNA content of each cell was then determined from the fluorescence of the DNA-bound dye by FCM as previously described (Kraemer et al., 1972). The fluorescence data from ~150000 cells were accumulated in the FCM multichannel pulse-height analyzer, producing a DNA histogram which was transferred to a magnetic disc for data processing. The fractions of cells in G_1, S, and G_2 plus M were derived from this DNA histogram with the computer program described by Dean & Jett (1974) using a PDP 11/40 computer (Digital Equipment Corp., Maynard, MA). The data analysis procedure fits a second-degree polynomial to describe S phase cells and Gaussian distributions to describe the G_1 and the G_2 plus M populations (Dean & Jett, 1974).

Isotopic Labeling. To examine acetate incorporation, we grew cultures for 48 h in F-10 medium containing [^{14}C]lysine (350 mCi/mmol; New England Nuclear) at an isotope concentration of 50 μCi/L. One hour prior to butyrate treatment, a parent culture [$(3.0–3.5) \times 10^5$ cells/mL] was divided into two identical 250- or 500-mL cultures. Sodium butyrate (1.0 M, pH 7.3) was added to one culture to give a final concentration of 10 mM. At the same time or 15 min later (see the text), sodium [^{3}H]acetate in ethanol (2.1 Ci/mmol; New England Nuclear) was added to a concentration of 20 mCi L^{-1}. Isotope incorporation was terminated 15 min after the addition of [^{3}H]acetate by pouring the culture over crushed, frozen F-10 medium. The cells were harvested by centrifugation in a cold room (4 °C), and all subsequent operations were performed there.

To determine phosphate incorporation into histones, a parent culture was labeled for 48 h with [^{3}H]lysine (8 Ci/mmol; Schwarz/Mann) at a concentration of 50 μCi L^{-1}. After dividing the parent culture into two cultures of equal volume, we treated one culture with sodium butyrate (10 mM). At the same time both cultures were treated with carrier-free $H_3{}^{32}PO_4$ (New England Nuclear) at a concentration of 20 mCi L^{-1}. The cultures were harvested 1 h later by centrifugation in the cold.

To compare relative phosphate incorporation between H1 from exponentially growing cultures and H1 from cultures arrested in G_1 by butyrate, we grew a parent culture for 72 h in the presence of [^{3}H]lysine (50 μCi L^{-1}). The culture was then divided so that each culture contained 1.4×10^5 cells/mL, and the isotope concentration of [^{3}H]lysine was 50 μCi L^{-1}. After treating one culture for 23.5 h with 15 mM sodium butyrate, we labeled both cultures for 1.0 h with $H_3{}^{32}PO_4$ (20 mCi L^{-1}) and harvested them by centrifugation in the cold.

Histone Isolation Procedures. CHO histones were isolated by three different procedures: (1) the first method of Johns (1964) from the chromatin of blended whole cells, (2) the first method of Johns from the chromatin of isolated nuclei, and (3) sulfuric acid extraction from the chromatin of isolated nuclei. Histones were isolated by the first method of Johns (1964) as described for cultured cells by Gurley & Hardin (1968), except that sodium bisulfite, mercaptoethanol, and 6 mM sodium butyrate were added to some of the solutions. Sodium bisulfite was originally used as a protease inhibitor

[1] Abbreviations used: BEP, butyrate-enhanced protein; FCM, flow cytometry; MD, microdensitometry; $NaDodSO_4$, sodium dodecyl sulfate; NP-40, Nonidet P-40, nonionic detergent; TCB, 15 mM Tris-HCl, 3 mM $CaCl_2$, and 6 mM sodium butyrate, pH 7.2; Tris, tris(hydroxymethyl)-aminomethane; UP, unidentified protein.

(Bartley & Chalkley, 1970); however, this laboratory has shown that the omission of sodium bisulfite also leads to extensive dephosphorylation of CHO histones H1 and H3 (Gurley et al., 1975). Therefore, sodium bisulfite was included in the saline wash solution and in the 5% perchloric acid solution (Gurley et al., 1975). Mercaptoethanol was present in all solutions used for the extraction of histones H2A, H3, and H4 to prevent oxidation of histone H3 (Smith et al., 1970). Sodium butyrate has been shown to inhibit deacetylation of histones during their isolation (Perry et al., 1979). We measure no increase in the proportion of modified histone species relative to controls if sodium butyrate (up to 12 mM) is included in solutions for preparing histones. Nevertheless, 6 mM butyrate (Perry et al., 1979), along with 0.050 M sodium bisulfite, was added to the isotonic saline wash solution as a precautionary measure. Extraction of histones by the first method of Johns (1964) separates histones into three fractions: (1) histone H1; (2) a mixture of histones H2A, H3, and H4; (3) histone H2B. Each fraction was dissolved in water and lyophilized for storage.

Nuclei from CHO cells were prepared by treating cells with 1.0% Nonidet P-40 (NP-40) in cold Tris–calcium buffer (TCB). TCB (Wigler & Axel, 1976) is 15 mM Tris-HCl and 3 mM $CaCl_2$ to which 6 mM sodium butyrate has been added and which has been adjusted to pH 7.2. Cells [$(1.5–2.0) \times 10^8$] were harvested by low-speed centrifugation and washed once with 10 mL of cold TCB. The cells were resuspended in 10 mL of cold TCB, and 1.0 mL of 10% NP-40 in TCB was added while vortexing to make the solution 1.0% in NP-40. Cells were homogenized in a 15-mL glass Dounce homogenizer (Kontes Glassware) with a tight B pestle for 9–12 strokes or until the cells were broken. The homogenate was transferred to centrifuge tubes, and the homogenizer was washed with 10 mL of 1.0% NP-40 in TCB. The wash was added to the original homogenate. After the nuclei were pelleted, they were washed twice with TCB without detergent (Wigler & Axel, 1976).

The pelleted nuclei were transferred to a Sorvall Omni-Mixer microblender cup (Du Pont Instruments) with 3.0 mL of isotonic saline containing 0.050 M $NaHSO_3$ and 6 mM sodium butyrate (from the 1.0 M solution, pH 7.3). The nuclei were blended in an iced water bath at a motor speed of 14 100 rpm for 1.25 min. The blended chromatin was pelleted by centrifugation and extracted with 5% perchloric acid and other reagents employed in the first method of Johns (1964) as described by Gurley & Hardin (1968).

For H_2SO_4 extraction, the pellet from the blended nuclei was extracted 3 times for 45 min with 0.13 mL of 0.20 M H_2SO_4. The pooled extracts were first clarified by centrifugation (30 min at 7700*g*). Then the histones were precipitated by the addition of 10 volumes of acetone. The next day, the histone precipitate was washed with acetone, dissolved in water, and lyophilized.

One-Dimensional Gel Electrophoresis. Histones were subjected to electrophoresis in two variations of the long (0.5×25 cm) cylindrical urea–acrylamide–acetic acid gels of Panyim & Chalkley (1969a). The first gel system, 6 M urea–12% acrylamide–acetic acid, was used for the H2A, H3, and H4 histone mixture and for histone H2B as previously described (D'Anna et al., 1977). The second gel system, 2.5 M urea–15% acrylamide–acetic acid, was used for histone H1, as well as for the other histone fractions. Electrophoresis of histone H1 was performed as previously described (Gurley et al., 1978a), while electrophoresis of the other histone fractions was carried out at 220 V for 23.0 h. Gels from both systems were stained overnight in 0.2% amido black in 30% methanol and 9% acetic acid and destained by diffusion in the same solvent without amido black.

Densitometer profiles of the one-dimensional gels were measured at 630 nm with a Gilford Model 240 spectrophotometer equipped with a gel linear transport device. When possible, the overlapping bands of each histone were resolved and quantified electronically by using a Du Pont Model 310 curve resolver.

In some cases, the stained gels contained radiolabeled histones. After the densitometer profiles were recorded, gels were sliced into 2.2-cm pieces with a Bio-Rad Model 190 gel slicer. The gel slices were dissolved overnight in 1.0 mL of 30% hydrogen peroxide (55–60 °C) and then counted in 15 mL of Aquasol II (New England Nuclear) or Hydrofluor (National Diagnostics) with a Packard Model 3320 scintillation spectrometer (Enger et al., 1979).

Two-Dimensional Electrophoresis. Two-dimensional electrophoresis was performed using a cylindrical (0.38×25 cm), 6 M urea–12% acrylamide–acetic acid gel (see above) in the first dimension and a 12.67% ($0.30 \times 13.3 \times 30$ cm) sodium dodecyl sulfate ($NaDodSO_4$) slab gel (Laemmli, 1970) in the second dimension. Approximately 8 μg of each histone was loaded on the first-dimension, cylindrical gel, and electrophoresis was performed at 200 V for 26 h. After electrophoresis, the cylindrical gel was equilibrated for 1.25–2.0 h in 40 mL of buffer 0 of O'Farrell (1975). The equilibrated gel was cut at 1.5 and 14.8 cm from its bottom, and the intervening slice was applied horizontally to the top of the second-dimension slab gel.

The second-dimension slab gel was poured in a Bio-Rad Model 221 vertical electrophoresis unit so that the top of the gel was only 1–4 mm below the bottom edge of the bevel of the beveled, notched plate which formed the inside containment wall of the slab gel. No stacking gel was used; therefore, the first-dimension gel was sealed in the beveled slot and connected to the $NaDodSO_4$ gel with 1.0% agarose in buffer 0 of O'Farrell (1975). These procedures are patterned after those of Davie & Candido (1978). Electrophoresis of a single second-dimension slab gel was carried out at 60 mA until the ion front had migrated 17–18 cm from the top of the $NaDodSO_4$ slab. Gels were stained and destained by the Coomassie blue–2-propanol system of Fairbanks et al. (1971).

Two-dimensional gels were photographed for printing by using Polaroid PN-55 film and a pair of photomicrographic glass filters which had a low-wavelength cutoff at 480 nm and maximum transmission at 550 nm. Gels were also photographed through the same filter with Kodak 35-mm Panatomic-X film. In some cases, the negatives were used for microdensitometer reading and computer analysis (see below).

Quantitation of Histone Species in the Two-Dimensional Gel System. Three methods were used to quantify the distribution of modified histone species separated by two-dimensional electrophoresis. Method 1 is a direct densitometer procedure employing a Gilford spectrophotometer and linear transport device. A rectangular section of the slab gel is excised so that the section contains all modifications of a given histone. The rectangular section is placed in a conventional gel cuvette, and densitometer profiles are measured at 570 nm. Since the dimensions of the spots vary with the protein load (O'Farrell, 1975), the whole spot must pass through the light beam to obtain an accurate relative measure of the protein in the spot. Therefore, care must be taken to cut and position the section so that all spots will pass through the light path. At the same time, the presence of nonhistone impurities or

other classes of histones on the section must be minimized.

To help meet the criteria for accuracy, the light entrance slit on the Gilford gel transport device was lengthened. The normal entrance slit of the Gilford transport device is only 2.4 mm in height, and its projection at the cuvette is ~2.8 mm. Lengthening the slit height to 4.0 mm increases the projection at the cuvette to ~4.25 mm. This is the maximum gel height that the Gilford can accommodate without reboring the entrance hole on the gel transport device or modifying the gel cuvette support. To determine the position of the spots in the light path, we marked two series of lines on the outer wall of the cuvette with a fine-lined marker pen. One series of lines of increasing length was marked from the base of the cuvette; the other series of lines started at the top of the cuvette. Each line that extends into the light path causes a pen deflection. From the number of lines which appears in the densitometer profile, one can determine which part of the cuvette and which spots are in the light path. After measurement of the densitometer profile, the overlapping bands of the individual spots are resolved and quantified with the Du Pont Model 310 curve resolver.

The second method (method 2) used to quantify the distribution of histone species employed isotopic labeling of the histones and scintillation spectrophotometry of dissolved gel slices. Stained regions of the gels which contained the radiolabeled histones were excised with a razor blade into 1.0 mL of 30% hydrogen peroxide and treated as described above (Enger et al., 1979). Control experiments show that gel slices from 5 to 110 mm^3 (and perhaps larger) can be dissolved in 1.0 mL of 30% H_2O_2; furthermore, the size of the gel slice does not alter the efficiency of tritium counting.

The third method (method 3) employs photography of the gel, followed by microdensitometry (MD) of the negative and computer processing. Existing hardware and software, owned by E. G. & G., Inc. (Los Alamos, NM), were used for the analyses. The microdensitometer was designed and built by John Carson of E. G. & G., Inc., and the analysis program was written by Newell Ramsey, also of E. G. & G., Inc. A 40–50-μm MD light beam (the "point" source) is used to scan a 35-mm negative, point by point, row by row, so that the absorbance at each point is read into a 512 × 512 row–column matrix (maximum field size) in computer memory. The density profile is then projected on a video screen. By use of an electronic marker on the video screen, the position of spots on the tube can be correlated with coordinates in memory. Two procedures have been used to quantify histone species. The first procedure (MD_1) is equivalent to a densitometer profile of an excised portion of the gel. This can be accomplished if the different species for a given histone can be aligned horizontally in a rectangular grid on the video screen and if the presence of other histones and nonhistone proteins is minimized. If the criteria are met, an average optical density is computed for each column position in the grid. The resulting densities are plotted as a function of column position (see Figure 14), and its appearance is the same as a direct densitometer scan of an excised gel slice. Overlapping histone species are resolved and quantified with the Du Pont Model 310 curve resolver.

The second procedure (MD_2) is equivalent to excising a portion of the gel and counting by scintillation spectrophotometry. Each spot is enclosed in a rectangular grid, and the integrated intensity of the region is computed. Continuing the process for each spot, the percentage of each histone species can be calculated. The usefulness of MD is that the photographic negative forms a permanent record of the gel; consequently, if the gel is damaged or destroyed by direct dissection, the negative can be analyzed by MD.

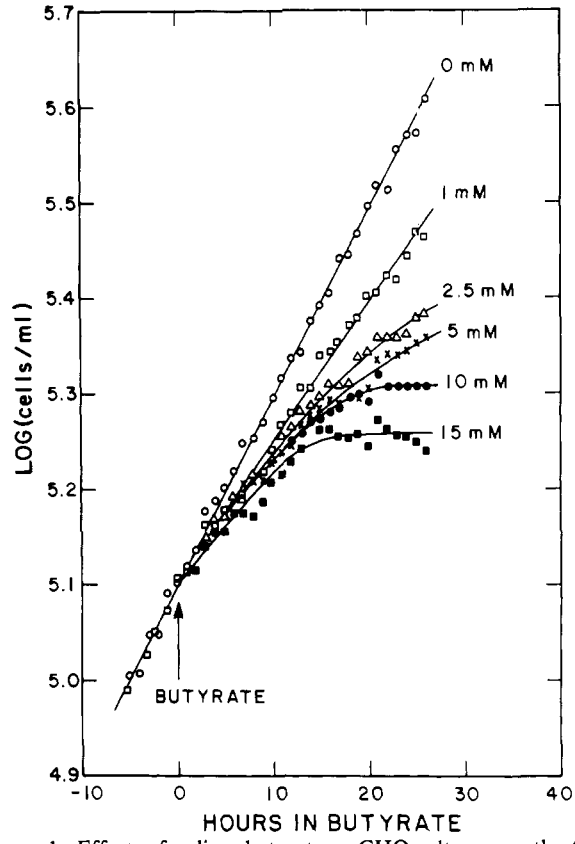

FIGURE 1: Effects of sodium butyrate on CHO culture growth: (○) culture in exponential growth, no butyrate; (□) 1 mM butyrate; (△) 2.5 mM butyrate; (×) 5 mM butyrate; (●) 10 mM butyrate; (■) 15 mM butyrate.

Results

Effects of Butyrate upon Cell-Cycle Progression. The effects of sodium butyrate upon cell culture growth are shown in Figure 1. A 24-h period was chosen for initial growth studies, because other laboratories had observed that butyrate induced large changes in the degree of histone acetylation during that time. The growth curves indicate that at the lower butyrate concentrations, cell growth continues throughout the 24-h monitoring period, but at 10 and 15 mM butyrate, cell populations stabilize after 20 h. FCM analysis of the cell-cycle distributions at the end of 24 h (Figure 2) shows that the proportion of G_1 cells in culture increases with butyrate concentration until ~5 mM butyrate, above which the proportion of cells in G_1 and the cell-cycle distribution remain the same.

Since some proliferating cultures (2.5 and 5.0 mM butyrate) and the arrested cultures (10 and 15 mM butyrate) in Figure 1 have a similar cell-cycle distribution at 24 h (Figure 2), we wanted to determine the way in which different concentrations of butyrate altered cell-cycle distribution during entrance into the G_1-enriched state at 24 h. Figure 3 shows examples of the changes in FCM histograms which occur after butyrate is added to a culture. The analyzed FCM histograms not only indicate the proportion of cells in different phases of the cell cycle but also provide insight as to the distribution of cells within S. This can be seen at 4 and 6 h in Figure 3 where the percentage of cells in S is 36% for both times; however, at 6 h most cells are in late S in contrast to the broader distribution at 4 h. Figure 4 shows changes in the cell-cycle distribution (computed from the histograms) of CHO cells

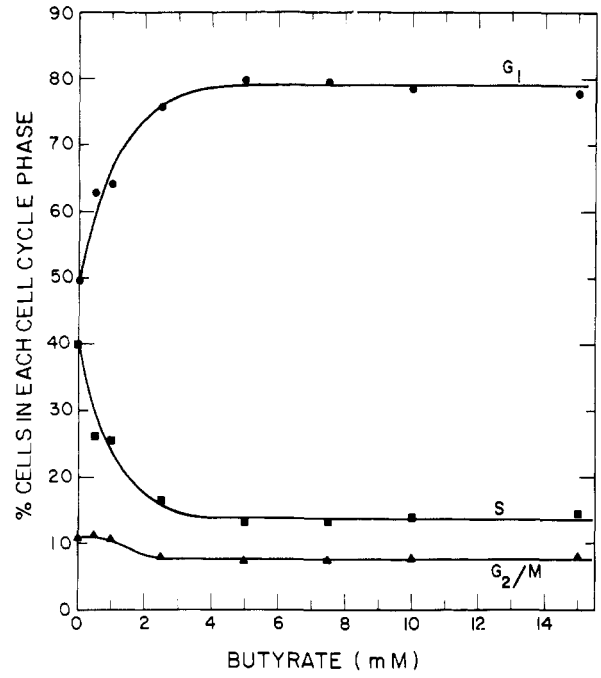

FIGURE 2: Cell-cycle analysis of CHO cultures treated for 24 h with various concentrations of sodium butyrate. Percentage of cells in G_1 (●), percentage of cells in S (■), and percentage of cells in G_2 plus M (▲) were determined by FCM as illustrated in Figure 3.

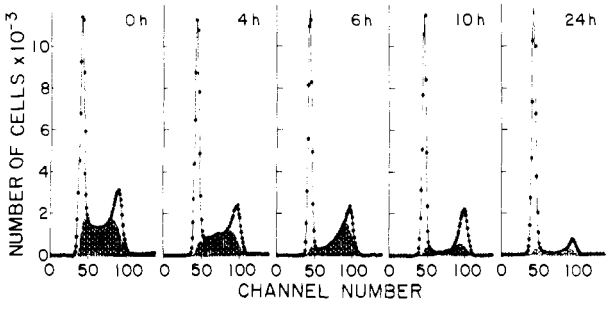

FIGURE 3: Flow cytometry (FCM) analysis of CHO cultures during exposure to 5 mM sodium butyrate. Exposure to sodium butyrate is continuous for 0, 4, 6, 10, and 24 h (left to right). The shaded area represents the population of cells in S phase as determined by computer analysis described by Dean & Jett (1974). Cells in G_1 and G_2 plus M are determined from the unshaded balance of the area of the peaks with modal channel numbers of ~43 and ~89, respectively.

during exposure to several concentrations of butyrate. The major observation is that at 2.5 mM butyrate a considerable portion of cells continues to enter S phase from 10–16 h after butyrate is added to the culture. As the concentration of butyrate is increased, however, the percentage of cells which enter S declines. At 15 mM butyrate, where there is no culture growth after 20 h, those cells which enter G_1 appear to be trapped in that phase of the cell cycle.

A second effect of butyrate treatment is that it slows cell-cycle progression through G_2 and/or M. This can be seen at all butyrate concentrations from the transitory increase of G_2 and M cells which occurs at 4 and 10 h (Figure 4). The increase in the G_2/M component is larger and decays more slowly with increasing butyrate concentration. In addition to increasing the G_1 phase of the cell cycle and slowing down progression through G_2/M, the shoulder in the S phase curves (between 4 and 7 h) suggests that butyrate is altering the rate at which some cells progress through S.

Notice that the butyrate-induced changes in cell-cycle distribution occur over a prolonged period of time. Therefore, examination of cell-cycle distributions at other than 24 h in

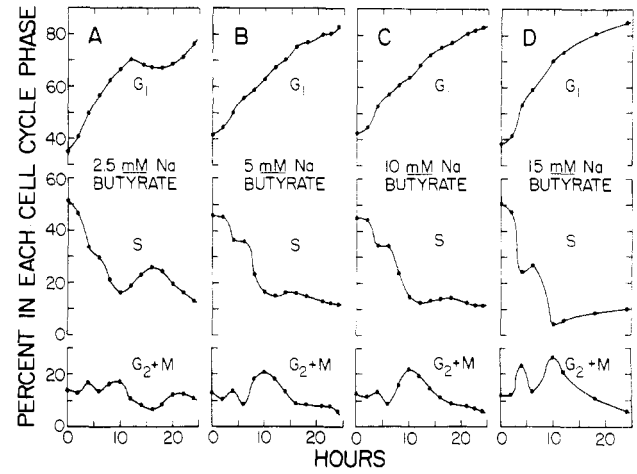

FIGURE 4: Changes in cell-cycle distribution during exposure of CHO cells to sodium butyrate. Left to right: cultures treated with (A) 2.5, (B) 5, (C) 10, and (D) 15 mM sodium butyrate, respectively. The percentage of cells in each phase of the cell cycle was determined by FCM as illustrated in Figure 3.

Figure 2 would present a different functional dependence of butyrate effects. That difference would be accentuated at times less than 20 h.

Since Hagopian et al. (1977) have found that butyrate treatment is partially reversible in HeLa cells, and Macher et al. (1978) have suggested that KB cells can resume proliferation in a synchronous wave, we have examined the possible utility of butyrate as a reversible synchronizing agent in CHO cells. Figure 5A shows plots of cell growth as a function of time after resuspension of cells exposed to butyrate treatment for 24 h. At 2.5 and 5.0 mM there is a long delay in the resumption of cell growth, but at 7.5 and 15 mM the cell populations actually decrease. Cell-cycle analysis of a culture following release from 2.5 mM butyrate also indicates poor synchronous release (Figure 5B). Therefore, while butyrate induces G_1 arrest, it is unsuitable for synchronizing CHO cells for subsequent release and cell-cycle traverse experiments.

Resolution of Inner Histones. Figure 6 shows electrophoretograms (one-dimensional gels) of the H2A, H3, and H4 histone mixture (Johns, 1964) isolated from the nuclei of exponentially growing control cells and from the nuclei of cells grown for 24 h in the presence of 5.0 and 15.0 mM sodium butyrate. Clearly, butyrate treatment of CHO cells induces changes in these inner histones. This is most easily seen in the H4 region of the gels where five bands are present. These results are similar to those observed for histones from other cell lines in one-dimensional gels (Candido et al., 1978; Sealy & Chalkley, 1978a; Cousens et al., 1979); however, the changes in Figure 6 appear to be greater than those previously reported.

While the one-dimensional gels of Figure 6 indicate that change is occurring in the H2A and H3 regions, they do not allow adequate separation for quantitation of either histone. Therefore, electrophoresis in two-dimensional gels was performed as described under Experimental Procedures. Parts a and c of Figure 7 show photographs of two-dimensional gels which contain the sulfuric acid extracts from the nuclei of exponentially growing cells (Figure 7a) and cells grown 24 h in the presence of 10 mM butyrate (Figure 7c). Parts b and d of Figure 7 present close-ups of those same gels in which the first-dimension cylindrical gel is superimposed upon the two-dimensional gel. This superposition facilitates comparison of the position of bands in the first dimension with the horizontal position of the spots in the second-dimension gel.

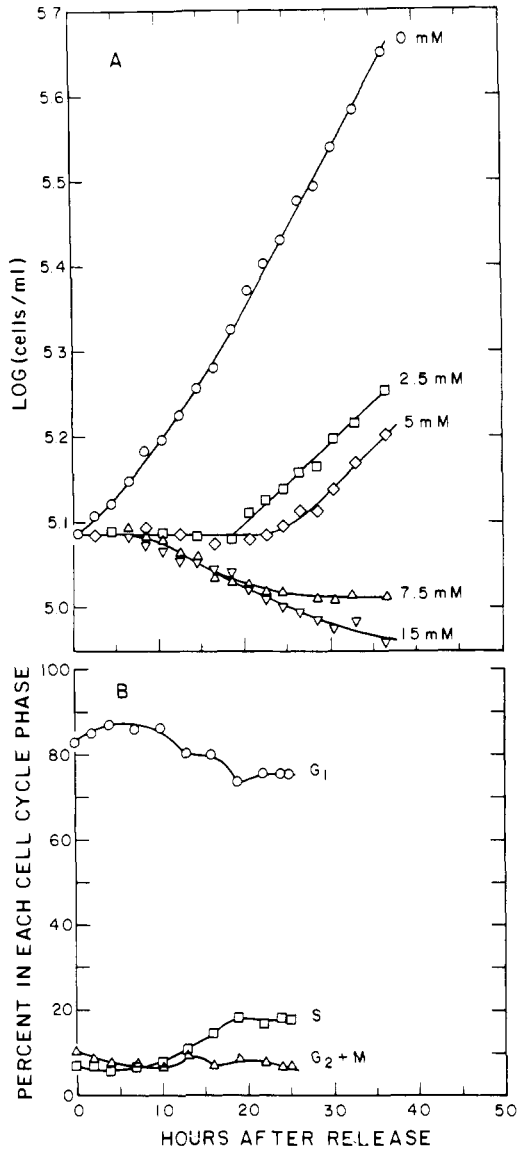

FIGURE 5: CHO culture growth following removal of sodium butyrate. (A) Culture growth following release from a 24-h treatment with no butyrate (○), 2.5 mM butyrate (□), 5 mM butyrate (◇), 7.5 mM butyrate (△), and 15 mM butyrate (▽). (B) FCM cell-cycle analysis of a culture following release from a 24-h treatment with 2.5 mM butyrate. Percent of cells in G_1 (○); percent of cells in S (□); percent of cells in G_2 plus M (△).

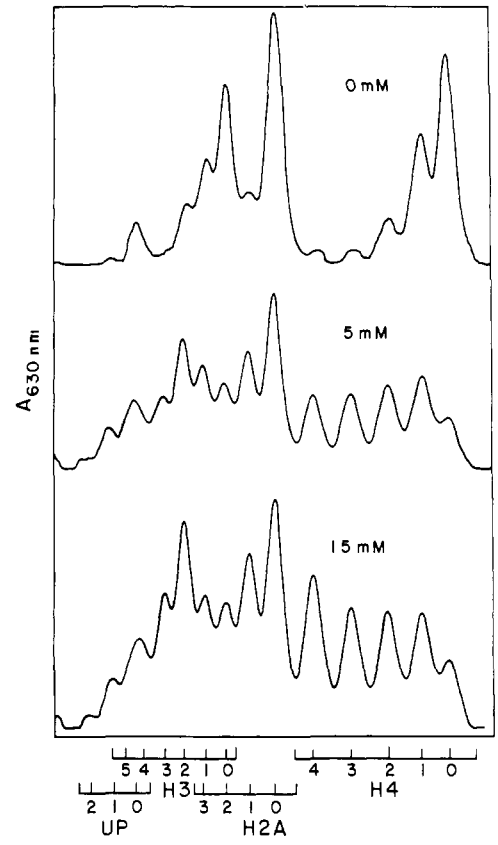

FIGURE 6: Electrophoretograms (one-dimensional gels) of the H2A, H3, and H4 histone mixture extracted by the first method of Johns (1964) from isolated nuclei of cells grown for 24 h in the presence of 0, 5.0, and 15.0 mM butyrate. The direction of migration of the histones is from left to right. In referring to the bands of a given histone and the UP, the fastest migrating species is given the subscript 0, and the other species are given the subscript 1, 2, 3, ..., etc. (right to left) in order of their decreasing mobility. The subscripts indicate the number of modifications (acetylation or phosphorylation) per molecule which retards migration of the parent species by approximately one band position in urea–acrylamide–acetic acid gels [see Ruiz-Carrillo et al. (1975)]. The assignments in this figure are based upon two-dimensional electrophoresis profiles (Figures 7 and 8) and conclusions in the text.

Assignment of the spots in Figure 7 is based upon the known migration of proteins in the first dimension and upon the well-established order of migration of mammalian histones in Tris–glycine–$NaDodSO_4$ gel systems (Laemmli, 1970). Those neighboring proteins which migrate as a row of spots in the second-dimensional gel are assumed to be of the same histone class. In referring to the spots of the inner hitones, we shall follow the convention used for one-dimensional gels (see Figure 6). The parent inner-histone species which migrates the fastest in the first dimension (and is farthest to the right in a given row of the second-dimension slab gel) is given the subscript 0. Other species are given the subscript i = 1, 2, 3, ..., etc. (right to left in each row) in order of their decreasing mobility in the first-dimension gel. Histones H4 and H2A are labeled in Figure 7b,d as examples. From comparison of parts b and d of Figure 7, it can be seen that treatment with butyrate induces remarkable changes in the distribution of modified species of each histone. The number of easily observable spots increases from 3 to 5 for histone H4, from 2 to 3 for histone H2A, from 2 to 5 for histone H2B, and from 3 to 6 for histone H3.

Histone H1 appears as two spots, H1(I) and H1(II), which differ in molecular weight by ~1000. The appearance of two spots for H1 agrees with previous one-dimensional $NaDodSO_4$ gels of purified CHO H1 which gave two bands (D'Anna et al., 1979).

Only three major "nonhistone proteins" are observed in the histone region in sulfuric acid extracts from isolated nuclei. One of these proteins (BEP) has a molecular weight of ~2.2 × 10^4 and migrates near H1. Since the quantity of this protein is enhanced by butyrate treatment (Figure 7b,d), the protein is referred to as the butyrate-enhanced protein, BEP. Quantitation of the increase in BEP as a function of butyrate concentration will be shown in a later section.

A second unidentified protein (UP) is found just above $H3_{4-5}$ in the two-dimensional gels. Butyrate treatment increases the number of easily visible spots of UP from one to two (parts b and d of Figure 7), and there is about a twofold increase in the amount of UP in butyrate-treated cells (Figure 7d) relative to that in untreated cells (Figure 7b). These changes also can be seen in the UP region of the one-dimensional electrophoretograms of Figure 6. There, a third species, UP_2, can be seen in the electrophoretograms from butyrate-treated

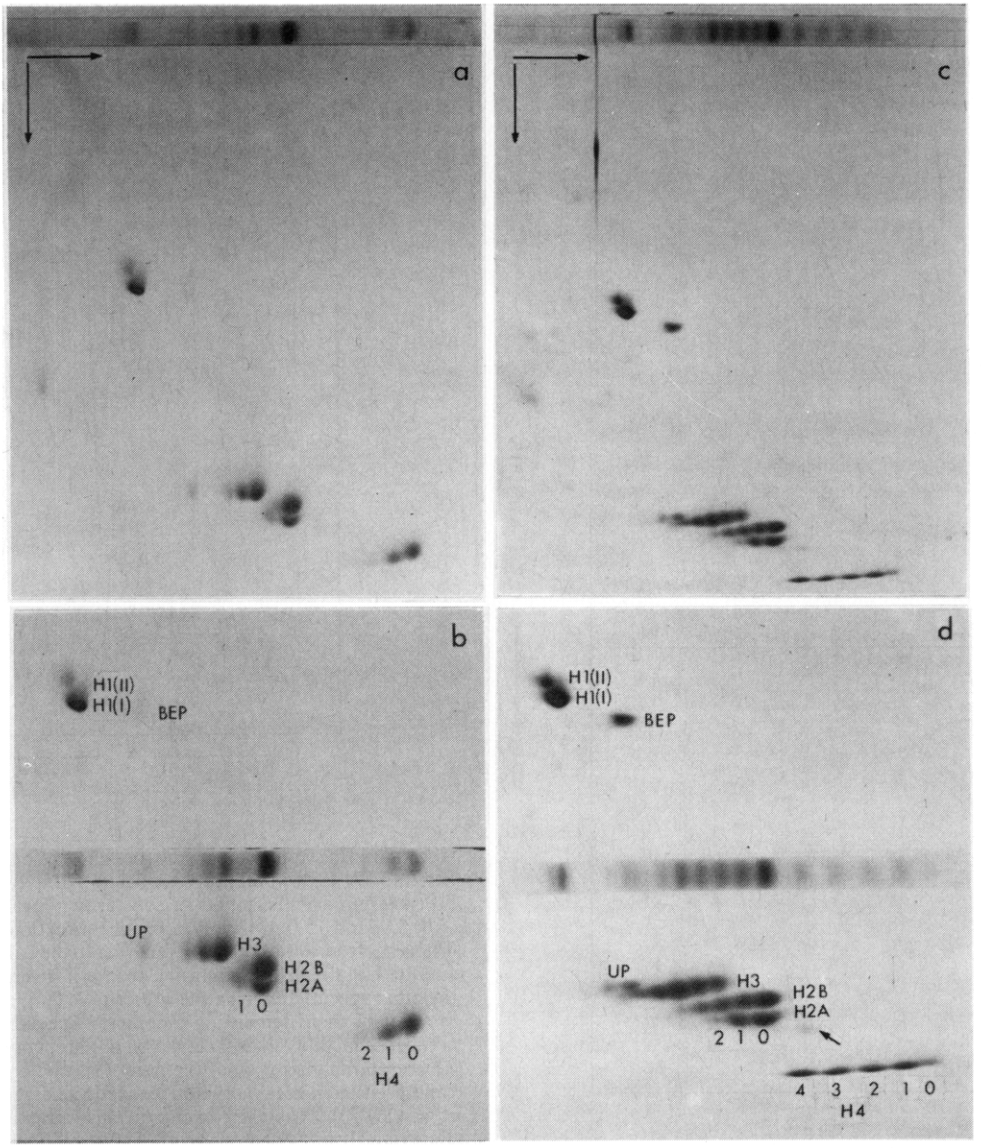

FIGURE 7: Two-dimensional electrophoresis of histones extracted with H_2SO_4 from isolated nuclei of control and butyrate-treated cultures. Histones from an untreated control culture are shown in (a) and (b), and histones from a culture grown for 24 h in the presence of 10 mM butyrate are shown in (c) and (d). (a) and (c) show the two-dimensional gel with the first-dimension cylindrical gel at the top of slab gel as it was during electrophoreses. (b) and (d) show a superposition of the first-dimension cylindrical gel upon the histone region of the two-dimensional gel. The direction of migration in the first dimension is from left to right; the direction of migration in the second dimension is from top to bottom. The distance from the top of the gel to H4 is about 16 cm. The arrow in (d) denotes an impurity which migrates in the H4 region.

cultures. Therefore, it appears that this protein is modified, and butyrate treatment alters the distribution of modified species. Other observations (data not shown) suggest that [^{32}P]phosphate is incorporated into UP_1 and UP_2, but this has not been examined in detail. It is noted that both UP and BEP migrate in the same horizontal region as $H3_{4-5}$; therefore, their presence confuses the analysis of H3 in conventional one-dimensional, acid–urea–polyacrylamide gels.

A third protein migrates above $H4_4$ in the two-dimensional gels (see arrow in Figure 7d). This protein is not observed in the H2A, H3, and H4 histone mixture extracted by the first method of Johns from isolated nuclei (Figure 8). BEP is also not found in that extract, because it is removed by prior extraction with 5% perchloric acid (Johns, 1964). Therefore, the one-dimensional gels of Figure 6 are apparently free of nonhistone contaminants in the H4 region, and $H3_{4-5}$ is contaminated only by UP.

From visual examination of the two-dimensional gels, we conclude that there are at least three molecular species of H2A, five species of H2B, six species of H3, and five species of H4

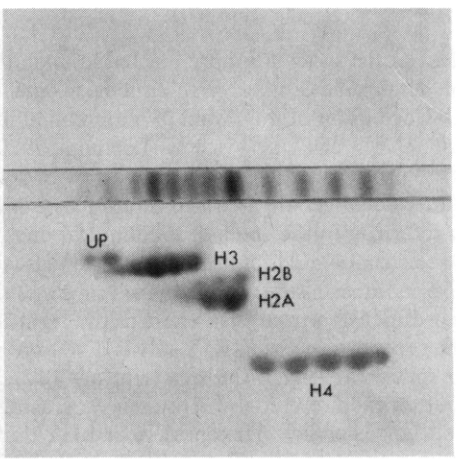

FIGURE 8: Two-dimensional electrophoresis of the H2A, H3, and H4 histone mixture extracted by the first method of Johns (1964) from the nuclei of cells grown 24 h in the presence of 2.5 mM sodium butyrate. The first-dimension cylindrical gel is superimposed on the histone region of the two-dimensional gel to facilitate comparison.

in butyrate-treated cells. Assuming that the modified species of the CHO inner histones arise from acetylation or phosphorylation, which are known to reduce histone positive charge and mobility in the first-dimension gel [see Panyim & Chalkley (1969a), Wangh et al. (1972), Ruiz-Carrillo et al. (1975), and Allfrey (1977)], then histone H2A is modified at a minimum of two sites, H2B is modified at a minimum of four sites, H3 is modified at a minimum of five sites, and H4 is modified at a minimum of four sites.

We note that in all two-dimensional gels, the modified species of a given histone class (except H2A) migrate slightly faster than the parent histone in the $NaDodSO_4$ second dimension. This suggests that the reduced positive charge of the modified histones (or some other effect of the modification) alters histone electrophoretic mobility in the second-dimension $NaDodSO_4$ slab as well as in the first-dimension urea–acrylamide–acetic acid gel. Such subtle differences in migration would go unnoticed in one-dimensional $NaDodSO_4$ systems.

Isotopic Labeling of the Inner Histones. To determine which modifications of inner histones in butyrate-treated cultures were due to acetylation and which were due to phosphorylation, we labeled cultures with either [^{3}H]acetate or [^{32}P]phosphate and subjected them to one-dimensional electrophoresis. Data from two gel systems are presented in this section, because each gel system possesses advantages. Electrophoresis in 6 M urea–12% acrylamide–acetic acid gels gives better resolution of acetylated H3 species and the UP protein, but electrophoresis in 2.5 M urea–15% acrylamide–acetic acid gels is more helpful for analyzing H2A and H2B modifications. Both systems are equally effective for analyzing modifications of H4. As we shall see, the labeling experiments verify the existence of several modified species, and they indicate that, except for H2A, acetylation is the most likely origin of the modifications in the inner histones from butyrate-treated cultures.

Figure 9 shows densitometer and labeling profiles of the H2A, H3, and H4 histone mixture separated by electrophoresis on long, 6 M urea–12% acrylamide–acetic acid gels. The histones in Figure 9a were extracted from the nuclei of cells grown 15 min in the presence of 10 mM butyrate and then pulse-labeled with [^{3}H]acetate for an additional 15 min. The histones of Figure 9b were extracted from untreated control cultures pulse-labeled with [^{3}H]acetate in the same fashion, except butyrate was omitted. Extensive acetate incorporation is seen in all protein-containing regions of the gels. Acetate is incorporated strongly into bands 1–4 and somewhat into band 0 of histone H4. This result agrees with the well-characterized acetylation of this histone in other species [e.g., see Dixon et al. (1975), Allfrey (1977), Candido et al. (1978), and Sealy & Chalkley (1978a)]. Acetylation also occurs at positions corresponding to $H2A_0$ and $H2A_1$ and extends into the $H2A_2$ position where $H3_0$ is also found (Figures 7 and 8). Since acetylation at the NH_2-terminal serine of H2A has been reported in several species (Dixon et al., 1975; Allfrey, 1977), it is not surprising that acetate is incorporated into $H2A_0$. On the other hand, acetylation of $H3_0$ at its N-terminal end has not been observed (Dixon et al., 1975; Allfrey, 1977). We therefore presume that the acetate in the $H3_0$ position is associated with $H2A_2$.

Five other acetate-labeled bands and shoulders can be observed in the H3 region of Figure 9. The positions and spacings of four of them indicate that they should be labeled $H3_{1-4}$. The position of the fifth (in the $H3_5$ region) also suggests the presence of acetylation at the position of the $H3_5$ species seen

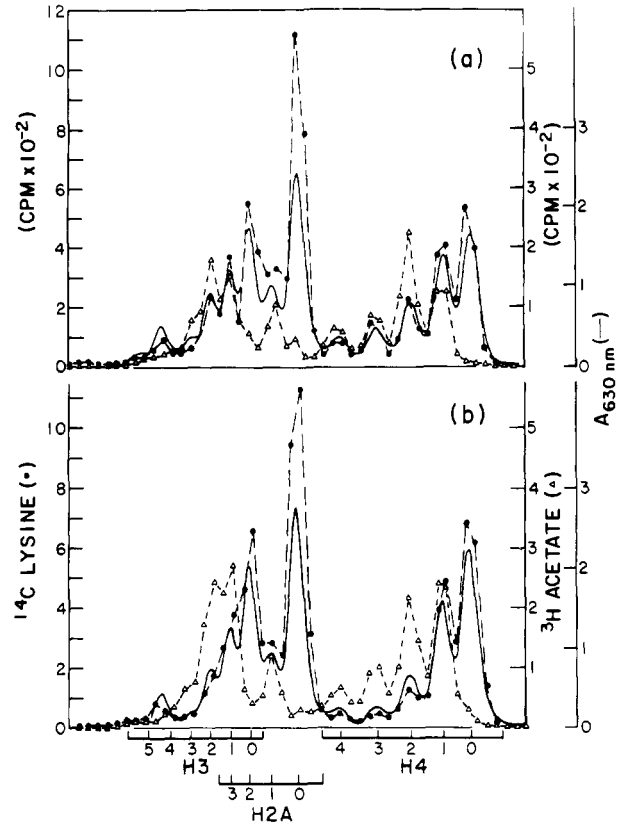

FIGURE 9: One-dimensional electrophoresis (6 M urea–12% acrylamide–acetic acid gels) of double-labeled histones H2A, H3, and H4 extracted by the first method of Johns from isolated nuclei: (●) [^{14}C]lysine, (▲) [^{3}H]acetate, and (—) absorbance profile at 630 nm. Cultures were grown 48 h in the presence of [^{14}C]lysine. One culture (a) was then treated for 15 min with 10 mM sodium butyrate and pulse-labeled an additional 15 min with [^{3}H]acetate. The control culture (b) was pulse-labeled in the same way, but it was not treated with sodium butyrate. The direction of histone migration is from left to right.

in the two-dimensional gels. In extracts from butyrate-treated cultures, there is a shoulder in the acetate profile at the expected $H3_5$ position. In extracts from untreated controls, the $H3_5$ acetate band is flattened in appearance and extends into the region of UP_1. Although acetate counts exceed background (which is negligible) and are reproducible, the lower counts in the $H3_5$ region preclude a conclusive assignment of the acetate to $H3_5$ and/or to the UP. Hence, the acetate incorporation profiles corroborate the existence of four of the five H3 species ($H3_{1-4}$) seen in the two-dimensional gels, and they are consistent with the existence of the fifth ($H3_5$).

If histones are isolated from blended whole cells in the presence of the phosphatase inhibitor sodium bisulfite (Gurley et al., 1975), the acetate profiles are the same (not shown). Therefore, the isolation of histones in the presence of sodium bisulfite does not increase the number of bands seen in acetate incorporation profiles, nor does it increase the number of spots seen in two-dimensional gels (data not shown). These results suggest (but, of course, they do not prove) that the presence of phosphorylation in H3 is unnecessary to observe an $H3_5$ species.

Figure 10 shows the acetate incorporation profiles of H2B's (Johns method I from blended whole cells) from butyrate-treated and control cultures which were pulse-labeled for 15 min with sodium [^{3}H]acetate. Although there are several absorbance and ^{14}C bands that arise from impurities in the H2B region (two-dimensional gels not shown), most acetate incorporation is confined to the left of the intensity maximum

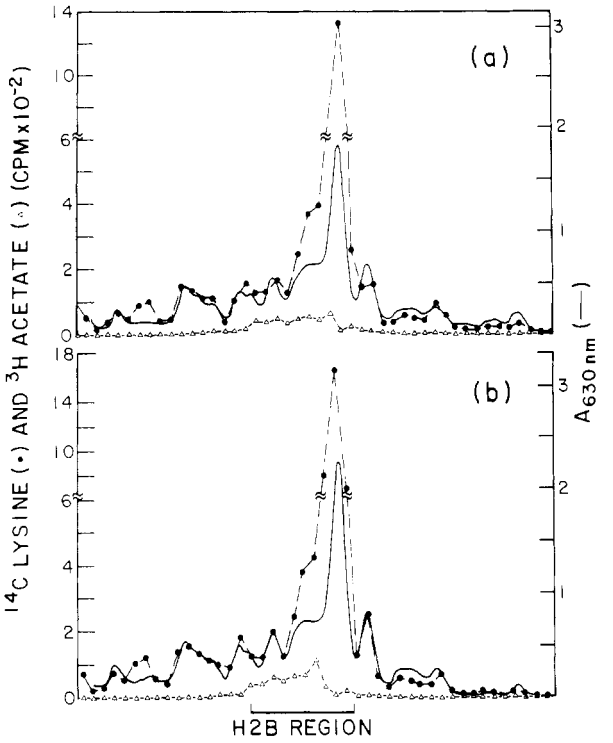

FIGURE 10: One-dimensional electrophoresis (2.5 M urea–15% acrylamide–acetic acid gels) of double-labeled H2B extracted by the first method of Johns from the chromatin of blended whole cells: (●) [^{14}C]lysine, (△) [^{3}H]acetate, and (—) absorbance profile at 630 nm. Cultures were grown 48 h in the presence of [^{14}C]lysine. One culture (a) was then pulse-labeled with [^{3}H]acetate for 15 min in the presence of 10 mM butyrate. The control culture (b) was pulse-labeled in the same way, but it was not treated with sodium butyrate. The direction of migration is from left to right.

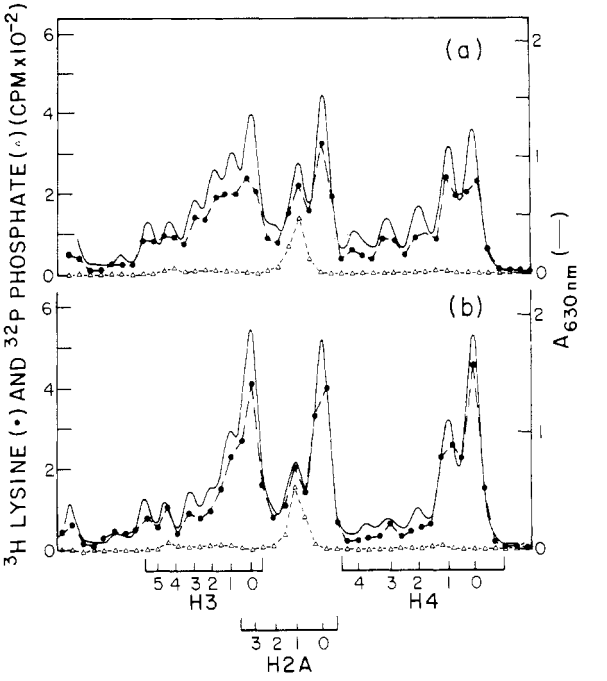

FIGURE 11: One-dimensional electrophoresis (2.5 M urea–15% acrylamide–acetic acid gels) of double-labeled histones H2A, H3, and H4 extracted by the first method of Johns (1964) from the chromatin of blended whole cells: (●) [^{3}H]lysine, (△) [^{32}P]phosphate, and (—) absorbance profile at 630 nm. Cultures were grown 48 h in the presence of [^{3}H]lysine. One culture (a) was then pulse-labeled with [^{32}P]phosphate in the presence of 10 mM butyrate for 1.0 h. The control culture (b) was pulse-labeled in the same way, but it was not treated with sodium butyrate. The direction of histone migration is from left to right.

in the H2B region. This region corresponds to that expected for more slowly migrating acetylated H2B species. In addition, there is a small acetate band on the right shoulder of the most intense H2B band; this position corresponds to that of residual H2A not extracted with the H2A, H3, and H4 histone mixture and further indicates that CHO H2A is acetylated on at least one internal site in addition to its N-terminal end (Dixon et al., 1975; Allfrey, 1977).

In contrast to the incorporation of acetate by all of the inner histones, the incorporation of phosphate is more selective. This can be seen from the 2.5 M urea–15% acrylamide–acetic acid gel profiles of Figures 11 and 12. The ^{32}P profiles of the butyrate-treated and control cultures are the same. Only H2A is appreciably phosphorylated. In Figure 11, H2A is strongly phosphorylated at $H2A_1$, and there is a weak shoulder at the position of $H2A_2$. In Figure 12, the small amount of phosphorylation on the right shoulder of the H2B maximum again corresponds to the position of $H2A_1$.

There is a broad band of ^{32}P incorporation in the H3 region of both parts a and b of Figure 11. Previous work from this laboratory has shown that H3 phosphorylation is confined to those stages of mitosis when chromosomes are maximally condensed (Gurley et al., 1978a). Since ~3% of the cells from an exponentially growing culture are mitotic, a low level of ^{32}P incorporation is expected. [We note that the small ^{32}P peak near $H3_4$ corresponds to the position of UP_1 as determined from two-dimensional gel electrophoresis in which a 2.5 M urea–15% acrylamide–acetic acid gel is used in the first dimension (unpublished observations).] In Figure 11a there is already a noticeable increase in the proportion of modified histones after exposure to butyrate for 1 h; yet, there is no increased phosphate incorporation. Hence, there is no sign

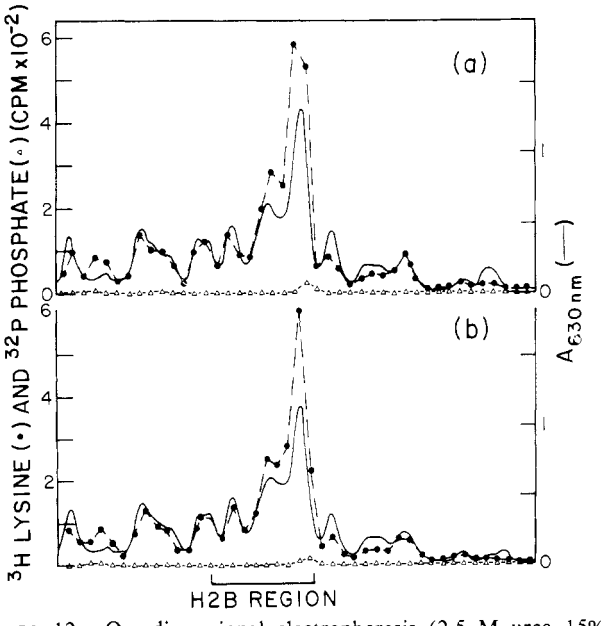

FIGURE 12: One-dimensional electrophoresis (2.5 M urea–15% acrylamide–acetic acid gels) of double-labeled H2B histones from butyrate-treated (a) and control (b) cultures: (●) [^{3}H]lysine, (△) [^{32}P]phosphate, and (—) absorbance profile at 630 nm. Cultures were labeled and histones were extracted by the same method as in Figure 11. The direction of histone migration is from left to right.

that butyrate induces aberrant H3 phosphorylation in interphase cells.

No phosphate incorporation is observed in the H2B region of Figure 12a,b. These data confirm previous results of Gurley & Walters (1973) that H2B is not phosphorylated in interphase CHO cells. The data also indicate that butyrate is not

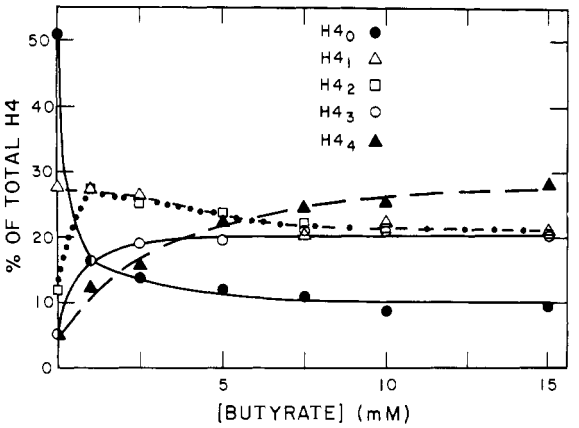

FIGURE 13: Distribution of histone H4 species as a function of butyrate concentration in the culture medium: $H4_0$ (●), $H4_1$ (△), $H4_2$ (□), $H4_3$ (○), and $H4_4$ (▲). Cells were grown for 24 h in the presence of different concentrations of butyrate. The H2A, H3, and H4 histone mixture was extracted by the first method of Johns (1964) from isolated nuclei and then subjected to one-dimensional electrophoresis in 6 M urea–12% acrylamide–acetic acid gels.

inducing abnormal H2B phosphorylation and acetylation in the origin of the four modified H2B species seen in the two-dimensional gels of Figures 7 and 8.

On the basis of these experiments, we conclude that (1) the modified species of H2B, H3, and H4 in butyrate-treated CHO cultures arise primarily from acetylation of these histones, (2) the modified species of H2A in butyrate-treated cultures arise from both acetylation and phosphorylation, and (3) the modified species of histones are present in exponentially growing cultures as well as the butyrate-treated cultures; however, their distribution is very different.

Quantifying Inner Histone Species. Another objective of this investigation was to quantify changes in the distribution of modified histone species after butyrate treatment. Figure 13 shows the percentages of the five histone H4 species plotted as functions of butyrate concentration following a 24-h treatment period. The values were obtained from analysis of one-dimensional gels (6 M urea–12% acrylamide–acetic acid gels) containing the H2A, H3, and H4 histones extracted by the first method of Johns from isolated nuclei. These procedures yield separated species of histone H4 which are free of nonhistone impurities (see Figure 8). While the quantity of unmodified $H4_0$ appears to plateau at about 10%, the percentage of $H4_4$ continues to increase with butyrate concentration. Similar trends have been observed by Cousens et al. (1979) in HTC cells. Cousens et al. (1979) have interpreted the persistence of unacetylated $H4_0$ as being characteristic of a portion of chromatin that is not accessible to acetylases. Our data do not bear upon that interpretation. It appears, however, that there is no obvious relationship between the cell-cycle distribution of CHO cells and the extent of CHO histone acetylation after 24 h of treatment with butyrate [correlation between histone acetylation and the cell cycle is observed in the absence of butyrate; see D'Anna et al. (1977) and Moore et al. (1979)]. This is concluded from comparison of Figures 2 and 13. Although the percentage of G_1 cells stabilizes at 5 mM butyrate (Figure 2), the percentage of highly acetylated $H4_4$ continues to increase with the concentration of butyrate in culture. Sealy & Chalkley (1978a) reached a similar conclusion regarding acetylation and the cell cycle from cold chase experiments of butyrate-treated cultures.

In order to overcome overlapping histone bands and the presence of nonhistone proteins, we determined species distributions of histones H2A, H2B, and H3 (as well as H4) from

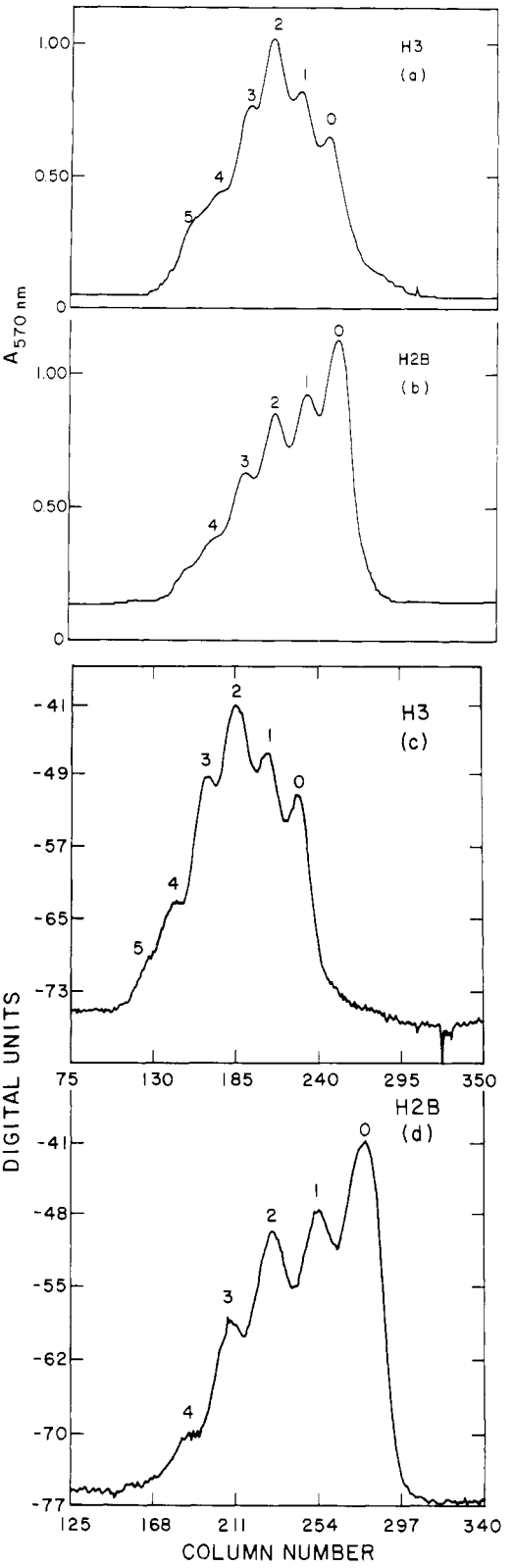

FIGURE 14: Densitometer profiles of histones H3 and H2B from the two-dimensional gel in parts c and d of Figure 7. Densitometer profiles were measured by method 1 on the Gilford spectrophotometer (a and b) or by method 3 (MD_1) using the microdensitometer and computer analysis (c and d). The cultures were grown in the presence of 10 mM butyrate for 24 h, and the histones were extracted as described in Figure 7c,d.

analysis of two-dimensional gels (see Experimental Procedures). Figure 14 gives examples of densitometer profiles of histones H3 and H2B as measured by method 1 with the Gilford spectrophotometer (Figure 14a,b) and as computed

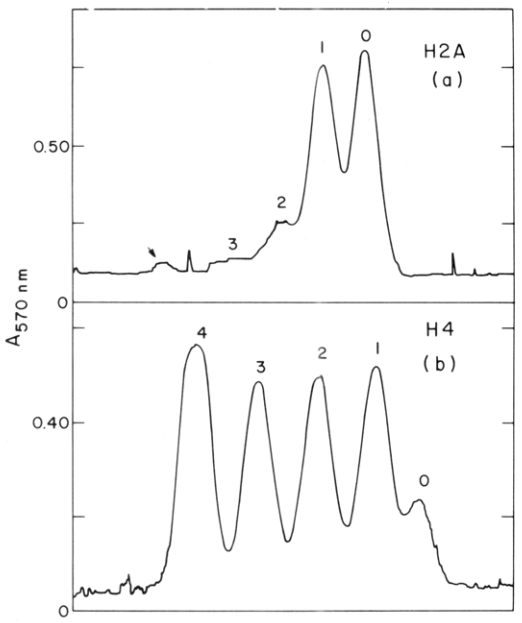

FIGURE 15: Densitometer profiles of histones H2A (a) and H4 (b) separated by two-dimensional electrophoresis. The profiles were measured by method 1 with the Gilford spectrophotometer. Cultures were grown 24 h in the presence of 15 mM butyrate, and the histone H2A, H3, and H4 mixture was extracted by the first method of Johns (1964) from the chromatin of blended whole cells. The arrow in (a) indicates an imperfection in the gel.

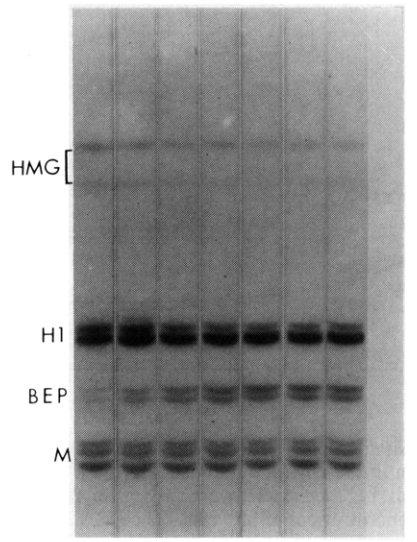

FIGURE 16: Electrophoresis (2.5 M urea–15% acrylamide–acetic acid gels) of the perchloric acid extracted proteins from blended whole cells grown for 24 h in the presence of different concentrations of sodium butyrate. From left to right the concentration of butyrate is 0, 1.0, 2.5, 5.0, 7.5, 10.0, and 15 mM. The marker bands (M) are calf thymus histone H1 which was loaded 4 h prior to the CHO perchloric acid extract.

by method 3 (MD_1) from microdensitometer analysis. Comparison of the H3 profiles in parts a and c of Figure 14 (and quantitation below) indicates reasonable agreement between the two methods. In the case of H2B, both profiles (parts b and d of Figure 14) show agreement, and they exhibit a shoulder (less pronounced in Figure 14d because electronic "gel slicing" is more precise) to the left of $H2B_4$ which suggests the existence of $H2B_5$. There is, however, a visible impurity on the gel slice which is not aligned with the other H2B species but which remains in the light path at the $H2B_5$ position. Consequently, if there is a small amount of $H2B_5$, we cannot resolve it from the impurity in the region.

Figure 15 shows gel profiles (method 1) of histones H2A and H4 measured from sections of two-dimensional gels in the Gilford spectrophotometer. We note that the H2A profile in Figure 15a indicates the existence of a minor species, $H2A_3$, which is not visible to the eye or by photography. Evidence for $H2A_3$ has been obtained repeatedly by direct densitometry (method 1) and by scintillation spectrometry of ^3H-labeled histones (method 2). On the other hand, histone overlap prevents its observance by acetate or phosphate radiolabeling in one-dimensional gels (see Isotopic Labeling of the Inner Histones). Therefore, the existence of the minor $H2A_3$ species is strongly suggested, but it is not conclusive. Gel profiles of H4 are easily measured in two-dimensional gels, and Figure 15b is shown solely as an example.

The distributions of various histone species from cells grown in 0, 10.0 and 15.0 mM butyrate are compiled in Table I. Sets of values obtained by two methods for the same sample are grouped together, and their agreement is surprisingly good. In going from 0 to 15.0 mM butyrate, the extent of modification increases from 34 to 60% for H2A, from 16 to 68% for H2B, from 43 to 89% for H3, and from 44 to 92% for H4. Only 4% of H2A has three or more modifications per molecule at 15 mM butyrate, but H2B has 26%, H3 has 37%, and H4 has ~50%.

Notice in Table I that $H3_5$ from 10 and 15 mM butyrate-treated cultures comprises 3–6% of total H3. Since only 2–3% of the cells are in mitosis after 24 h of treatment with butyrate, a maximum of only 3% of the total H3 should be phosphorylated (Gurley et al., 1978a), and they should be distributed [see Figure 11 and Gurley et al. (1978a)] among the five modified H3 species. Phosphorylation of 3% of the $H3_4$ molecules, however, would shift only 0.2–0.4% of total H3 to the $H3_5$ position. This is 1 order of magnitude less than the 3–6% actually measured at 10 and 15 mM butyrate. On the basis of these considerations and the acetate-labeling profiles, we conclude that CHO histone H3 can be acetylated at five different internal sites; furthermore, H3 can be phosphorylated at an additional site(s).

If fully acetylated $H3_5$ can be phosphorylated, why isn't an $H3_6$ species observed? If 3% of $H3_5$ were phosphorylated in the butyrate-treated cultures, only 0.1–0.2% of the total H3 would be shifted from $H3_5$ to the $H3_6$ position. Such a small percentage of $H3_6$ is beyond the limits of detection by our methods.

Dephosphorylation of Histone H1 in Butyrate-Arrested Cultures. Electrophoresis of the perchloric acid extracted proteins (Johns H1) from cultures treated for 24 h with butyrate reveals two prominent changes that occur with increasing butyrate concentration (Figure 16). First, there is a change in the relative intensity of H1 bands, and, second, there is an increase in the integrated intensity (relative to H1 and HMG proteins) of a set of bands (BEP) whose electrophoretic mobility is between those of H1 and the marker bands in Figure 16.

With regard to changes in the H1 subfraction distribution, increasing the concentration of butyrate in the culture leads to a reduction in the relative amount of H1 that is found in the slower migrating bands, numbered 2–5 in Figure 17. This type of change in the electrophoretic pattern of histone H1 has been observed for cultures arrested in G_1 by isoleucine deprivation, and it has been correlated with dephosphorylation of histone H1 (Hohmann et al., 1976). Both preparative electrophoresis (Gurley et al., 1975) and peptide mapping (Hohmann et al., 1976) have shown that phosphate is not incorporated into H1 for CHO cultures arrested in G_1 by isoleucine deprivation (Tobey & Ley, 1971).

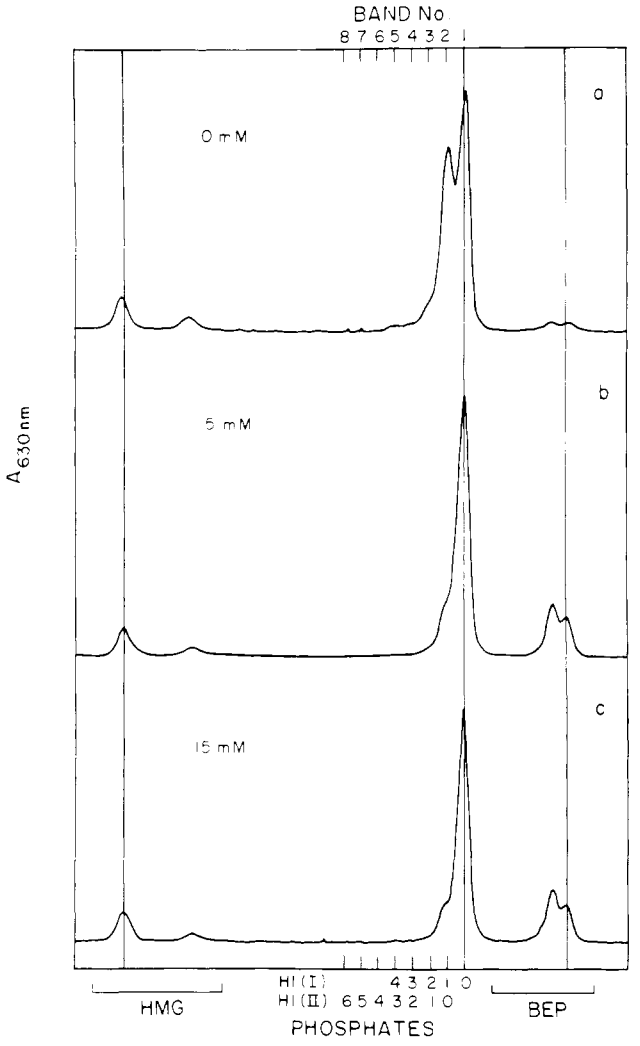

FIGURE 17: Electrophoretograms (630 nm) of perchloric acid extracted proteins from cells grown 24 h in the presence of 0, 5.0, and 15.0 mM sodium butyrate. The "band numbers" at the top of the figure are used simply to identify different H1 bands. H1(I) and H1(II) refer to the major and minor subfractions seen in the two-dimensional gel of Figure 7d. The numbers 0, 1, 2, 3, ..., etc. at the bottom of this figure refer to the number of phosphates per molecule in the major and minor subfractions at that band position.

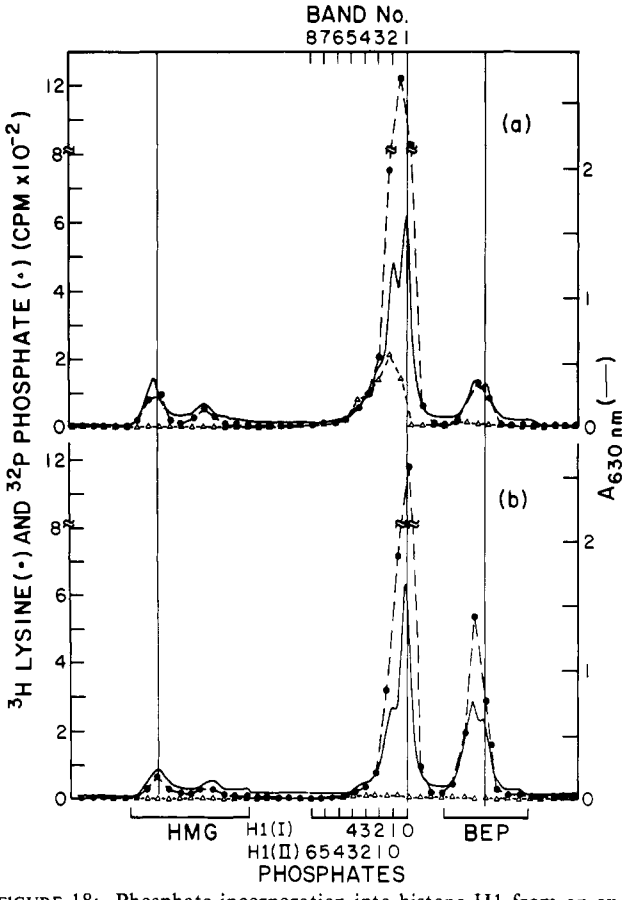

FIGURE 18: Phosphate incorporation into histone H1 from an exponentially growing culture (a) and from a culture treated 24.5 h with 15 mM sodium butyrate (b): (●) [^{3}H]lysine, (△) [^{32}P]phosphate, and (—) absorbance at 630 nm. Histone H1 was extracted from blended whole cells with 5% perchloric acid. Electrophoresis was performed in 2.5 M urea–15% acrylamide–acetic acid gels. As in Figure 17, the band numbers at the top of the figure simply identify the different H1 bands, and H1(I) and H1(II) refer to the major and minor subfractions of histone H1.

Since the electrophoretic pattern of H1 from butyrate-arrested G_1 cultures (Figure 17c) is very similar to that of H1 from cells arrested in G_1 by isoleucine deprivation (Hohmann et al., 1976; Gurley et al., 1978a; D'Anna et al., 1978), we have examined the ability of butyrate-treated cultures to incorporate [^{32}P]phosphate into H1. Analytical gel electrophoresis of H1 from pulse-labeled cultures (Figure 18) shows that H1 incorporates ^{32}P at very low levels in the butyrate-treated culture, compared with an untreated control. The $^{32}P/^{3}H$ ratio is only 0.025 in the butyrate-treated culture (Figure 18b), but it is 0.23 in the control (Figure 18a). Therefore, cells arrested in G_1 by butyrate treatment, like cells arrested in G_1 by isoleucine deprivation (Hohmann et al., 1976; Gurley et al., 1973, 1975), incorporate phosphate at very low levels.

We note that the positions and relative intensities of H1 bands 1 and 2 in Figures 17b,c and 18b (unphosphorylated H1 from butyrate-treated cultures) are comparable with the first-dimension positions and relative intensities of the two H1 subfractions, H1(I) and H1(II), in the two-dimensional gel of Figure 7d (also from butyrate-treated cultures). Thus, these data indicate that bands 1 and 2 in Figures 17b,c and 18b are really the unphosphorylated species $H1(I)_0$ and $H1(II)_0$ of two different H1 subfractions which are described in the *abscissa* of Figures 17 and 18. This conclusion confirms the previous assignment of Hohmann et al. (1976). The data also verify the assumption that an increase in the percentage of H1 in band position 1 corresponds to an increase in the quantity of unphosphorylated histone H1 (Gurley et al., 1978a,b; D'Anna et al., 1978).

Using the electrophoretic pattern of unphosphorylated H1 from cells arrested in G_1 by isoleucine deprivation as a reference and previous assumptions (Gurley et al., 1978a), we have estimated the percentage of unphosphorylated H1 in the perchloric acid extracts of butyrate-treated cultures (Figure 19). The percentage of unphosphorylated H1 increases from ~52% in control cultures to ~93% at butyrate concentrations greater than or equal to 5 mM. At the same time, the percentage of G_1 cells increases from 52 to 88% (Figure 19). Although the percentages of unphosphorylated H1 are not the same as the percentages of cells in G_1, both sets of data exhibit increases which plateau at 2.5 mM butyrate. It therefore appears that in butyrate-treated cultures the dephosphorylation of H1 is correlated with the increased proportion of cells in G_1 and G_1 arrest. Such a correlation is consistent with previous observations that H1 becomes dephosphorylated in early G_1 during normal cell-cycle progression (Gurley et al., 1975) and in cultures arrested in G_1 by isoleucine deprivation (Gurley et al., 1973, 1975).

Table I: Quantitation[a] of Inner Histone Species Separated on Two-Dimensional Gels

[butyrate] (mM)	method[b]	origin[c]	extract[d]	% of each histone			
				$H2A_0$	$H2A_1$	$H2A_2$	$H2A_3$
0	OD	cell	Eth	65	29	5	2
0	^3H	cell	Eth	68	24	6	2
10	OD	nuc	WH	50	33 ± 2	15 ± 2	2
10	MD_1	nuc	WH	49	38	12	1
10	MD_2	nuc	WH	50	35	11	4
15	OD	cell	Eth	41	43	13	4
15	^3H	cell	Eth	40 ± 2	46 ± 2	11	4

[butyrate] (mM)	method[b]	origin[c]	extract[d]	% of each histone				
				$H2B_0$	$H2B_1$	$H2B_2$	$H2B_3$	$H2B_4$
0	OD	cell	H2B	83	14	3	0	0
0	^3H	cell	H2B	85	12	3	0	0
10	OD	nuc	WH	33	24	20	15	7
10	MD_1	nuc	WH	34 ± 2	26	22 ± 2	13	6
10	MD_2	nuc	WH	36	25	23	12	4
15	OD	cell	H2B	31	24	18	14	13
15	^3H	cell	H2B	34	22	20	14	11

[butyrate] (mM)	method[b]	origin[c]	extract[d]	% of each histone					
				$H3_0$	$H3_1$	$H3_2$	$H3_3$	$H3_4$	$H3_5$
0	OD	cell	Eth	55 ± 2	29	13 ± 3	2	0	0
0	^3H	cell	Eth	59 ± 2	28 ± 2	11	2	1	0
10	OD	nuc	WH	19	19 ± 2	27	17 ± 2	11	6
10	MD_1	nuc	WH	17	21 ± 2	34 ± 2	15 ± 2	10 ± 2	3
10	MD_2	nuc	WH	17	23	27	20	8	4
15	OD	cell	Eth	14	17	33 ± 3	19 ± 2	14	4
15	^3H	cell	Eth	9	18	37 ± 3	23 ± 3	9	5

[butyrate] (mM)	method[b]	origin[c]	extract[d]	% of each histone				
				$H4_0$	$H4_1$	$H4_2$	$H4_3$	$H4_4$
0	OD	cell	Eth	56	32 ± 2	9	2	1
0	^3H	cell	Eth	55	32	7	4	2
10	OD	nuc	WH	9	21	23	21	25
10	MD_1	nuc	WH	6	24	24	22	24
10	MD_2	nuc	WH	7	23	22	22	26
15	OD	cell	Eth	8	22	22	20	28
15	^3H	cell	Eth	8	18	21	20	33

[a] Grouped values indicate that two or more methods were used for the same sample. The errors in the computed numbers refer to measured variation between two separate runs and/or differences in the analyses on the curve resolver. No error is listed if it is ±1. [b] Method refers to the method of quantitation. OD is method 1 which is direct absorbance on the Gilford spectrophotometer. ^3H is method 2 which is scintillation spectrometry of radiolabeled histones. MD_1 is microdensitometry procedure 1 and MD_2 is microdensitometry procedure 2. [c] Origin refers to histones extracted from isolated nuclei (nuc) or from the chromatin of blended whole cells (cell). [d] Extract refers to the method of extraction. WH is whole histone extracted by H_2SO_4. Eth refers to the H2A, H3, H4 histone mixture extracted with ethanol by the first method of Johns (1964), and H2B is the H2B fraction extracted by the first method of Johns.

Butyrate-Enhanced Protein. In addition to hyperacetylation of the inner histones and dephosphorylation of histone H1 (because of the induction of G_1 arrest), butyrate also stimulates an increase in a protein which we have called the butyrate-enhanced protein (BEP). BEP can be extracted by 5% perchloric acid (Figure 16) and by 0.20 M H_2SO_4 (Figure 7). It also can be extracted with equal efficiency from both the chromatin of blended whole cells and isolated nuclei. Thus, it is unlikely that the large quantities of extracted BEP arise from cytoplasmic contamination.

In long urea–acrylamide–acetic acid gels (Panyim & Chalkley, 1969a), BEP migrates in front of histone H1 (Figures 16 and 17) as four bands of which the two major bands comprise ~85% of BEP. In $NaDodSO_4$ gels (Figure 7) BEP also migrates in front of H1, but it migrates as a single band (M_r 2.2×10^4). The amount of induced BEP (after 24 h in butyrate) increases as a function of butyrate concentration (Figure 19). In going from 0 to 10 mM butyrate, there is approximately a fourfold increase in BEP so that its band area increases from 6% of that of H1 in control cultures to 32% of H1 at 7.5 mM butyrate. While the H1/BEP ratio is greater at the higher butyrate concentrations (where the cells have a reduced proliferative capacity), there is no direct correspondence between the increase in the BEP/H1 ratio and the percentage of cells in G_1 at the end of 24 h in the presence of butyrate.

The presence of four BEP bands in urea–acrylamide–acetic acid gels (Figures 17 and 18) as compared with only one band in $NaDodSO_4$ gels (Figure 7) suggests that BEP contains four sequence subfractions of the same molecular weight or that one or more BEP subfractions are modified so as to alter their charge. Examination of the phosphate incorporation profiles of Figure 18 indicates that the two major bands (~85%) of BEP do not arise from phosphorylation in butyrate-treated cultures. This is concluded from comparison of the $^{32}P/^3H$ ratios of H1 and BEP from butyrate-treated cultures (Figure 18b). Since (1) the $^{32}P/^3H$ ratios of H1 and BEP are similar in Figure 18b, (2) H1 and BEP contain similar numbers of

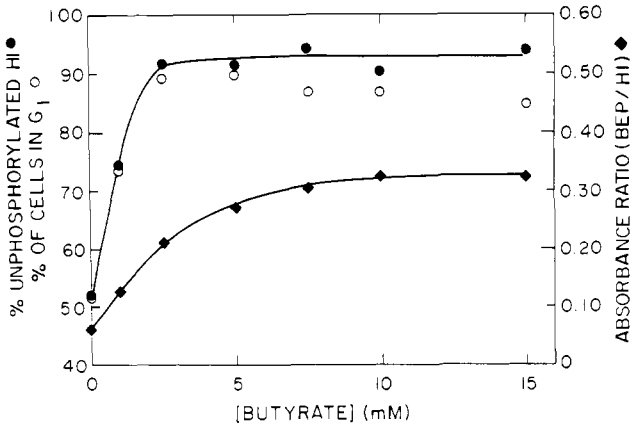

FIGURE 19: Percentage of unphosphorylated H1 (●), the ratio of the integrated absorbance of BEP to that of H1 (◆), and the percentage of cells in G_1 (○) plotted as functions of butyrate concentration. The percentages of cells in G_1 were determined from FCM analyses of samples taken when the cultures were harvested. Lines are drawn through the percentages of unphosphorylated H1 and the ratio of BEP to H1.

lysines per molecule (unpublished results), and (3) only ~7% of the H1 molecules are phosphorylated in butyrate-treated cultures (Figure 19), it follows that only ~7% of the BEP molecules are phosphorylated in the butyrate-treated cultures. [Recent unpublished results support this conclusion. Alkaline phosphatase treatment (Lake et al., 1972) of BEP from butyrate-treated cultures does not alter the electrophoretic mobilities or distribution of the BEP bands.] Although very little phosphate incorporation can be seen in BEP from exponentially growing cultures (Figure 18a), other unpublished results indicate that BEP, like histone H1, can be phosphorylated in a cell cycle dependent fashion.

The extraction properties of BEP and its mobility in urea–acrylamide–acetic acid gels (Figures 16–18) suggest that BEP is an analogue of calf lung histone $H1_0$ (Panyim & Chalkley, 1969b). The increased proportion of BEP induced by butyrate and its mobility on $NaDodSO_4$ gels suggest that it is also related to protein IP_{25} which is induced in erythroleukemia cells by treatment by dimethyl sulfoxide and hexamethylenebisacetamide (Keppel et al., 1977, 1979) and by butyrate (Candido et al., 1978). Although $H1_0$ and IP_{25} do not appear to be degradation products (Panyim & Chalkley, 1969b; Keppel et al., 1977), we cannot yet ignore such a possibility for BEP. However, if BEP is an H1 degradation product, there is no concomitant loss of H1 as the amount of BEP increases. This has been determined from electrophoretic analysis of the sulfuric acid extracts from isolated nuclei of butyrate-treated and untreated control cultures. The ratio of the integrated intensity of H1 relative to H4 is 0.42 ± 0.02 in both exponentially growing control cultures and cultures treated 24 h with 10 mM sodium butyrate. This constancy of H1 mass relative to other histones has also been reported during induction of protein IP_{25} (Keppel et al., 1977). On the other hand, Riggs et al. (1977) have found an apparent increase in the amount of H1 in HeLa cells and Friend cells treated with butyrate. The origin of the discrepancy is not known.

Discussion

In these investigations we have sought to define the effects of sodium butyrate upon cell-cycle progression in CHO cells, its effect upon histone modifications, and its possible effect upon other observed proteins. We also have looked for correlations between changes in cell-cycle progression and changes in molecular species.

Our data demonstrate that butyrate treatment decreases the probability that cells will traverse from G_1 into S and that the effect is dependent upon butyrate concentration. At low butyrate concentrations, some cells continue to traverse through G_1, and cell growth continues at least during the first 24 h of exposure. These results extend previous studies which show that mouse erythroleukemia cells become arrested in G_1 after 31 h of treatment with 1.0–1.5 mM butyrate (Rastl & Swetly, 1978). Our data show that in addition to G_1 arrest, butyrate also decreases the rate of cell-cycle progression through G_2 and/or M and, perhaps, S phase as well. Although butyrate may slow cell progression in all phases of the cell cycle, it does not arrest CHO cells indiscriminately; rather, the cells become arrested predominantly in G_1.

Since these cell-cycle studies were completed, Fallon & Cox (1979) have reported effects of butyrate upon cells initially located in different phases of the cell cycle. Their data indicate that butyrate also arrests HeLa cells in G_1; however, when S phase HeLa cells (obtained by double thymidine block) are exposed to butyrate, a large proportion of HeLa cells become arrested in G_2. Although butyrate causes transient increases in the proportion of CHO cells in G_2 (Figure 4), our data do not indicate permanent arrest. Therefore, it appears that (S phase) HeLa cells are more susceptible than CHO cells to G_2 block.

Butyrate treatment induces both hyperacetylation and dephosphorylation of CHO histones. The inner histones become hyperacetylated (Riggs et al., 1977; Hagopian et al., 1977; Candido et al., 1978; Sealy & Chalkley, 1978a; Vidali et al., 1978), while H1 is dephosphorylated, because of cell-cycle arrest in G_1. Two-dimensional polyacrylamide electrophoresis and radiolabeling in butyrate-treated CHO cultures indicate the presence of at least one site of internal acetylation in histone H2A, four sites of internal acetylation in H2B, five sites of internal acetylation in H3, and four sites of internal acetylation in histone H4. Histone H2A is appreciably phosphorylated, so that it is acetylated and phosphorylated at a total of up to three sites. Histone H3 is phosphorylated to a small degree in butyrate-treated cultures as would be expected for cultures in which only 2–3% of the cells were in mitosis (Gurley et al., 1978a). We emphasize that while we have deduced the presence of several possible sites of histone modification, other sites may exist in quantities which are below our limits of detection.

The numbers of possible histone acetylation sites detected in this work agree remarkably well with those obtained from sequence studies of radiolabeled histones. Candido & Dixon (1971, 1972a,b) have identified the sites of ϵ-NH_2-acetylation in lysine residues of trout testes histones. They found at least one site of internal acetylation in H2A, four sites in H2B, four sites in H3, and four sites in H4. Thwaits et al. (1976a,b) have determined the internal sites of in vitro enzymatic acetylation in calf thymus histones H3 and H4. They found five sites of internal acetylation in H3 and four sites in H4. Since our labeling experiments indicate that all modified histone species are present (in miniscule amounts in some cases) in control cultures, it appears that butyrate does not induce acetylation at novel sites. This result corroborates this deduction from other studies which show that butyrate inhibits deacetylation of inner histones (Boffa et al., 1978; Candido et al., 1978; Reeves & Candido, 1978; Sealy & Chalkley, 1978a; Vidali et al., 1978).

Butyrate treatment decreases H1 phosphorylation in a cell cycle dependent fashion. At 2.5 mM and higher concentrations of butyrate, the percentage of cells in G_1 and the percentage

of unphosphorylated H1 remain constant. These results are consistent with previous suggestions from this laboratory (Gurley & Walters, 1971; Gurley et al., 1974, 1975, 1978a,b; Hohmann et al., 1976) and the suggestions of others (Ajiro et al., 1975; Balhorn et al., 1972, 1975; Bradbury et al., 1973a,b; Lake & Salzman, 1972; Lake et al., 1972; Marks et al., 1973; Ord & Stocken, 1968; Stevely & Stocken, 1968) that H1 phosphorylation is closely associated with cell-cycle progression (rather than with G_1 arrest).

In addition to changes in histone modifications, we have observed quantitative changes in two other proteins which, coincidentally, migrate in the H3 region of one- and two-dimensional gels. The amount of one of these proteins, BEP, increases as a function of butyrate concentration during a 24-h treatment period. In contrast to the dephosphorylation of histone H1, the increase in BEP is not directly correlated with an increased proportion of cells in G_1. On the other hand, the BEP/H1 ratio is greater at the higher butyrate concentrations where cell growth has essentially ceased. Hence, it is possible that BEP may play some role in curtailing cell proliferation or that BEP may be a specific protein whose synthesis is peculiar to butyrate-induced G_1 arrest. Detailed studies of BEP synthesis may resolve these questions and provide insight into the functional role of BEP.

As stated under Results, BEP seems to be similar to both $H1_0$ (Panyim & Chalkley, 1969b) and an inducible protein IP_{25} (Keppel et al., 1977, 1979; Candido et al., 1978). Keppel et al. (1977) have argued that IP_{25} cannot be $H1_0$, because IP_{25} contains methionine which is not found in H1 or $H1_0$. However, Medvedeva et al. (1975) have reported finding methionine-containing species of both H1 and putative $H1_0$ (Medvedev et al., 1977) in rat tissues. Therefore, it is conceivable that $H1_0$, IP_{25}, and BEP are the same protein with, perhaps, minor species variations. It is also noteworthy that Davie & Candido (1978) observed in their two-dimensional gels an "unidentified protein" that migrates like BEP does in Figure 7. Their unidentified protein appears to be associated with the template active fraction of chromatin separated by the method of Gottesfeld & Butler (1977). Very recently Keppel et al. (1979) have reported that IP_{25}, like H1, appears to be associated with nucleosomes and is located on internucleosomal regions. They suggest, however, that IP_{25} is not associated with active chromatin. Until there is sufficient data on the composition of BEP, IP_{25}, $H1_0$ from rat, and the unidentified protein of Davie & Candido (1978), their relationship to calf lung $H1_0$ and to one another must remain speculative.

The other nonhistone protein which undergoes change in the presence of butyrate is our own unidentified protein UP. It has an apparent molecular weight of ~15 000, it migrates in the $H3_{4-5}$ region of 6 M urea–12% acrylamide–acetic acid gels, it also appears to be enhanced in butyrate-treated cultures, and our unpublished observations indicate that it is phosphorylated. Very recently Palmer et al. (1979), West et al. (1979), and Glover et al. (1979) have reported the isolation of H2A-related proteins whose electrophoretic properties are similar to those of UP. Since our data indicate that UP is phosphorylated, UP also may be an H2A variant. This remains to be determined, however.

Finally, the resolution and quantitation of the species distribution of histones H2A, H2B, and H3 from butyrate-treated cultures are, to our knowledge, the first reports of their kind. Three different methods of analysis have been used, and their agreement is good. Presently, quantitation is achieved more easily when histones are isolated by the first method of Johns (1964). The removal of H2B from the inner histone fraction by this method prevents crowding of H2B with H2A and H3 so that better resolution is achieved. Ultimately, further separation of the histones and reduced spot diffusion would be helpful; nevertheless, the current two-dimensional gel system allows the resolution and quantitation of species which have not been possible with one-dimensional gels.

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