

Different Accessibilities in Chromatin to Histone Acetylase*

(Received for publication, July 20, 1978)

Lawrence S. Cousins, Dieter Gallwitz,‡ and Bruce M. Alberts

From the Department of Biochemistry and Biophysics, University of California at San Francisco, San Francisco, California 94143

The addition of millimolar concentrations of *n*-butyrate to tissue culture media has been shown to cause a dramatic buildup of the acetylated ϵ -aminolysine forms of cellular histones, without causing cell death (Riggs, M. G., Whittaker, R. G., Neumann, J. R., and Ingram, V. M. (1977) *Nature* 268, 462-464). We find that *n*-butyrate acts by strongly inhibiting histone deacetylation, without any apparent effect on the acetylation reaction, *per se*. In a rat hepatoma cell line (HTC cells), the presence of high concentrations of *n*-butyrate causes the *in vivo* half-life of the normally labile histone acetyl groups to be extended over 150-fold to more than 24 h. All histones naturally acetylated *in vivo* (H2a, H2b, H3, and H4) are affected, and deacetylation is inhibited regardless of the number of acetyl groups present on any particular histone. *n*-Butyrate can also be shown to be an effective *in vitro* inhibitor of partially purified histone deacetylating enzymes ($K_i \sim 60 \mu\text{M}$, noncompetitive). This *in vitro* inhibition shows the same fatty acid specificity as the *in vivo* inhibition of deacetylation, and its magnitude is sufficient to account for the full *in vivo* effect. An intriguing finding is that a substantial subclass of each acetyltable histone remains totally unacetylated, even at the highest concentrations of *n*-butyrate. At the other extreme, we show that a readily acetylated class of histones responds to *n*-butyrate treatment much more rapidly, and to a larger extent, than do the bulk histones. More generally, the manner in which the acetylated histones accumulate suggests the existence of special nucleosome environments within the cell, which differ markedly in their accessibility to the histone acetylase enzyme(s).

Two each of the histones H2a, H2b, H3, and H4 complex together with 140 base pairs of DNA to form the basic subunit of chromatin, the nucleosome (for reviews, see Refs. 1 and 2). The amino acid sequences of these four proteins have been unusually highly conserved during evolution (particularly H3 and H4), indicating that even a slight change in their amino acid side chains has a major effect on their biological function (3). Yet these same proteins undergo a variety of postsynthetic covalent modifications which substantially alter their local charge and structure (4, 5). Because these modifications are incomplete (only a small fraction of the potential sites are modified at any one time), they introduce a heterogeneity in

* This work was supported by Grant 23928 from the National Institutes of Health (to B. M. A.) and, in part, by a grant from the Deutsche Forschungsgemeinschaft (to D. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address, Institut für Physiologische Chemie I, Institutsgruppe Lahnberge der Medizinischen Fakultät der Philipps-Universität, Marburg (Lahn), West Germany.

nucleosome structure into the genome. This heterogeneity is assumed to be of major biological consequence (4-6), but its real significance is unknown.

The most extensive of the known modifications of the nucleosomal histones is their partial acetylation at specific ϵ -amino groups of lysines located near each histone NH_2 -terminus (4, 5). These acetyl groups turn over rapidly (7, 8), presumably due to the action of specific deacetylating enzymes (9-11). Yet, in spite of the recent dramatic advances in our knowledge of chromatin structure, nothing is known about the reason for this rapid turnover of acetyl groups or the role that these modifications play in chromatin function. Suggestive correlations can be made between histone acetylation and gene activity (reviewed in Ref. 4). However, it is not certain that acetylation is directly involved in gene expression, as has been postulated for many years.

Recently, Riggs *et al.* (12) showed that addition of millimolar concentrations of sodium *n*-butyrate to tissue culture medium dramatically increases the level of histone acetylation in cultured cells. The potential usefulness of *n*-butyrate as a tool in the study of the biological role of histone acetylation depends on an understanding of its mode of action. For example, *n*-butyrate might cause histone acetylation at sites not normally modified *in vivo*, the problem encountered in chemical acetylation of chromatin by acetic anhydride (13, 14). In this report, we present evidence that *n*-butyrate causes accumulation of properly acetylated histones, since it acts by specifically inhibiting their deacetylation.

In addition, our studies suggest that there is a marked heterogeneity in nucleosomal environments within the cell. For example, there appears to be a minor class of nucleosomes which is essentially inaccessible to modification by the histone acetylase, as well as another class which is unusually susceptible.

MATERIALS AND METHODS

[³H]Acetate Labeling Protocol—HTC¹ cells, a permanent line of rat hepatoma cells, were grown on the surface of 100-mm Petri dishes in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. When the cells were at a density of 10^7 cells/plate, all but 2 ml of medium was removed and 2 mCi sodium [³H]acetate (4 Ci/mmol from Amersham, kept frozen as a 50 mCi/ml stock solution) was added for the desired time. In pulse-chase experiments, the radioactive medium was removed, the adhered cells were quickly washed three times with 2 ml of 37°C medium, and the incubation was continued at 37°C with 20 ml of nonradioactive, prewarmed medium. In all cases, the incubation was terminated by removing the medium, washing the plate with 0°C phosphate-buffered saline (0.8% NaCl, 0.02% KCl, 0.22% $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.02% KH_2PO_4 , 0.01% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01% CaCl_2), and then immediately placing the plate in a -80°C freezer. Controls show that cells frozen in this way yield histones with extents of acetylation identical to those from unfrozen cells and that there is no significant histone deacetylation following the initial chilling of the cells.

¹ The abbreviation used is: HTC, rat hepatoma tissue culture cell line.

For large scale preparation of labeled histones used as substrate for *in vitro* deacetylase assays, 1 liter of HTC cells grown in suspension (5×10^6 cells/ml in Swins S-77 medium plus 10% calf serum) was centrifuged, resuspended in 50 ml of the supernatant, and labeled for 15 min with 40 mCi of [^3H]acetate (8). The cells were then chilled by addition of a large volume of 0°C phosphate-buffered saline, centrifuged, and washed once with 0°C phosphate-buffered saline before isolation of the histones.

Histone Isolation—Histones were isolated using the basic procedure of Jackson *et al.* (8). The washed cells were detached from Petri dishes and suspended in 0°C lysis buffer (10 mM Tris, 50 mM sodium bisulfite, 1% Triton X-100, 10 mM MgCl_2 , 8.6% sucrose, pH 6.5) by scraping with a rubber policeman. After Dounce homogenization (four strokes, tight pestle), the nuclei were washed three times in the above lysis buffer, and then once in 10 mM Tris-HCl, 13 mM Na_3EDTA (pH 7.4). The pellet was either blended on a Vortex mixer or sonicated into suspension in 0.5 ml of 0°C H_2O (using the lowest power of a Biosonik sonicator (Bronwill Scientific) for one or two short blasts, just until the pellet was in suspension). After addition of concentrated H_2SO_4 to 0.4 N, the preparation was incubated at 0°C for 1 h and then centrifuged for 10 min at 10,000 rpm in a Sorvall SS-34 rotor. The supernatant was removed and mixed with 10 volumes of acetone. After the precipitate had coagulated overnight at -20°C , it was collected by centrifugation and air-dried. This histone was either dissolved in water (for determination of radioactivity per μg of protein) or in a buffer consisting of 6 M urea, 0.9 M acetic acid, and 5% β -mercaptoethanol (for gel electrophoresis).

Analytical Methods—Protein was quantitated by a modification of the procedure of Lowry (15). Radioactivity was determined by scintillation counting in Biofluor scintillant (New England Nuclear). For acid-urea-acrylamide gel electrophoresis (0.9 M acetic acid, 2.5 M urea, 15% acrylamide) on a slab gel ($\frac{1}{2} \times 6.5 \times 6.5$ inch), the procedure of Panyim and Chalkley (16) was modified to include an upper gel above the separating gel they described. This gel, made with half the concentration of acrylamide and *N,N'*-bisacrylamide, was deaerated prior to polymerization and then used to form sample wells so that the sample migrated 0.7 cm before reaching the separating gel. The slab was pre-electrophoresed at constant voltage until the amperage remained constant, and then 5 to 20 μl of sample was loaded into each well. Triton acid-urea gel electrophoresis was with 8 M urea and 0.37% Triton X-100, as described by Cohen *et al.* (17), and modified for slab gels as above. After electrophoresis for 13 h at 120 V, gels were stained in a mixture of water/acetic acid/methanol (5:1:5) containing 0.25% Coomassie blue for 30 min and then destained in this solution without Coomassie blue for 2 to 3 h. Gels were photographed, prepared for fluorography (18, 19), and dried under heat and vacuum onto Whatman SG-81 paper. Dried gels were exposed at -70°C to hypersensitized Kodak X-Omat R film (19). When fluorography was unnecessary, the destained gel was dried between sheets of dialysis membrane (Bio-Rad). To do this, a sandwich was made consisting of a glass plate, a sheet of wet membrane, the gel, and another wet membrane. Water was poured between each layer to prevent formation of air bubbles. Four plastic strips were placed on top of the final membrane, along the edges of the gel. These were clamped onto the glass plate to seal the edges, and the gel was then left to dry at room temperature and pressure in a fume hood for 2 days.

Preparation of Histone Deacetylases—A detailed description of the purification of calf thymus histone deacetylases used (20) will be published elsewhere.² Briefly, frozen calf thymus tissue was homogenized in buffer (75 mM Tris-HCl, 1 mM MgCl_2 , 0.25 mM Na_3EDTA , 5 mM β -mercaptoethanol, 10% v/v glycerol, pH 7.9) containing 1 M $(\text{NH}_4)_2\text{SO}_4$. The viscous homogenate was sonicated, and the deacetylases were precipitated by raising the concentration of $(\text{NH}_4)_2\text{SO}_4$ to 3.5 M. The precipitated protein was dissolved in a buffer consisting of 15 mM Tris-HCl, 10 mM NH_4Cl , 0.25 mM Na_3EDTA , 5 mM β -mercaptoethanol, and 10% v/v glycerol, pH 7.9, and then desalted by chromatography on Sephadex G-25 in this buffer. The preparation was then loaded onto a DEAE-cellulose column equilibrated with the same buffer and eluted with a linear 10 to 500 mM gradient of NH_4Cl . Two peaks of deacetylase activity were recovered: "deacetylase I" eluted at about 250 mM NH_4Cl and "deacetylase II" eluted between 300 and 350 mM NH_4Cl . The eluates were then adsorbed to hydroxylapatite columns equilibrated with 20 mM sodium phosphate, 0.25 mM Na_3EDTA , 5 mM β -mercaptoethanol, and 10% v/v glycerol, pH 6.8, and eluted with linear 20 to 600 mM sodium phosphate gradients. Deacetylase I eluted between 250 and 300 mM sodium phosphate, and

deacetylase II eluted between 200 and 400 mM sodium phosphate. The enzymes were each concentrated in an Amicon concentrator (Minicon B-15) before storage in small aliquots at -80°C . Deacetylase I was estimated to be purified 60-fold in specific activity, and deacetylase II about 150-fold, from the initial soluble extract.

Histone Deacetylase Assay—In order to measure initial rates for kinetic studies, an assay was needed which accurately measured conversion of a small fraction of the substrate to product. The following method was therefore developed. In a volume of 20 μl , the standard assay contained 9 μg of *in vivo*-labeled [^3H]acetate histone (3300 cpm) in a buffer consisting of 15 mM potassium phosphate (pH 7), 4% v/v glycerol, and 0.2 mM Na_3EDTA . After 1.5 min at 37°C , the reaction was stopped by the addition of an equal volume of a cold solution containing 0.5 N HCl, 0.1 M acetic acid, and 1 mg/ml of carrier histone (Worthington Biochemicals). To this was added 40 μl of a 0°C saturated solution of ammonium reinecke salt (ammonium tetrathiocyanodiammonochromate, Sigma). After 30 min at 0°C, the histone substrate was quantitatively precipitated (21), leaving released [^3H]acetate in the supernatant. The precipitate was removed by centrifugation for 5 min in an Eppendorf 3200 centrifuge. Fifty microliters of the supernatant were counted directly in scintillation fluid. Controls were performed to assure that the assay conditions determined enzyme activity in the linear range of response with respect to both added enzyme and incubation time.

RESULTS

n*-Butyrate Inhibits Histone Deacetylation *in Vivo—The fact that a large increase in acetylated histones is observed after treatment of tissue culture cells with *n*-butyrate (12) might suggest that this compound increases the rate of histone acetylation. However, since histone acetyl groups are normally subject to rapid turnover (7, 8), this observation could equally well be explained if *n*-butyrate inhibits histone deacetylation. To test the first possibility, [^3H]acetate incorporation into histone was measured during a 1-min [^3H]acetate pulse of HTC cells with and without 5 mM *n*-butyrate present (*n*-butyrate added 15 min prior to the pulse). Equivalent incorporation was observed under both conditions.³ This concentration of butyrate was also found to have no significant effect on the *in vitro* acetylation of histones in a standard histone acetylase (22) assay.³

Because of the above results, it seemed unlikely that *n*-butyrate functions by increasing histone acetylation rates. To test whether the *n*-butyrate effect is instead due to decreased histone deacetylation, HTC cells were labeled for 15 min with [^3H]acetate and the label incorporated into histone was then measured as a function of the time of further incubation in nonradioactive chase medium. Results obtained with 0, 5, and 100 mM *n*-butyrate present in the chase medium are plotted in Fig. 1 as per cent [^3H]acetate label in histone remaining at various times. From these data, it is clear that *n*-butyrate drastically reduces [^3H]acetate turnover; in fact, the rate of histone deacetylation appears to be decreased 40-fold in 5 mM *n*-butyrate, and over 150-fold in 100 mM *n*-butyrate.

In order to determine the effect of *n*-butyrate inhibition of acetate turnover on each individual histone, HTC cells were pulsed for 6 min and then chased for 10 and 60 min in the presence or absence of 5 mM *n*-butyrate. After purification of the histones from each time point, they were electrophoresed on an acid-urea gel, which allows both the individual histones and their variously acetylated species to be resolved (16, 23). The gel was then fluorographed to determine the distribution of [^3H]acetyl groups among the various histones. As shown in Fig. 2, the fastest running histone in this system is H4, with mono-, di-, tri-, and tetraacetylated H4 forms migrating progressively slower than the unacetylated form of H4, which is not visible on this fluorogram since it is unlabeled by [^3H]acetate (although it is the predominant species on a stained gel). Monoacetylated forms of H2a and H2b are also clearly

² S. Bürger, D. Meyer zu Steighorst, and D. Gallwitz, in preparation.

³ L. Cousins, unpublished results.

seen, along with what appear to be mono-, di-, and triacetylated forms of H3, which are the closely spaced bands near the top of the gel section shown. Histone H4 is the most evolutionarily conserved of the histones (3), is situated at the core of the nucleosome (24–27), and is the easiest to quantitate from gels because it is best resolved. For this reason, we shall tend to focus on its behavior in discussing gel profile results; however, the other histones can be assumed to behave analogously, unless specifically stated otherwise.

Lane A of Fig. 2 shows how a brief [^3H]acetate pulse distributes into the various histone forms without a chase in unlabeled media. This is to be compared with Lanes B and C, which show the label remaining in histones after a 10-min chase without and with 5 mM butyrate present, respectively, and with Lanes D and E, which show analogous results after a 60-min chase period. Clearly, *n*-butyrate strongly inhibits the turnover of [^3H]acetate incorporated into all four of the nucleosomal histones (H2a, H2b, H3, and H4). Note also that, after the 10- and 60-min chase incubations in 5 mM *n*-butyrate (Fig. 2, C and E), the original pulse label tends to move to higher levels of acetylation. In the case of H4, for example,

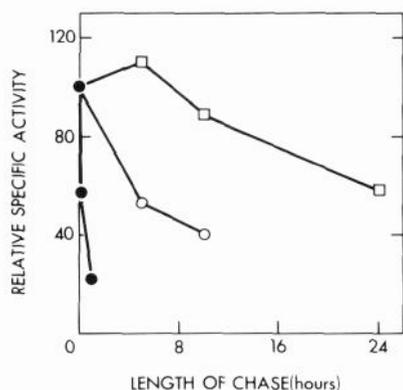


FIG. 1. Kinetics of turnover of [^3H]acetate incorporated into histones. HTC cells pulsed for 15 min with [^3H]acetate were chased for various times in medium containing 0 (●), 5 (○), and 100 (□) mM *n*-butyrate. The histones were isolated and assayed for protein and radioactivity. A relative specific activity of 100 is equivalent to 532 cpm/ μg of histone.

much of the label moves from the mono- and diacetylated positions to the tetraacetylated position. This reflects the further addition of unlabeled acetyl groups to these histones, and indicates that the deacetylation of each of the differently acetylated forms of H4 is inhibited by *n*-butyrate.

In order to determine how quickly butyrate acts, HTC cells were pretreated for 0 min (Fig. 3A), 20 min (Fig. 3B), or 60 min (Fig. 3C) with 5 mM *n*-butyrate (Fig. 3C) and then labeled with a 15-min pulse of [^3H]acetate with butyrate present. In all three cases, fluorography of an acid-urea gel shows that addition of *n*-butyrate to the medium causes a dramatic increase in the label incorporated into the highly acetylated forms of H3, and more clearly, H4. This effect is apparent even when *n*-butyrate and [^3H]acetate are added simultaneously for a 15-min pulse (in Fig. 3, compare A with D, where no butyrate was added). In fact, preincubation in *n*-butyrate for 60 min does not greatly enhance its effect (in Fig. 3, compare C and A). The changes in the labeling pattern observed in Fig. 3 will be discussed subsequently (see Fig. 8). We wish to note here only that *n*-butyrate is active inside the cell within a few minutes after its addition to the medium. This extremely rapid action would eliminate any mechanism of *n*-butyrate action requiring *de novo* RNA or protein synthesis, as in these cases there should be a significant lag period preceding any effect on deacetylation.

Histone Deacetylase Enzymatic Activity Is Strongly Inhibited by *n*-Butyrate in Vitro—*A priori*, the observed inhibition of deacetylation by *n*-butyrate *in vivo* could result from a number of different mechanisms. One of these is that *n*-butyrate could directly inhibit the deacetylating enzymes themselves. This possibility was tested by assaying two partially purified histone deacetylases prepared from calf thymus (denoted deacetylases I and II), with and without *n*-butyrate present. The assay measures the amount of [^3H]acetate removed from an *in vivo*-labeled total histone substrate, which is determined as radioactivity-soluble in reinecke salt, as described under "Materials and Methods." The results presented in Table I demonstrated a striking inhibition of this reaction by low concentrations of *n*-butyrate, 50% inhibition being observed at somewhat less than 60 μM butyrate for both deacetylases.

A detailed kinetic analysis of the *n*-butyrate inhibition of

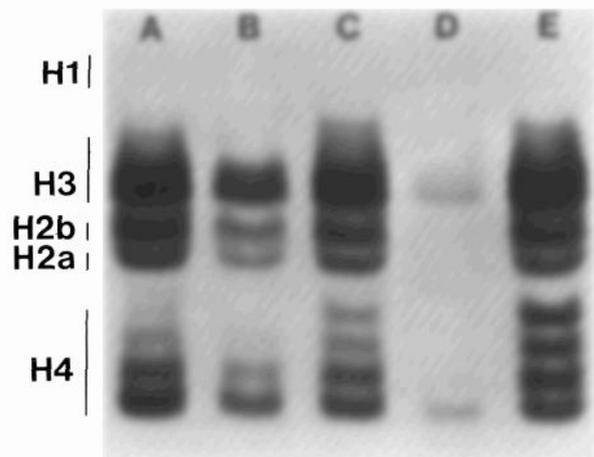


FIG. 2 (left). Acid-urea gel electrophoretic analysis of [^3H]acetate retained by histones after chase incubation. HTC cells, pulse-labeled for 6 min (A), were washed free of labeled acetate and chased for 10 min (B) or 60 min (D). Similarly treated cells were chased by adding unlabeled medium containing 5 mM *n*-butyrate for 10 min (C) or 60 min (E). The histones were isolated, electrophoresed on an acid-urea gel, and fluorographed. In this and other figures, only the histone region of the gel or fluorogram is shown.

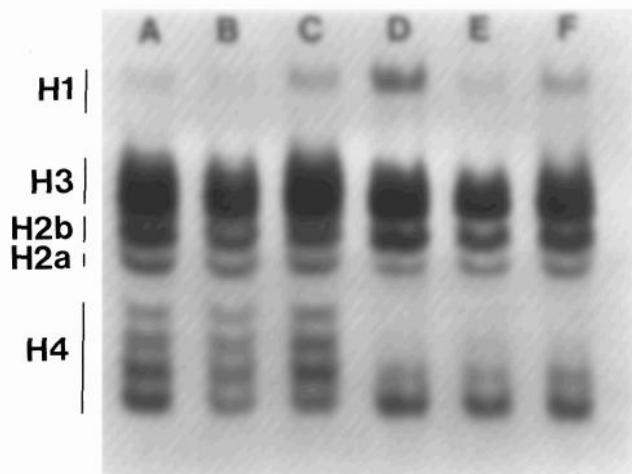


FIG. 3 (right). Effect of *n*-butyrate on [^3H]acetate uptake into histones. *n*-Butyrate was added to 5 mM simultaneously with a 15-min pulse of [^3H]acetate (A). Otherwise, it was added 20 (B) or 60 (C) min prior to labeling. Control cells received water simultaneously with labeling (D), or 20 (E) or 60 (F) min prior to labeling. The histones were isolated, electrophoresed on an acid-urea gel, and fluorographed.

TABLE I

Inhibition of calf thymus histone deacetylases by *n*-butyrate

The histone deacetylating enzymes were assayed as described under "Materials and Methods" in the presence and absence of various concentrations of *n*-butyrate. The data is presented as per cent inhibition relative to activity in the absence of *n*-butyrate.

<i>n</i> -Butyrate μM	Per cent inhibition	
	Deacetylase I	Deacetylase II
0	0	0
10	31	30
20	47	41
60	56	63
90	64	71

histone deacetylase I is shown in Fig. 4A as a Lineweaver-Burk plot of $1/v$ versus $1/S$, and in Fig. 4B as a Dixon plot of $1/v$ versus I (28). From Fig. 4A, it can be seen that the x -intercept is invariant as the concentration of *n*-butyrate is varied, whereas the y -intercept increases with increasing concentrations of this inhibitor. Thus, *n*-butyrate has no effect on the apparent K_m but decreases V_{max} , as expected for a "noncompetitive" inhibitor which acts at a site on the enzyme other than that which binds substrate. The apparent K_m of this deacetylase for histone can be calculated from the x -intercept of Fig. 4A (equal to $-1/K_m$) to be 270 $\mu\text{g}/\text{ml}$. In terms of acetyl groups (whose concentration in the substrate was determined from measurement of the amount of acetylated histone detected on a stained Triton acid-urea gel), the K_m is about 10^{-6} M. This low K_m is consistent with the claim that this enzyme is one which acts on histones *in vivo*. The K_i for *n*-butyrate can be determined from the x -intercept in Fig. 4B (equal to $-K_i$) to be about 58 μM . Extrapolation of these data to 5 mM *n*-butyrate predicts 99% inhibition of deacetylase. This value is compatible with the 96 to 98% inhibition of *in vivo* turnover observed at 5 mM butyrate (Fig. 1), and it strongly suggests that the entire *n*-butyrate effect on *in vivo* acetylation is due to this cause (note that, since the inhibition is noncompetitive, the very different concentrations of substrates *in vivo* and *in vitro* can be ignored).

To further cross-correlate *in vivo* and *in vitro* effects, we have compared the fatty acid specificity of the *in vivo* and *in vitro* deacetylation inhibitions. Fig. 5 shows the results of an experiment in which various straight chain fatty acids were added to the medium used to grow HTC cells (for 13 h at a concentration of 3 mM). From the stained acid-urea gel of histones isolated from these cells, it can be seen that *n*-butyrate leads to the greatest buildup of acetylated forms (Fig. 5D). However, *n*-propionate (Fig. 5C) and *n*-pentanoate (Fig. 5E) are also quite effective in this regard. The *in vitro* inhibition of the two partially purified deacetylases displays the same specificity, as shown in Fig. 6, where per cent inhibition is plotted against the number of carbons in the fatty acid added to the enzyme reactions. Again, although *n*-butyrate is the most potent inhibitor, *n*-propionate and *n*-pentanoate are also effective inhibitors. Note also that both deacetylase enzymes show very similar extents of inhibition by the various fatty acids.

Incidentally, these two experiments reveal a fatty acid specificity which mimics that observed for induction of Friend cell differentiation (29). However, other Friend cell inducers tested, (5 mM hexamethylenediacetamide (30), 2% dimethylsulfoxide (31), and 5 mM hypoxanthine (32), have no effect after 21 h on the HTC cell histone modifications detectable on stained acid urea gels³ (see also Ref. 12).

Nucleosomal Heterogeneity Suggested by the *in Vivo* Response to *n*-Butyrate—As an increasing concentration of *n*-

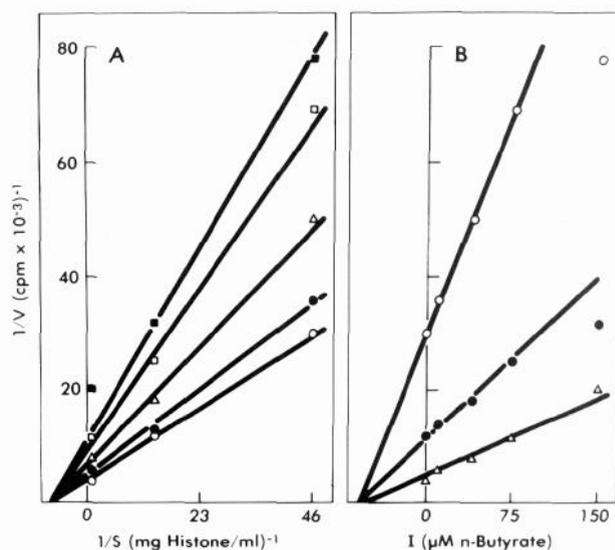


FIG. 4. Kinetic analysis of *n*-butyrate inhibition of calf thymus deacetylase I. The enzyme was assayed in reactions 5-fold larger than normal to increase the counts per min released at low substrate concentrations. Otherwise, the procedure used was that described under "Materials and Methods." In Part A, the data is presented as a $1/v$ versus $1/S$ plot at several different inhibitor concentrations. The concentrations of *n*-butyrate added were 0 (\circ), 10 (\bullet), 40 (Δ), 75 (\square), and 150 (\blacksquare) μM . In Part B, the same data are replotted as a Dixon plot (or $1/v$ versus I plot) for three substrate concentrations. Histone concentrations used were 880 (Δ), 73 (\bullet), and 22 (\circ) $\mu\text{g}/\text{ml}$. In comparing this kinetic data with *in vivo* inhibitions of histone acetyl turnover, it should be remembered that the histone substrate here contains all four histones in both acetylated and unacetylated forms, plus histone H1. This mixed "substrate" is present as free histone, rather than in the nucleosomes which serve as the substrate for the deacetylase *in vivo*. Furthermore, the *in vitro* work used calf enzymes, while the *in vivo* data are from experiments on rat cells. Nevertheless, the extent of agreement between *in vitro* and *in vivo* data is remarkable.

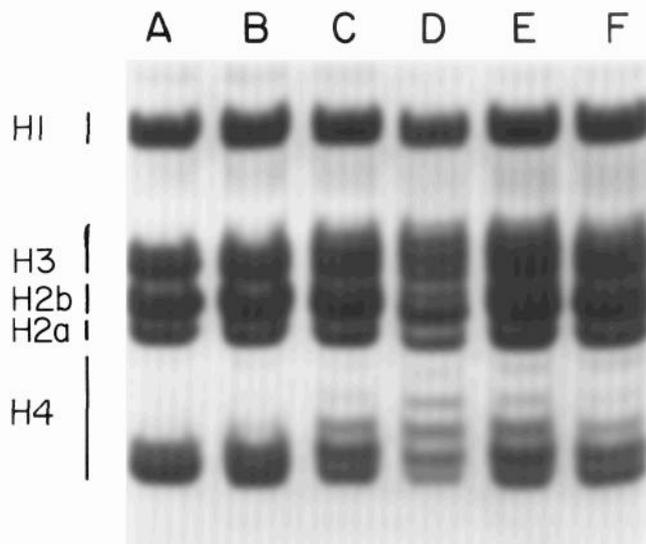


FIG. 5. Fatty acid specificity of inhibition of deacetylation *in vivo*. HTC cells were treated for 13 h in the absence of any addition (A), or in the presence of 3 mM concentrations of acetate (B), *n*-propionate (C), *n*-butyrate (D), *n*-pentanoate (E), and *n*-hexanoate (F). The histones were isolated, electrophoresed on an acid-urea gel, and stained with Coomassie blue.

butyrate is added to the culture media, the rate of histone acetyl turnover in HTC cells decreases to the point where turnover is only barely detectable (*i.e.* see Fig. 1). To concentrate on the behavior of a single type of histone, note that

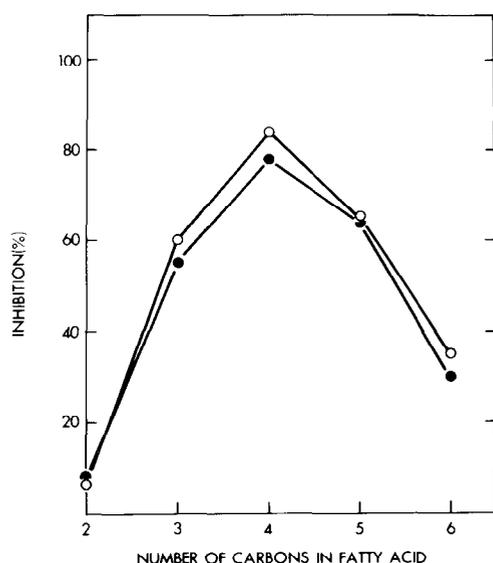


FIG. 6. Fatty acid specificity of *in vitro* inhibition of calf thymus deacetylases I (●) and II (○). The deacetylases were assayed in the presence of the indicated fatty acids at 0.25 mM and histone at 440 μ g/ml. The per cent inhibition is with respect to activity in the absence of any addition. Activity was determined in the "reinecke salt" assay described under "Materials and Methods."

normally most of the H4 molecules in HTC cells completely lack ϵ -aminolysine acetyls, and that 27% of them are monoacetylated and about 3% are diacetylated (*top panel* of the scans of the H4 region of strained acid-urea gels in Fig. 7A). Given this equilibrium point and the uninhibited rate constant for deacetylation, one can calculate that the greater than 150-fold inhibition of deacetylation which is observed at a high butyrate concentration (Fig. 1) should reduce the amount of nonacetylated H4 molecules to less than a few per cent of the total within an hour or so, if all the H4 molecules within a cell are equally accessible to acetylase enzyme(s).

Fig. 7B shows the actual proportions of each acetylated H4 species observed after a prolonged incubation, as a function of the *n*-butyrate concentration used. These appear to be equilibrium values since they do not change if the incubation time in *n*-butyrate is prolonged or if fresh media with *n*-butyrate is added.³ Note that, even for the highest *n*-butyrate concentrations, the amount of nonacetylated H4 never falls below 18% of the total. This suggests that roughly one-sixth of the nucleosomes within the cell contain H4 molecules which are essentially inaccessible to the acetylase. This conclusion is supported by the fact that a [³H]acetate pulse given after long times in *n*-butyrate barely labels the monoacetylated peak of H4, while the more highly acetylated forms are proportionately more highly labeled.³ The existence of such nucleosomal heterogeneity within the cell is also implied by the fact that total H4 acetate saturates at a relatively low concentration of *n*-butyrate, being 2.0 acetates/H4 at 15 mM butyrate (compared to a theoretical maximum of 4.0).

It is also important to note that the limit pattern of acetylation contains substantial amounts of each acetylated histone form (as illustrated for H4 by the gel scan in the *bottom panel*, Fig. 7A), suggesting that there may be several different nucleosomal environments within the cell, each with a different accessibility to acetylase.

We believe that the heterogeneity in the limit pattern of histone acetylation seen in the presence of butyrate reflects a nucleosomal heterogeneity which pre-exists prior to the actual addition of butyrate. Direct evidence for this assertion can be obtained by pulse-labeling normally growing HTC cells with

[³H]acetate, and then incubating for an extended "chase period" in nonradioactive medium containing 50 mM *n*-butyrate. Fluorographic analyses of Triton acid-urea polyacrylamide gels can then be used to follow the behavior of those histone molecules which initially labeled with [³H]acetate. These results are shown in Fig. 8B. They are to be compared with the stained gel results in Fig. 8A, which reveal the behavior of the total histones in these samples. Without *n*-butyrate present, the [³H]-labeled H4 molecules are mostly mono- and diacetylated, and these are rapidly converted in *n*-butyrate-containing medium to the tetraacetylated form. It is striking that this conversion is half-complete by 2 h and reaches a limit with an average of 3.6 acetates/acetylated H4 molecule (Fig. 8B). In marked contrast, the bulk of the H4 molecules are seen to reach their limit pattern with a half-time greater than 8 h (Fig. 8A) and never exceed an average of 2.1 acetates/H4 molecule. (If the unacetylated H4 molecules are omitted from this calculation, the average number of acetyls is still only 2.7).

In Fig. 9, we plot the per cent of H4 as the tetraacetylated species as a function of time in butyrate, for the two populations of histones in Fig. 8. Whereas 75% of the [³H]acetate prelabeled H4 histone became tetraacetylated in this experiment, only 28% of the bulk H4 histone reached this form. Such differences suggest that those H4 molecules which are acetylated in normal HTC cells are located in special chromosomal environments, where they are unusually accessible to histone acetylase. A similar conclusion with regard to the presence of a pre-existing nucleosomal heterogeneity can be reached by analyzing the behavior of histones other than H4 (see gels of Fig. 8). In particular, note that virtually all of the [³H]acetate-prelabeled H2b becomes tetraacetylated, whereas the total H2b population still contains much of the nonacetylated and partially acetylated species.

It should be noted that heterogeneity of the cellular population provides a possible alternative explanation for these results. Although we cannot eliminate this possibility, we feel that it is not likely to be the major factor. Thus, trypan blue exclusion reveals less than 1% nonviable cells after 24 h in 50 mM *n*-butyrate, and similar heterogeneous *n*-butyrate limit

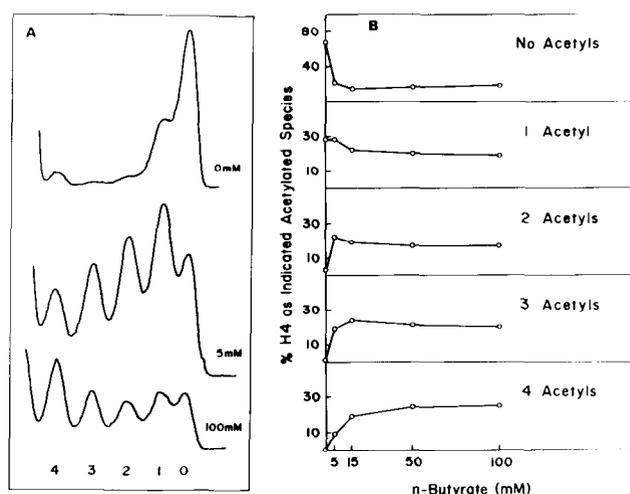


FIG. 7. High concentrations of *n*-butyrate reveal the limit of H4 acetylation. In *Part A* gel scans are shown of the H4 regions of stained acid-urea gels. Cells were treated at the indicated concentration of *n*-butyrate for 20 h before isolation and acid-urea gel electrophoresis of the histones. The numbers beneath the peaks of the gel scans indicate the number of acetyl groups present. In *Part B*, the per cent H4 as each differently acetylated species is plotted versus the concentration of *n*-butyrate. These data have been calculated from peak heights of the gel scans of acid-urea gels (some of which are shown in *Part A*).

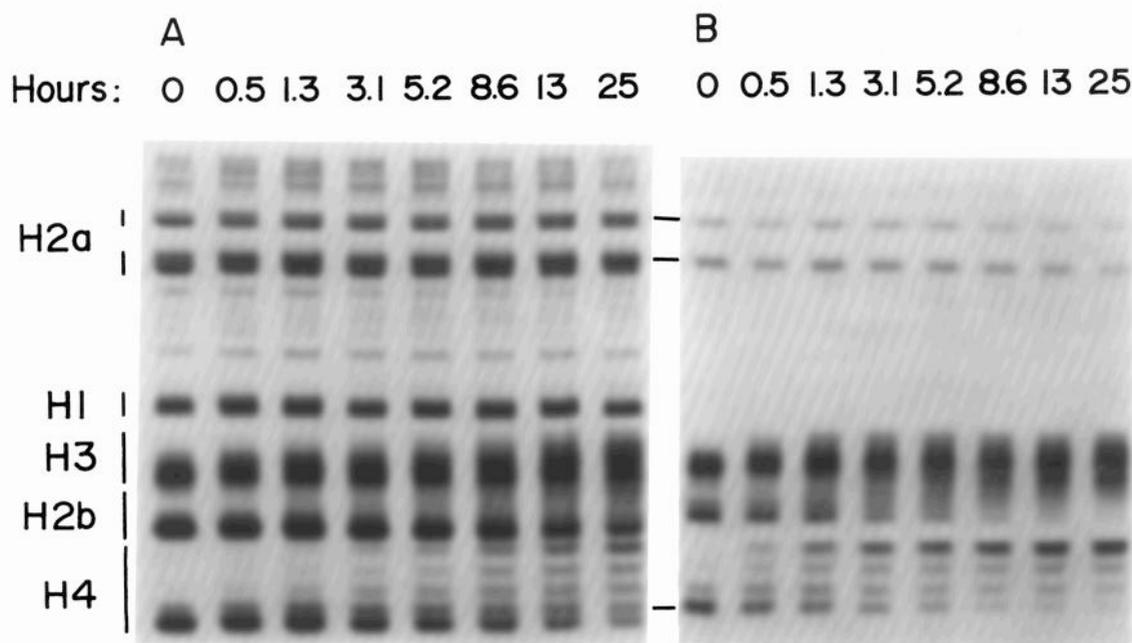


FIG. 8. During a chase incubation in *n*-butyrate, [^3H]acetate-pre-labeled histones reach higher levels of acetylation, and respond more rapidly, than does the total population of histones. HTC cells, pulse-labeled for 10 min, were incubated further in nonradioactive medium containing 50 mM *n*-butyrate. The histones were isolated as described

under "Materials and Methods." From each time point, equal amounts of protein were electrophoresed on the Triton acid-urea gel in Part A (Coomassie-stained), and equal amounts of ^3H label on the gel shown in Part B (fluorographed). The length of the chase incubation for each sample is indicated in hours above each gel lane.

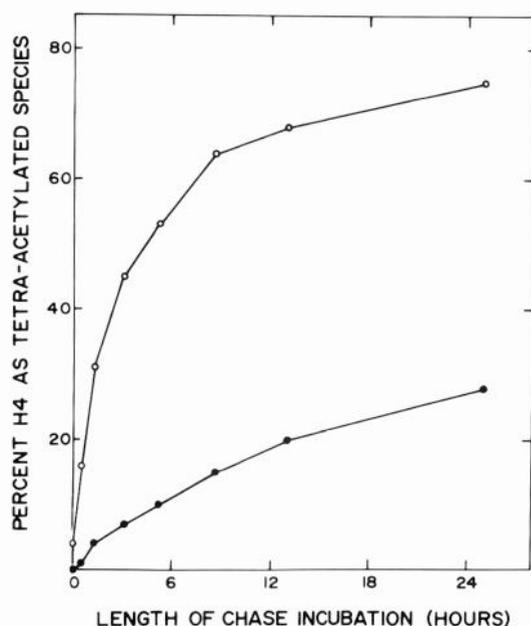


FIG. 9. When *n*-butyrate is added, [^3H]acetate-pre-labeled H4 histones become tetraacetylated more rapidly, and to a greater extent, than does the total population of H4 molecules. Gel scans of each lane of the gels shown in Fig. 8 are used to quantitate the per cent protein (●) and the per cent [^3H]label (○) as the tetraacetylated species of histone H4, plotted as a function of time of incubation in *n*-butyrate.

patterns are obtained for the histones isolated from freshly recloned sublines of these HTC cells.³ In addition, the cells containing nonacetylated H4 molecules must remain metabolically active, since pulse-labeling *n*-butyrate-treated cells with $^{32}\text{P}_4$ shows that the remaining nonacetylated class of H4 molecules are freely accessible to histone phosphorylating enzymes.³ Finally, preliminary results reveal that at least some

of these differences in *in vivo* nucleosome accessibility to histone acetylase are maintained in isolated cell nuclei.⁴

DISCUSSION

In this report, we have shown that *n*-butyrate strongly inhibits the histone deacetylating enzymes, and thereby blocks the normal rapid turnover of histone ϵ -aminolysine acetyls in living cells. Since the inhibition of the deacetylase reaction is of the noncompetitive type, we can calculate the turnover expected at any concentration of *n*-butyrate *in vivo*, despite the unknown accessibility of substrate within the cell. The agreement between the expected and observed inhibitions of histone turnover rates is remarkable. This fact, combined with the similar fatty acid chain length dependence of the *in vitro* and *in vivo* inhibitions (Figs. 5 and 6), and the acetylated lysine site specificities previously reported by others (9–11), convinces us that the deacetylase enzymes assayed *in vitro* are responsible for histone acetate turnover *in vivo*. Moreover, from results such as those shown in Fig. 1, which shows greater than 99% inhibition of histone deacetylation, we conclude that cells have no significant alternative mechanism for removing histone acetyls (*i.e.* no *n*-butyrate-resistant pathway).

After this work was completed, several reports appeared nearly simultaneously which likewise conclude that *n*-butyrate exerts its effect by inhibiting the turnover of histone acetyls (33–35). Our data on this point are in agreement with these studies from other laboratories and extends them by showing quantitative agreement between the extent of *in vitro* and *in vivo* deacetylase enzyme inhibitions, as well as by determining the noncompetitive nature of the inhibition. In this connection, it should be noted that, despite the marked heterogeneity in their chromatographic behavior (11, 20), the histone deacetylases I and II used here need not be products of different genes. Certainly these two enzyme fractions are indistinguishably inhibited by fatty acids (Fig. 5). An exami-

⁴ R. Garcea, unpublished results, this laboratory.

nation of the altered deacetylases in *n*-butyrate-resistant cell mutants, if such are found, should decide this question.

With regard to the types of histones whose turnover is affected, the combination of [³H]acetate fluorography and high resolution acid-urea gel electrophoresis has enabled us to determine that *n*-butyrate inhibits acetyl turnover for histones H2a and H2b, as well as for H3 and H4. Moreover, our pulse-chase experiments reveal that turnover is similarly inhibited by *n*-butyrate for all of the electrophoretically separable, acetylated histone forms of each histone.

Inhibition of deacetylases by *n*-butyrate would seem to be a general phenomenon among mammalian cells. In our work,⁵ we have found an *n*-butyrate-induced accumulation of acetylated histones in cell lines derived from rat liver tumor (HTC cells), rat pituitary (GH3), bovine lens (MDBK), and mouse myoblasts (L8); other investigators have reported similar results on human (HeLa, Ref. 12) and mouse erythroleukemia cell lines (Friend cells, Ref. 12).

The relative lack of specificity for fatty acid chain length (Figs. 5 and 6), coupled with the noncompetitive nature of the inhibition (Fig. 4), suggest that *n*-butyrate may be acting as a tight binding detergent in inhibiting these enzymes. This, in turn, should caution against automatically interpreting all of the effects seen when *n*-butyrate is added to cells as due to the changes in histone acetylation which ensue. The induction of Friend erythroleukemia cell differentiation by *n*-butyrate (29) is a case in point, as is the general inhibition of tissue culture cell growth by *n*-butyrate (see Ref. 36 for review). Once again, the isolation of *n*-butyrate-resistant mutant cells should be useful in deciding whether there are any important effects of high *n*-butyrate concentrations on cellular enzymes besides those studied here.

One might have hoped that the *n*-butyrate-induced accumulation of acetylated histone forms could be used to directly confirm the suggested role that acetylated histones play in gene activation. Specifically, the idea that the nucleosomes of active genes must be acetylated to allow efficient polymerase readthrough over bound nucleosomal histones has been an appealing one (4, 6, 27, 37); if it is correct, one might expect that *n*-butyrate-treated cells would begin to express many genes which were formerly silent. But when two-dimensional, polyacrylamide gel electrophoresis was used to analyze the HTC cell proteins synthesized after 13 h in 5 mM *n*-butyrate, very few differences were found from the normal proteins made.⁶ Although we cannot rule out the possibility that transcription is markedly changed in some nonproductive manner, it seems likely from this result that histone acetylation is not sufficient in itself (although it may be required) to induce gene activity. In order to decide whether acetylation is required for active RNA polymerase readthrough, it will be necessary to drastically reduce the normal level of acetylation of histones in cells, either through mutation or by designing specific inhibitors for the acetylation reaction.

The tentative new conclusion which we wish to draw from our results is the existence of a marked heterogeneity in the accessibility of different nucleosomes within the cell to histone acetylase(s). Special condensed and noncondensed chromatin environments have long been suspected from classical cytological observations (38, 39). More recently, such heterogeneity has been revealed by the greatly enhanced nuclease susceptibility of transcriptionally active genes (40, 41) and by electron microscopy of chromatin (42, 43). Possibly, the differential accessibility of nucleosomes to histone acetylase seen here reflects a biologically significant variation in chromatin structure along the genome, which is related to the differential

accessibility of genes for transcription in cells of different tissues. Thus, our data suggest that the susceptibility of histones to histone acetylase can be exploited as both an *in vivo* and an *in vitro* probe for biologically significant alterations in chromatin structure.

Acknowledgments—We would like to thank Ibrahim Tuet and the Cell Culture Facility for expert preparation of the tissue culture cells and Tim Mitchison for assistance during the early stages of this work as well as for suggesting the use of reinecke salt to precipitate histones. We also thank Rae Lyn Burke, Don Cleveland, and Chung-Cheng Liu for critically reading the manuscript.

REFERENCES

- Felsenfeld, G. (1978) *Nature* **271**, 115-122
- Elgin, S. C. R., and Weintraub, H. (1975) *Annu. Rev. Biochem.* **44**, 725-774
- DeLange, R. J., and Smith, E. L. (1975) *Ciba Found. Symp.* **28**, 59-70
- Allfrey, V. G. (1977) in *Chromatin and Chromosome Structure* (Li, H. J., and Eckhardt, R. A., eds) pp. 167-192, Academic Press, New York
- Dixon, G. H., Candido, E. P. M., Honda, B. M., Louie, A. J., MacLeod, A. F., and Sung, M. T. (1975) *Ciba Found. Symp.* **28**, 229-258
- Alberts, B., Worcel, A., and Weintraub, H. (1977) in *The Organization and Expression of the Eukaryotic Genome* (Bradbury, E. M., and Javaherian, K., eds) pp. 165-191, Academic Press, New York
- Sanders, L. A., Schechter, N. M., and McCarty, K. S. (1973) *Biochemistry* **12**, 783-791
- Jackson, V., Shires, A., Chalkley, R., and Granner, D. K. (1975) *J. Biol. Chem.* **250**, 4856-4863
- Inoue, A., and Fujimoto, D. (1970) *Biochim. Biophys. Acta* **220**, 307-316
- Vidali, G., Boffa, L. C., and Allfrey, V. G. (1972) *J. Biol. Chem.* **247**, 7365-7373
- Kikuchi, H., and Fujimoto, D. (1973) *FEBS. Lett.* **29**, 280-282
- Riggs, M. G., Whittaker, R. G., Neumann, J. R., and Ingram, V. M. (1977) *Nature* **268**, 462-464
- Simpson, R. T. (1971) *Biochemistry* **10**, 4466-4470
- Malchy, B., and Kaplan, H. (1974) *J. Mol. Biol.* **82**, 537-545
- Bailey, J. L. (1967) in *Techniques in Protein Chemistry*, 2nd Ed, p. 340, American Elsevier, New York
- Panyim, S., and Chalkley, R. (1969) *Arch. Biochem. Biophys.* **130**, 337-346
- Cohen, L. H., Newcock, K. M., and Zweidler, A. (1975) *Science* **190**, 994-997
- Bonner, W. M., and Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83-88
- Laskey, R. A., and Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335-341
- Bürger, S. (1976) Inaugural dissertation, University of Marburg
- Lindh, N. O., and Brantmark, B. L. (1965) *Anal. Biochem.* **10**, 415-420
- Horiuchi, K., and Fujimoto, D. (1975) *Anal. Biochem.* **69**, 491-496
- Panyim, S., and Chalkley, R. (1969) *Biochemistry* **8**, 3972-3979
- Camerini-Otero, R. D., Sollner-Webb, B., and Felsenfeld, G. (1976) *Cell* **8**, 333-347
- Sollner-Webb, B., Camerini-Otero, R. D., and Felsenfeld, G. (1976) *Cell* **9**, 179-193
- Bina-Stein, M., and Simpson, R. T. (1977) *Cell* **11**, 609-618
- Weintraub, H., Worcel, A., and Alberts, B. (1976) *Cell* **9**, 409-417
- Segel, I. H. (1975) *Enzyme Kinetics*, pp. 125-136, John Wiley & Sons, New York
- Leder, A., and Leder, P. (1975) *Cell* **5**, 319-322
- Reuben, R. C., Wife, R. L., Breslow, R., Rifkind, R. A., and Marks, P. A. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73**, 862-866
- Friend, C., Scher, W., Holland, J. G., and Sato, T. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68**, 378-382
- Gusella, J. F., and Housman, D. (1976) *Cell* **8**, 263-269
- Boffa, L. C., Vidali, G., Mann, R. S., and Allfrey, V. G. (1978) *J. Biol. Chem.* **253**, 3364-3366
- Sealy, L., and Chalkley, R. (1978) *Cell* **14**, 115-121
- Candido, E. P. M., Reeves, R., and Davie, J. R. (1978) *Cell* **14**, 105-113

⁵ L. Cousens, R. Garcea, and H. M. Blau, unpublished results.

⁶ P. H. O'Farrell, unpublished results, this laboratory.

36. Prasad, K. N., and Sinha, P. K. (1976) *In Vitro* **12**, 125-131
37. Yamamoto, K. R., and Alberts, B. M. (1976) *Annu. Rev. Biochem.* **45**, 721-746
38. Frenster, J. H. (1971) in *Handbook of Molecular Cytology* (Lima-de-Faria, A., ed) p. 251, American Elsevier, New York
39. Yunis, J. J., and Yasmineh, W. G. (1971) *Science* **174**, 1200-1209
40. Weintraub, H., and Groudine, M. (1976) *Science* **193**, 848-856
41. Garel, A., and Axel, R. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73**, 3966-3970
42. Foe, V. E., Wilkinson, L. E., and Laird, C. (1976) *Cell* **9**, 131-146
43. Foe, V. E. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **42**, 725-741

