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PHOSPHORYLATION OF THE BUTYRATE-ENHANCED PROTEIN, HISTONE $H1^{o}$, IN CHINESE HAMSTER CELLS

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Running Title: Phosphorylation of CHO $H1^{o}$

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In 1969 Panyim and Chalkley (1969a) reported the observance of a minor histone band which migrated between histone H1 and histone H4 in one-dimensional acid-urea-polyacrylamide gels. The protein (or proteins) which gave the characteristic electrophoetic band was extractable by both perchloric and sulfuric acids, and, most interestingly, it was found only in mammalian tissues which proliferated slowly or not at all. The amino acid analysis of the protein isolated from calf lung showed that it was similar to histone H1 in that it was highly lysine rich and that it did not contain tryptophan or cysteine. In contrast to H1, however, it did contain histidine, and its alanine content was much lower than that of histone H1. Since the report of Panyim and Chalkley (1969a) many workers have referred to acid extracted proteins which migrate like the calf lung protein as "histone H1a" (Marsh and Fitzgerald, 1973) or as "Histone $H1^{0}$" (Carter and Chae, 1975).

Despite the potential importance of "histone $H1^{0}$," only a few studies of $H1^{0}$ appeared during the 1970s, and those studies dealt primarily with the relative quantities of $H1^{0}$ in proliferating and nonproliferating tissues and cultured cells. $H1^{0}$ was observed to decrease during degeneration and regeneration of rat pancreas following a starvation-ethionine regimen (Marsh and Fitzgerald, 1973, Varricchio et al., 1977): $H1^{0}$ decreased prior to DNA synthesis, and, following cessation of DNA synthesis, it gradually increased to pretreatment levels. A similar inverse relationship between increased quantities of $H1^{0}$ and DNA synthesis was observed during development in rat pancreas (Varricchio, 1977): $H1^{0}$ was undectable in 15 day embryos; it increased to 5% of H1 at 5 weeks; and at 8 weeks, it increased to 27% of histone H1. Increased quantities of $H1^{0}$ also have been correlated with ontogeny in certain rat tissues (Medvedev et

al., 1977) and in bovine liver (Piha and Valkonen, 1979). Although (1) the quantity of $H1^{o}$ appears to increase as cells and tissues leave the proliferating state, and (2) Lea et al. (1974) have observed an inverse relationship between the quantity of $H1^{o}$ and growth rate in 4 rat hepatomas, Marks et al. (1975) have found that the amount of $H1^{o}$ varies greatly from tissue-to-tissue and from tissue-to-cell line. Hence, in general, there is no obvious relationship between the relative quantities of $H1^{o}$ and growth rate between different tissues or different cultured cell lines.

In addition to changes in the relative amount of histone $H1^{o}$, Marsh and Fitzgerald (1973) reported that rat pancreas $H1^{o}$ (H1a) incorporated radioactive acetate, lysine, phosphate, and methionine; and Medvedev et al. (1977) showed that methionine is present in $H1^{o}$ from rat liver and rat spleen. The detection of methionine in rat $H1^{o}$, but not in calf lung $H1^{o}$ (Panyim and Chalkley, 1969a), raised the possibility that rat $H1^{o}$ was a different protein from calf lung $H1^{o}$ or that the presence of methionine was variable.

Thus far in 1980, Smith and Johns (1980) have reported the amino acid analysis and composition of two $H1^{o}$ fractions from bovine liver; Pehrson and Cole (1980) have reported the isolation and amino acid analysis of $H1^{o}$ from mouse neuroblastoma cells; and we (D'Anna et al., 1980b) have reported the isolation of $H1^{o}$ (BEP) from cultured Chinese hamster (line CHO) cells. In CHO cells, $H1^{o}$ becomes greatly enhanced during treatment with sodium butyrate (D'Anna et al., 1980a). Besides these reports, there is strong circumstantial evidence that IP_{25}, a protein which is enhanced during chemical-induced differentiation of Friend erythroleukemia cells (Keppel et al. 1977; Candido et al. 1978), is related to histone $H1^{o}$.

In this paper we shall describe some of our results dealing with the induction, isolation, composition, and cell-cycle-dependent phosphorylation of histone $H1^{o}$ (BEP) from CHO cells. Whenever possible, we shall compare our results with the results of others, and we shall discuss potential roles of histone $H1^{o}$ and $H1^{o}$ phosphorylation in chromatin structure and function.

EXPERIMENTAL PROCEDURES

Cell Growth and Cell Synchrony.

Suspension cultures of Chinese hamster cells (line CHO) were grown and maintained in suspension cultures as previously described (D'Anna et al., 1980a,b). CHO cells for preparative isolation of histone H1 were grown in 4 L suspension cultures. Unphosphorylated $H1^{o}$ was obtained from cultures treated with 10 mM sodium butyrate for 24 hr (D'Anna et al., 1980b), and phosphorylated $H1^{o}$ was obtained from 4 L suspension cultures synchronously enriched in metaphase cells (D'Anna et al., 1979). The mitotic fraction of the metaphase enriched cultures, determined by phase contrast microscopy, was typically 65-70%.

To determine effects of sodium butyrate upon cell cycle distribution and $H1^{o}$ cellular content, we treated exponentially growing cultures (1.4×10^{5} cells ml^{-1}) with various concentrations of butyrate. After 24 hr, $\sim 4 \times 10^{6}$ cells were removed for cell cycle analysis by flow cytometry (FCM), and $\sim 1.6 \times 10^{8}$ cells were harvested for isolation of histones.

Experimental procedures to synchronize cells and monitor $H1^{o}$ and H1 phosphorylations during entry and exit of cells from mitosis have been described in detail elsewhere (Gurley et al., 1978a; D'Anna et al., 1980b) and briefly in the text.

To determine phosphate incorporation into $H1^{o}$, cultures were grown for several generations in the presence of [H^3] lysine (50 μCi/L). Then, immediately before harvest, the cells were labeled for 1.0 hr with $H_3{}^{32}PO_4$ (D'Anna et al., 1980a,b).

Cell-Cycle Analysis.

The distribution of cells in the cell cycle was determined from FCM (Holm and Cram, 1973). Cells were dispersed by trypsin treatment and, subsequently, stained with the fluorescent dye, mithramycin (Crissman, et al., 1977). The DNA content of each cell was determined from its mithramycin fluorescence (Kraemer et al., 1972). Fractions of cells in G_1, S, and G_2 plus M were computed by the method of Dean and Jett (1974).

Isolation and Amino Acid Analyses of $H1^{o}$.

Histones H1 and $H1^{o}$ were extracted from blended CHO cells with 0.83 M $HClO_4$ (PCA) by the first method of Johns (1964) as previously described (Gurley et al., 1975). A fraction enriched in $H1^{o}$ was isolated by step-gradient chromatography on Bio•Rex 70 ion exchange resin (D'Anna et al., 1980b). $H1^{o}$ fractions, $H1^{o}a$ and $H1^{o}b$, also were separated by Bio•Rex 70 ion exchange chromatography in which the proteins were eluted with a shallow gradient of guanidine hydrochloride (8-14%) in 0.10 M phosphate buffer. Combined fractions were desalted in G-25 and concentrated by lyophilization as described previously (D'Anna et al., 1980b).

Amino acid analyses were performed with a Beckman/Spinco 120B modified automatic analyzer using standard procedures (Spackman et al., 1958). Hydrolysis was carried out in constant-boiling HCl at 110°C for 22 hr in sealed evacuated tubes.

Electrophoresis.

Electrophoresis of $H1^{o}$ and PCA extracted proteins was performed in 0.5 x 25 cm, 5.2% acetic acid-2.5 M urea-15% polyacrylamide gels (Panyim and Chalkley, 1969b) as previously described (Gurley et al., 1978a). Gels were stained overnight in 0.2% amido black-30% methanol-9% acetic acid and destained by diffusion. Densitometer profiles of analytical gels were measured with a Gilford Model 240 spectrophotometer equipped with a gel linear transport device and different $H1^{o}$ and H1 bands were resolved electronically with a Dupont Model 310 curve resolver.

Radio-labeled histones were separated by electrophoresis. They were then analyzed by optical densitometry and by scintillation spectrometry. After densitometry the gels were cut into 2.2 mm pieces and dissolved overnight in 1.0 ml 30% hydrogen peroxide (55-60°C). They were counted in 15 ml of Aquasol II or Hydrofluor (D'Anna et al., 1980a).

RESULTS

$H1^{o}$ Enhancement and Cell Cycle Effects Induced by Butyrate

Butyrate treatment greatly increases the proportion of G_1 cells in culture (Rastl and Swetly, 1978; Fellon and Cox, 1979; D'Anna et al., 1980a; Prugnell et al., 1980). This can be seen in Fig. 1 where the percentages of CHO cells in different phases of the cell cycle are plotted as functions of butyrate concentration. In those experiments, exponentially growing cultures were treated for 24 hr with different concentrations of butyrate, and their cell cycle distributions were determined by flow cytometry. The proportion of G_1 cells in culture (Fig. 1) increases sharply as a function of butyrate concentration up to about 2.5 mM, above which all of the values are within 5% of one another. At

7.5–15 mM there is very little culture growth after 24 hr so that the cultures are essentially in a state of G_1 arrest (D'Anna et al., 1980a).

When we examined the perchloric acid-extracted proteins from butyrate-treated cultures in long acid-urea polyacrylamide gels (Fig. 2), we observed two prominent effects: (1) first, in going from 0–15 mM butyrate there was a change in the distribution of H1 species whose electrophoretic mobilities (and other data) indicate a dephosphorylation of histone H1 (D'Anna et al., 1980a); and (2) there was an increase in the relative intensity of a set of bands that migrates in front of histone H1. Although the electrophoretic mobilities of these bands and their doublet character (Varricchio et al., 1977) suggested that they were related to histone $H1^0$ (Panyim and Chalkley, 1969) and to protein IP_{25} (Keppel et al., 1977, 1979; Candido et al., 1978), we had no proof of the identity of the bands, so we originally designated them as the butyrate-enhanced protein, BEP. For reasons designated below, in this manuscript we have designated them as CHO $H1^0$.

Plots of the percentage of unphosphorylated H1 (D'Anna et al., 1980a) and the ratio of the integrated intensity of $H1^0$ to that of H1 (Fig. 3) show that both variables increase as a function of butyrate concentration. Comparison of each variable with the percentage of cells in G_1 indicates that the increased percentage of unphosphorylated H1 is correlated with an increased proportion of cells in G_1; however, there is no direct correspondence between the increase in the $H1^0$/H1 ratio and the percentage of cells in G_1. Nevertheless, the increased $H1^0$/H1 ratio in the G_1 arrested cultures raises a number of questions. (1) Does $H1^0$ induction precede G_1 arrest so that $H1^0$ plays a role in the turn-off of cell proliferation? (2) Is the enhanced synthesis of $H1^0$ determined solely by

the point of G_1 arrest induced by butyrate treatment? (3) Does butyrate treatment enhance $H1^o$ synthesis throughout the cell cycle so that the enhancement of $H1^o$ in G_1 cells is seen only because cells become arrested there?

While we cannot yet answer all of the above questions explicitly, we have begun to unravel some aspects of the problem. (1) When cells are arrested in G_1 by isoleucine deprivation, the $H1^o/H1$ ratio increases from 0.08 in exponentially growing cultures to 0.15 after 60 hr in isoleucine deficient medium (J. A. D'Anna et al. unpublished). We, therefore, see a small increase in $H1^o$ which appears to be strictly a cell cycle effect. (2) In addition, however, butyrate treatment of cells arrested in G_1 by isoleucine deprivation causes additional $H1^o$ synthesis (J. A. D'Anna et al., unpublished). For example, addition of butyrate (15 mM) to cultures arrested in G_1 by isoleucine deprivation (36 hr) causes the $H1^o/H1$ ratio to increase to 0.37 after 24 hr (D'Anna et al., unpublished). The value of 0.37 is 2.5 times as large as the ratio of 0.1 measured in the absence of butyrate (60 hr). (3) When cells are released from isoleucine deprivation into fresh F-10 medium containing 15 mM butyrate, the cells, for the most part (85%), remain in G_1 and the $H1^o/H1$ ratio increases to about 0.47. It appears, therefore, that (a) the point of G_1 arrest induced by butyrate is the same or later in G_1 than arrest induced by isoleucine deprivation, and (b) cells do not have to pass through S phase in the presence of butyrate to accumulate in G_1. We note that autoradiography in which very high levels of high specific activity [^{3}H] thymidine are employed shows that treatment with butyrate truly arrests cells in G_1 and not in early S phase as with hydroxyurea (Walters et al., 1976). More detailed studies of $H1^o$ induction are in progress.

Amino Acid Analysis of CHO $H1^{o}$; Comparison with $H1^{o}$s From Other Species

To determine if "CHO $H1^{o}$" might be the equivalent of ca... lung $H1^{o}$ (the only $H1^{o}$ whose amino acid composition had been reported), we isolated the butyrate-enhanced protein by guanidine hydrochloride step gradient chromatography on Bio•Rex 70 ion exchange resin (D'Anna et al., 1980a). About 1.1 mg of CHO $H1^{o}$ was purified from 8.8 mg of PCA extracted proteins from butyrate-treated cultures which typically contain 80-90% G_1 cells and are 4-5 fold enriched in $H1^{o}$. Further, about 0.17 mg of CHO $H1^{o}$ was isolated from 10 mg of PCA-extracted proteins from cultures which were synchronously enriched (65-70%) in metaphase cells (no butyrate present).

Electrophoresis of $H1^{o}$ from G_1-enriched and from metaphase-enriched cultures in sodium dodecyl sulfate ($NaDodSO_4$) gels gives rise to single bands of identical electrophoretic mobility (D'Anna et al., 1980b). From comparison of the electrophoretic mobility of $H1^{o}$ with those of other histones, we estimate a molecular weight of 20-22,000 or about 1500 less than histone H1.

In contrast to single bands in $NaDodSO_4$ gels, electrophoresis in 25 cm acid-urea-polyacrylamide gels shows striking differences between the two preparations (Fig. 4). $H1^{o}$ from the G_1 enriched, butyrate-treated cultures exhibits two major bands as we would expect from Fig. 2; however, $H1^{o}$ from metaphase-enriched cultures is split into six bands. A priori, the multiple bands of $H1^{o}$ from metaphase-enriched cultures may arise for a number of reasons: (1) $H1^{o}$ from metaphase cells may contain impurities not extracted from G_1 cultures; (2) $H1^{o}$ may be modified during mitosis so that its electrophoretic mobility is retarded in acid-urea-poly acrylamide gels; or (3) CHO $H1^{o}$ really may be an H1 degradation product so that the multiple bands of $H1^{o}$ from metaphase cells are degradation products of

phosphorylated H1. As we shall see, CHO $H1^{o}$ is truly distinct from histone H1, and it is phosphorylated in a cell cycle dependent fashion which mimics that of authetic histone H1.

In addition to the isolation of whole CHO H1 (D'Anna et al., 1980a), we recently have separated CHO H1 from G_1-enriched (butyrate-treated) cultures into two fractions by use of guanidine hydrochloride gradient chromatography on Bio•Rex 70 ion exchange resin (J. A. D'Anna and R. R. Becker, unpublished). The two fractions have been designated as $H1^{o}a$ (slower migrating band in gel 2 of Fig. 4) and $H1^{o}b$ (faster migrating band in gel 2 of Fig. 4) in accordance with the precedent of Smith and Johns (1980), and their amino acid analyses are given in Table I. Comparison of the amino acid analyses of CHO $H1^{o}$ with those of bovine liver $H1^{o}$ (Smith and Johns, 1980), mouse neuroblastoma $H1^{o}$ (Pehrson and Cole, 1980), and calf lung $H1^{o}$ (Panyim and Chalkley, 1969) in Table I indicates that all the $H1^{o}$s possess common features which distinguish them from histone H1: (1) they contain 10-13% less alanine than H1; (2) they contain more isoleucine and less leucine than H1; (3) they contain more tyrosines and phenylalanines than H1; and (4) they contain histidine, but H1 does not.

Examination of the amino acid analyses in Table I also suggests that the presence of methionine may be variable in $H1^{o}$ from different species: the analysis of CHO $H1^{o}b$ exhibits only a trace of methionine, and calf lung $H1^{o}$ has none. Although only a trace of methionine is detected in the amino acid analysis of CHO $H1^{o}b$, cyanogen bromide treatment (Gross and Witkop 1962) and electrophoretic analysis (Fig. 5) shows that both $H1^{o}a$ and $H1^{o}b$ are cleaved with equal facility. Other experiments (D'Anna et al., 1980b) have shown that cyanogen bromide cleaves about 20 residues from the $H1^{o}$s of both G_1-enriched and metaphase-enriched cultures, but it does not cleave

histone H1. Therefore, cyanogen bromide treatment indicates that: (1) methionine is truly present in both CHO $H1^{o}a$ and CHO $H1^{o}b$; (2) $H1^{o}$ cannot be an H1 degradation product; and (3) $H1^{o}$ from G_1-enriched and from metaphase-enriched cultures are very likely the same protein.

Phosphate Incorporation into CHO $H1^{o}$

[^{32}P] Phosphate incorporation into $H1^{o}$ has been measured from preparative gel electrophoresis (D'Anna et al., 1980b), analytical gel electrophoresis (D'Anna et al., 1980a,b), and Bio•Rex 70 chromatography (Gurley et al., 1975; J. A. D'Anna and R. R. Becker, unpublished). Figure 6 shows examples of ^{32}P phosphate incorporation into $H1^{o}$ as determined from long analytical acid-urea-polyacrylamide gel electrophoresis. Although some ^{32}P phosphate is incorporated into $H1^{o}$ and H1 from cells arrested in G_1 (80-90%) by butyrate treatment, the relative phosphate incorporation is only about 10% of that measured for exponentially growing cultures. In the exponentially growing cultures most [^{32}P] phosphate is incorporated into bands 3-5 of $H1^{o}$ and in bands 2-5 of histone H1.

In metaphase enriched cultures (Fig. 6c), large portions of both H1 and $H1^{o}$ are shifted to positions of lower electrophoretic mobility, and the more slowly migrating bands are accompanied by phosphate incorporation. Since the scale of phosphate incorporation in Fig. 6c is only 1/4 of those in Fig. 6a and 6b, it is clear that the net phosphate incorporation is much higher in the metaphase-enriched cultures. Based on these experiments and others (D'Anna et al., 1980b), we conclude that histone $H1^{o}$ becomes phosphorylated and that phosphate incorporation retards its electrophoretic mobility in acid-urea-polyacrylamide gels.

Further details of H1 phosphate incorporation have been obtained from reexamination of the H1 phosphorylation studies of Gurley et al. (1975).

In those experiments, Bio•Rex 70 chromatography was used to examine phosphate incorporation into the PCA-extracted proteins from synchronized CHO cells. Comparison of the Bio•Rex 70 chromatograms of Gurley et al. (1975) with more recent data (J. A. D'Anna and R. R. Becker, unpublished) indicates that the fractions originally designated as H1(III) and H1(IV) are really the respective $H1^{o}$ fractions $H1^{o}a$ and $H1^{o}b$. The phosphate incorporation studies of Gurley et al. (1975) confirm our results from analytical and preparative gel electrophoresis (D'Anna, 1980a,b), and they add several pieces of additional information. (1) Little or no phosphate is incorporated into $H1^{o}a$ or $H1^{o}b$ in cultures arrested in G_1 by isoleucine deprivation; this result is the same as we observed for cells arrested in G_1 by treatment with butyrate. (2) Both $H1^{o}a$ and $H1^{o}b$ incorporate [^{32}P] phosphate following release from G_1 arrest (isoleucine deficient medium); hence, it appears that the phosphorylation of $H1^{o}a$ and $H1^{o}b$, like phosphorylation of H1(I) and H1(II), begins in late G_1 prior to DNA synthesis and increases throughout interphase. (3) Both $H1^{o}a$ and $H1^{o}b$ are phosphorylated at similar rates, and the fractions of $H1^{o}a$ and $H1^{o}b$ which become phosphorylated are similar to one another but somewhat less than those of H1(I) and H1(II): (1) when synchronized cultures are released from G_1-arrest, about 10-15% of $H1^{o}a$ and $H1^{o}b$ [15-18% of H1(I) and H1(II)] become phosphorylated just prior to the appearance of S phase cells in culture; (2) by 8 hr after release from G_1 arrest, when 50% of the cells are in S phase, 30-35% of $H1^{o}a$ and $H1^{o}b$ (50-60% of H1(I) and H1(II)] become phosphorylated.

<u>$H1^{o}$ Phosphorylation During Entry and Exit from Mitosis</u>

Because of the similarities between $H1^{o}$ and H1 phosphate incorporation, we wondered if the details of $H1^{o}$ phosphorylation during

entry and exit of cells from mitosis were the same as those of histone H1. Previous studies from our laboratory (Gurley et al., 1978a) have shown that by late interphase 55-60% of all H1 molecules become phosphorylated at 1-3 sites. During mitosis all H1 molecules become "superphosphorylated" at 4-6 sites per molecule, and the superphosphorylation is restricted to those stages of mitosis—prophase, metaphase, and anaphase—where chromosomes are maximally condensed.

To compare changes in $H1^{o}$ phosphorylation with changes in chromatin structure, we examined electron micrographs and unpublished regions of densitometer profiles which previously had been used to study H1 phosphorylation (Gurley et al., 1978a). For studies involving entry into mitosis, cells were synchronized near the G_1/S boundary by sequential use of isoleucine deprivation and hydroxyurea blockade. They were then released into drug free medium, and, four hours later, Colcemid was added to arrest the cells in metaphase.

Cells having 4 types of chromatin structures were observed by electron microscopy (Gurley et al., 1978a): (1) cells having small amounts of heterochromatin located near the periphery of the nucleus (interphase cells), (2) cells having many loci of heterochromatin interspersed throughout the nucleus (preprophase cells), (3) cells having fully condensed chromosomes within an intact nuclear membrane (prophase cells), and (4) cells having fully condensed chromosomes free in the cytoplasm (metaphase cells). These chromatin structures and changes in their distribution have been reported (Gurley et al., 1978a) and will not be reproduced here. For our purposes, the feature of interest from those data is that prophase and metaphase cells do not appear until 7 hr after release from hydroxyurea blockade.

Figure 7 shows examples of densitometer profiles of $H1^{o}$ from long acid-urea-polyacrylamide gels following release from hydroxyurea blockade. During the early part of the experiment, $H1^{o}$ is found almost exclusively in bands 1-4; however, at 7.0-7.5 hr, $H1^{o}$ begins to appear in bands 5-7 as well, and the proportion of $H1^{o}$ in those bands continues to increase with time. To determine if the appearance of $H1^{o}$ in bands 5-7, like H1 superphosphorylation, was correlated with chromosomal condensation (Gurley et al., 1978a), the percentage of total $H1^{o}$ in bands 5-7 ($H1^{o}_{M}$) was plotted as a function of time after release from hydroxyurea blockade (Fig. 8). On the same figure, we plotted the percentages of superphosphorylated H1 ($H1_{M}$) and the percentages of cells in prophase plus metaphase. The agreement between all three sets of data indicates that phosphorylation of $H1^{o}$ to $H1^{o}_{M}$ is temporally correlated with H1 superphosphorylation and with chromosomal condensation.

To examine $H1^{o}$ phosphorylation during exit from mitosis, cells were synchronized by mitotic selection in the absence of Colcemid (Gurley et al., 1978a). Cell cycle traverse was then monitored at various times after mitotic selection by electron microscopy, and the extent of $H1^{o}$ phosphorylation was determined from analysis of long acid-urea-polyacrylamide gels.

Electron microscopy reveals four types of chromatin structures during exit from mitosis: (1) cells having fully condensed chromosomes free in the cytoplasm (metaphase and anaphase cells), (2) cells having partially unraveled chromosomes in contact with or fully surrounded by nuclear membrane (early telophase cells), (3) cells having dense patches of heterochromatin in the nucleus but no chromosomes (late telophase cells), and (4) typical interphase cells. Examples of these chromatin structures

and changes in their distribution during exit from mitosis also have been reported (Gurley et al., 1978a). We note that, by 30 minutes after mitotic selection, more than 90% of the cells have progressed from metaphase and anaphase to telophase and G_1.

Figure 9 shows examples of the $H1^o$ densitometer profiles at various times after mitotic selection. $H1^o$ in bands 5-7 ($H1_M$) becomes shifted back to band positions 1-4. Plots of $H1^o_M$, $H1_M$, and the percentage of cells in metaphase plus anaphase as functions of time after mitotic selection (Fig. 10) also exhibit good agreement. We therefore conclude that the presence of $H1^o_M$ is correlated with H1 superphosphorylation and with the presence of fully condensed chromosomes during metaphase and anaphase. It follows that as cells exit from mitosis, $H1^o_M$, like $H1_M$, becomes dephosphorylated near the anaphase/telophase transition.

If we assume that the acquisition of n phosphate groups will retard the electrophoretic mobility of an $H1^o$ molecule by n band positions in Fig. 7 and 9 (see Chalkley et al., 1974; D'Anna et al., 1980b), then, by use of the profile of unphosphorylated $H1^o$ from G_1 cells as a reference (Fig. 9), we can estimate the extent of $H1^o$ phosphorylation in late interphase and during mitosis.

As cells approach mitosis (late interphase), phosphorylation of 30-35% of $H1^o$a and of $H1^o$b (bands 2 and 3 in the G_1 profile of Fig. 9) at one site per molecule or the phosphorylation of 30% of $H1^o$b (band 2 in the G_1 profile of Fig. 9) at two sites per molecule would account for the pattern seen at 6 hours after release from hydroxyurea blockade (Fig. 7). Because it has been shown that $H1^o$a and $H1^o$b become phosphorylated at similar rates (Gurley et al., 1975; J. A. D'Anna and R. R. Becker, unpublished), we conclude that by late interphase 30-35%, each, of the $H1^o$a

and $H1^{o}b$ molecules become phosphorylated so that they contain one phosphate per molecule.

Comparison of the electrophoretic pattern of $H1^{o}$ from cells arrested in G_1 with the pattern of $H1^{o}$ from cells mitotically selected in the presence of Colcemid (Fig. 9) indicates that most $H1^{o}$ is shifted from bands positions 2 and 3 in G1 to band positions 6 and 7 in metaphase-enriched cultures. We, therefore, estimate that during mitosis all $H1^{o}$ molecules become phosphorylated so that they contain 4 phosphates per molecule.

DISCUSSION

$H1^{o}$ Composition and Function.

Comparison of the amino acid analyses of "$H1^{o}$" from CHO cells, bovine liver, neuroblastoma cells, and calf lung indicates that they all have a similar composition which distinguishes them from histone H1. Thus far, methionine has been detected in all mammalian $H1^{o}$s except $H1^{o}$ from calf lung. We have seen, however, that methionine appeared only in trace quantities in the amino acid analysis of CHO $H1^{o}b$. In this regard it is possible that methionine also is present in calf lung $H1^{o}$, but it became oxidized during repeated chromatography of purification (Panyim and Chalkley, 1969a) or during hydrolysis for amino acid analysis.

Two $H1^{o}$ fractions, $H1^{o}a$ and $H1^{o}b$, have been isolated from butyrate-treated (G_1-arrested) CHO cells and from bovine liver (Smith and Johns, 1980). $H1^{o}a$ and $H1^{o}b$ from these two sources have essentially the same amino acid composition; they possess the same electrophoretic mobility in $NaDodSO_4$ gels; and they are both cleaved by cyanogen bromide. $H1^{o}a$ and $H1^{o}b$ are, nevertheless, separable by chromatography on Bio•Rex 70 ion exchange resin and by electrophoresis in long acid-urea-polyacrylamide

gels, so that the two fractions appear to differ by the equivalent of one charge unit in 5% acetic acid (D'Anna et al., 1980b).

Presently, the difference(s) between $H1^{\circ}a$ and $H1^{\circ}b$ fractions has not been resolved. It is known, however, that: (1) both $H1^{\circ}a$ and $H1^{\circ}b$ are unphosphorylated in butyrate-treated CHO cells; (2) both $H1^{\circ}a$ and $H1^{\circ}b$ from bovine liver are blocked in their NH_2-terminal end (Smith and John, 1980); and (3) neither $H1^{\circ}a$ nor $H1^{\circ}b$ from bovine liver appears to contain diphosphoribosyl moities (Smith and Johns, 1980). Although, tryptic peptide maps of bovine $H1^{\circ}a$ and $H1^{\circ}b$ exhibit only minor differences (Smith and Johns, 1980), the existence of two $H1^{\circ}$ fractions (excluding phospohorylation) raises the possibility that there exist at least two subsets of chromatin structures involving $H1^{\circ}$.

Very recently, Smith et al. (1980) have reported the partial sequence of 20 amino acids of bovine liver $H1^{\circ}a$ beginning at methionine, which they estimate is located ~30 residues from the NH_2-terminal end. Comparison of the partial sequence of $H1^{\circ}a$ with residues 32-52 in chicken H5 and residues 45-65 in bovine H1 shows homology between 12 residues of $H1^{\circ}a$ and chicken H5, but only 7 between $H1^{\circ}a$ and bovine H1. (There is also homology between 7 of the 20 residues in chicken H5 and bovine H1). Although H5 and $H1^{\circ}$ differ significantly in lysine and arginine content, sequence studies and trends in H5 composition between birds, fish, and reptiles have led Smith et al., (1980) to suggest that "Avian H5 and mammalian $H1^{\circ}$ possibly represent two extremes of a family of proteins which share similar structures in their central regions."

We note that similarities in the properties between CHO $H1^{\circ}$ and IP_{25} led us to speculate that CHO $H1^{\circ}$ and IP_{25} might be equivalent proteins (D'Anna et al., 1980b). Recent information, however, indicates that this

is not the case. While $H1^{o}$ and IP_{25} may ultimately be shown to belong to the same family of proteins, the amino acid analysis of IP_{25} (R. Reeves, personal communication) shows that IP_{25} contains 13.5 mole % alanine, 3.3 mole % arginine, but only 21.4 mole % lysine, indicating that it's composition is intermediate between those of H1 and H5.

There is only limited information regarding $H1^{o}$ enhancement; however, the available data indicate that synthesis of $H1^{o}$, like synthesis of H5 (Appels and Wells, 1970; Ruiz-Carrillo et al., 1976) and HMG proteins (Kuehl, 1979), can occur in the absence of DNA synthesis. As noted in the introduction, studies of $H1^{o}$ levels during pancreas regeneration (Marsh and Fitzgerald, 1973) indicate that $H1^{o}$ does not begin to return to pretreatment levels until after DNA synthesis has ceased. Our own data indicate that $H1^{o}$ can be greatly enhanced in G_1 when cells arrested in G_1 are treated with butyrate or when cells are released from isoleucine deprivation (G_1 arrest) into 15 mM butyrate (where they remain blocked at the same or a later point in G_1). Therefore, it is clear that $H1^{o}$ synthesis can occur in G_1-arrested cells.

Other recent reports indicate that: (1) $H1^{o}$ becomes enhanced in temperature sensitive mutants of Balb/C-3T3 cells grown at their nonpermissive temperature where DNA synthesis is blocked (Naha and Sorrentino, 1980); (2) $H1^{o}$ becomes enhanced in mouse neuroblastoma cells grown in the absence of serum and in "density inhibited" HeLa cells (Pehrson and Cole, 1980); and (3) "$H1^{o}$" (more likely IP_{25}) synthesis continues unabated when Friend cells are treated 15 minutes with hydroxyurea (Zlatanova, 1980).

We do not as yet know whether: (1) $H1^{o}$ synthesis normally occurs in G_1 of proliferating cells or in other phases of the cell cycle as well; (2)

$H1^{o}$ can be enhanced in S, G_2 or M by treatment by butyrate; or (3) there are modulations in the cellular content of $H1^{o}$ during the cell cycle of untreated cultures.

The inverse relationship between $H1^{o}$ content and DNA synthesis (and cell division) has led to a number of hypotheses by which changes in $H1^{o}$ content might be directly related to changes chromatin structure and function. (1) Marsh and Fitzgerald (1973) have suggested $H1^{o}$ may serve a repressor function and that its removal or decrease may be a necessary prelude to the onset of DNA synthesis. (2) Varricchio (1977) has suggested that $H1^{o}$ may be involved in some final condensation of chromatin. (3) Keppel et al. (1979) have suggested that the protein IP_{25} may play a role either in the secondary structure of chromatin or in the turnoff of genes not required for the final post-replicative stages of erythroid differentiation. The second hypothesis of Keppel et al. (1979) might be broadened to apply to differentiation in general and to the maintenance of G_1 arrest. While all of these hypotheses are plausible, eventual detailed explanations must also account for the great variability of $H1^{o}$ content in proliferating, as well as, nonproliferating cells (Marks et al., 1975).

Currently, little is known about the molecular properties of $H1^{o}$ and its interactions in chromatin. Is the conformation of $H1^{o}$ in solution similar to the nose-head-tail models of H1 and H5 (Hartman et al., 1977; Aviles et al., 1978)? Is $H1^{o}$, like H1 and H5, located on the outside of the 140 DNA base pair-inner histone core of nucleosomes (see Simpson, 1978)? Does $H1^{o}$, like H1 and H5, preferentially bind to superhelical DNA (Vogel and Singer, 1975; Bina-Stein et al., 1976)? Does $H1^{o}$ also cause compaction of oligonucleosomes into solenoid-like structures (Finch and Klug, 1976)? Since the partial sequence of $H1^{o}$ suggests that it is a

homolog of H5, which itself is regarded as a variant of H1 (Yaguchi et al., 1979; Isenberg, 1979), we would expect the answer of all these questions to be "yes." Thus far, Keppel et al. (1979) have reported that IP_{25}, like H1 and H5, is attached primarily to internucleosomal spacer DNA. IP_{25} does not appear, however, to be located in transcriptionally active regions of chromatin. Answers to these questions for the closely related $H1^{o}$s of Table I have not been reported.

$H1^{o}$ Phosphorylation.

Our studies (D'Anna et al., 1980a,b; Gurley et al., 1975; this communication) indicate that histone $H1^{o}$ is phosphorylated in a cell cycle dependent fashion. When cells are arrested in G_1 by isoleucine deprivation or by treatment with butyrate, little or no phosphate is incorporated into $H1^{o}$. As cells are released from G_1 arrest, $H1^{o}$ becomes phosphorylated in late G_1, and phosphorylation continues throughout S and G_2. By late interphase, 30-35% of the $H1^{o}$a and $H1^{o}$b molecules become phosphorylated so that they contain an estimated one phosphate per molecule.

As cells enter mitosis, all $H1^{o}$ molecules become highly phosphorylated so that they contain an estimated four phophates per molecule ($H1_M$); the occurence of $H1_M$ is temporally correlated with chromosomal condensation at prophase, metaphase, and anaphase. As cells exit from mitosis $H1^{o}$ is rapidly dephosphorylated near the anaphase/telophase boundary.

Temporally, the cell cycle dependent phosphorylations of CHO $H1^{o}$ are identical to those of histone H1; however, we do not know (1) the sites of $H1^{o}$ phosphorylation; (2) the amino acids which are phosphorylated, nor (3) the regional locations of phosphorylation in $H1^{o}$. In CHO H1, phosphorylations in different parts of the molecule can be correlated with distinct phases of the cell cycle (Hohmann, 1978; Gurley, 1978a,b). In

late G_1, following release from G_1 arrest, a portion of the H1 molecules become phosphorylated at a serine located in the COOH-terminal part (relative to tyrosine 73) of the molecule. As cells enter S phase, H1 becomes phosphorylated at two additional sites in the COOH-terminal part so that by late interphase 55-60% of the H1 molecules contain 1-3 phosphates. During mitosis, all H1 molelcules become phosphorylated at additional sites which involve serine and threonine in both their NH_2- and COOH- terminal parts.

Our only information pertaining to the location of phosphorylation in $H1^o$ involves phosphorylation during mitosis ($H1^o_M$). Previous cyanogen bromide cleavage experiments (D'Anna et al., 1980b) indicate that most or all of the 4 phosphates of $H1_M$ are located in the large fragment which remains after cleavage of approximately 20 residues. Since Smith et al. (1980) find methionine in the NH_2-terminal end of bovine liver $H1^o$, we estimate that at least 3, and possibly all 4, of the phosphates of $H1_M$ are located in the large fragment which begins at methionine (residue 20-30) and continues to the COOH-terminus of $H1^o$.

We note that Marsh and Fitzgerald (1973) have measured acetate incorporation into rat pancreas $H1^o$. In experiments designed to monitor acetate incorporation into the inner histones (D'Anna et al., 1980a), we observed little or no acetate incorporation into $H1^o$ (J. A. D'Anna et al., unpublished observations). The levels observed were much less than those measured for modified species of inner histones, and, at most, the levels were similar to those observed for histone H1 whose NH_2-terminal is blocked by an acetyl group which does not turn over (Dixon et al., 1977). Since the NH_2-terminal of $H1^o$ is blocked (Smith and Johns, 1980), it also may be blocked by an acetyl group that does not turn over. On the other hand, if

acetate is incorporated into $H1^{o}$ in such a way that it can turn over, then our estimates of $H1^{o}$ phosphorylation will be high. Those estimates would then become, in fact, estimates of total modifications which alter $H1^{o}$ charge.

Temporal correlations of H1 phosphorylations with the cell cycle have led to suggestions that H1 phosphorylation may play a role in initiation of cell proliferation, DNA synthesis, separation of newly synthesized daughter chromosomes during the cell cycle, and chromosomal condensation (see Marks et al., 1973; Gurley et al., 1978a, b; Hohmann, 1978; Matthews and Bradbury, 1978; D'Anna et al., '79 for extensive lists of references). Since histone $H1^{o}$ appears to be a variant of the H1 class of proteins, and since it is phosphorylated in a cell cycle dependent fashion which mimics that of histone H1, it seems reasonable to postulate that the phosphorylations of H1 and $H1^{o}$ serve the same functions (whatever that may be) in chromatin.

A number of mechanisms have been suggested by which H1 phosphorylation might alter chromatin structure: (1) H1 phosphorylation may induce changes in H1 conformation which could alter the orientation of nucleosomes and higher orders of chromatin structure; (2) phosphorylation may induce H1:H1 interactions which could effect chromosomal condensation; (3) H1 phosphorylation may alter H1 interaction with DNA to cause (a) compaction of chromatin or (b) localized changes in accessibility to DNA; (4) H1 phosphorylation may modulate interaction of H1 with other chromosomal proteins (see Gurley et al., 1978a, b, Hohmann, 1978; Matthews and Bradbury, 1978 and D'Anna et al. 1979 for references).

Of several physical studies employing phosphorylated histone H1 (see Matthews and Bradbury, 1978; D'Anna et al., 1979; Hoffman et al., 1980

for references) we are aware of only 3 which have employed H1 phosphorylated *in vivo* from synchroninzed culture or H1 phosphorylated *in vitro* by a "growth associated" kinase whose H1 phosphorylation sites also have been detected *in vivo* (Langan, 1978). One of these studies (D'Anna *et al.*, 1979) indicates that: (1) phosphorylation does not affect the sensitivity of H1 folding or the net secondary structure of the folded H1 molecule in solution; (2) it does not induce H1-H1 interaction in solution; and (3) it does not greatly affect the sedimentation properties of H1-superhelical DNA complexes in solution. Phosphorylation, however, does affect (1) the circular dichroism of H1-superhelial DNA complexes (D'Anna *et al.*, 1979); (2) the extent of H1 binding to superhelical DNA at low ionic strength (Singer and Singer, 1978) and (3) the turbidity of H1-linear DNA complexes (Matthews and Bradbury, 1978).

Although the model studies focus attention upon the ability of H1 phosphorylation to alter H1-DNA interactions, the molecular effects of H1 phosphorylation in chromatin remain unresolved. Part of the difficulty has been technical. For example, most H1 molecules become dephosphorylated during the isolation of chromatin and chromosomes (see Gurley *et al.*, 1981); therefore, it has not been possible to compare the properties of fully phosphorylated chromatin or chromatin subunits with those of their unphosphorylated counterparts. Recent reports (e.g. Simpson, 1978; Strätling, 1979; Hoffman *et al.*, 1980), however, suggest that it may be possible to replace unphosphorylated H1 with phosphorylated H1 in oligonucleosomes, nucleosomes, and chromatosomes. Hence, in the near future, we anticipate significant advances in this area.

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TABLE I

Amino Acid Analyses of CHO $H1^{o}a$ and $H1^{o}b$: Comparison with bovine $H1^{o}a$ and $H1^{o}b$, mouse neuroblastoma $H1^{o}$, calf lung $H1^{o}$ and CHO histone H1(I).

	CHO $H1^{o}a$	CHO $H1^{o}b$	B. Liver[a] $H1^{o}a$	B. Liver[a] $H1^{o}b$	M. N.[b] $H1^{o}$	C. Lung[c] $H1^{o}$	CHO H1(I)
Asx	3.9	4.6	3.3	3.2	3.9	3.3	2.2
Thr	5.9	5.7	6.7	6.6	5.4	7.7	5.3
Ser	7.6	8.1	9.3	9.4	9.1	8.5	7.0
Glx	4.9	5.9	5.4	5.3	6.1	4.2	3.6
Pro	9.7	7.7	7.8	7.2	8.2	9.4	9.4
Gly	3.5	5.6	4.4	4.0	4.3	4.3	6.6
Ala	14.0	14.1	14.2	13.6	16.6	16.8	27.0
Cys	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Val	5.5	5.5	5.7	5.7	6.5	5.2	5.6
Met	0.3	trace	0.4	0.4	0.7	0.0	0.0
Ile	2.9	3.1	2.6	2.5	2.4	1.9	1.0
Leu	2.0	2.5	2.3	2.0	2.1	2.1	4.5
Tyr	1.3	1.2	1.3	1.5	0.8	1.1	0.6
Phe	0.9	1.4	1.2	1.3	0.9	0.9	0.6
Lys	32.6	29.9	31.1	32.7	28.8	31.1	24.5
His	1.5	1.0	1.0	1.0	0.5	0.6	0.0
Arg	3.4	3.8	3.1	3.2	3.3	2.6	2.2

[a] Bovine liver (Smith and Johns, 1980)

[b] Mouse neuroblastoma (Pehrson and Cole, 1980)

[c] Calf lung (Panyim and Chalkley, 1969a)

FIGURE LEGENDS

Fig. 1. Cell-cycle analysis of CHO cultures treated for 24 hr with various concentrations of sodium butyrate. Percentage of cells in G_1 (●), percentage of cells in S (■), and percentage of cells in G_2 + M (▲) were determined by FCM.

Fig. 2. Electrophoresis (long acid-urea-polyacrylamide gels) of the PCA-extracted proteins from cultures grown for 24 hr 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 hr prior to the CHO PCA extract.

Fig. 3. Percentage of unphosphorylated H1(●), the ratio of the integrated absorbance of $H1^{o}$ to 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 at the time of harvest (see Fig. 1). Lines are drawn through the percentages of unphosphorylated H1 and the ratio, $H1^{o}/H1$.

Fig. 4. Long acid-urea-polyacrylamide gel electrophoresis of whole $H1^{o}$ purified by Bio•Rex 70 chromatography (D'Anna *et al.*, 1980b): (1) major PCA-extracted proteins from G_1-enriched, butyrate-treated cultures; (2) purified $H1^{o}$ from G_1-enriched, butyrate-treated cultures; (3) major PCA-extracted proteins from metaphase-enriched

cultures; (4) purified $H1^{o}$ from metaphase-enriched cultures. Direction of protein migration is from top to bottom of gels.

Fig. 5. Long acid-urea-polyacrylamide gel electrophoresis of $H1^{o}a$ and $H1^{o}b$ (1) before and (2) after cleavage with cyanogen bromide. Direction of protein migration is from top to bottom.

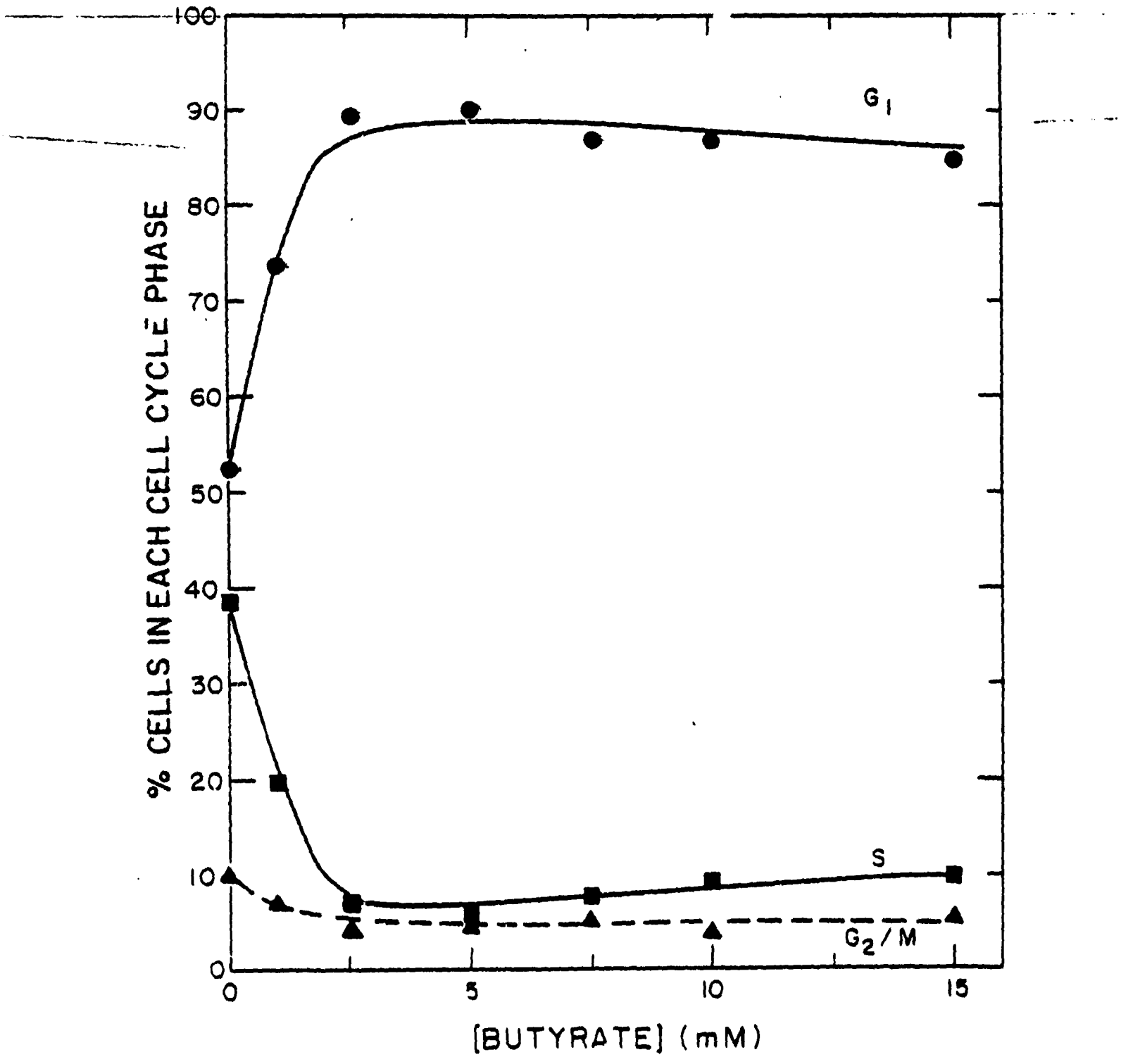
Fig. 6. ^{32}P-Phosphate incorporation (▲) into $H1^{o}$ and H1, determined from electrophoresis in long (0.5 x 25 cm) acid-urea-polyacrylamide gels: (a) the major PCA-extracted proteins from butyrate-treated (80-90% G_1) cultures; (b) the major PCA-extracted proteins from exponentially growing cultures; (c) the major PCA-extracted proteins from cultures synchronously-enriched in metaphase cells. All cultures were labeled with ^{3}H-lysine (●) for several generations and then labeled for 1 hr with $H_3{}^{32}PO_4$ before harvest. The direction of migration is from left to right. Numbers on the abscissa simply identify bands for comparison.

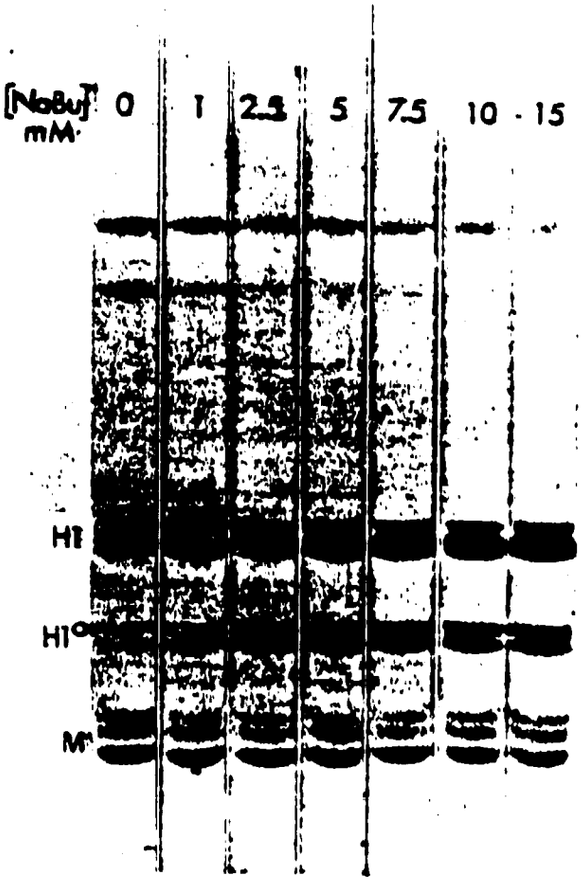
Fig. 7. Densitometer profiles from long acid-urea-polyacrylamide gels of $H1^{o}$ isolated from cells at various times after release from hydroxyurea blockage. M refers to $H1^{o}$ extracted from a culture enriched (90%) in metaphase cells by mitotic selection in the presence of Colcemid. Direction of migration is from left to right. Numbers on the abscissa imply identify bands for comparison. (Reprinted from D'Anna et al., 1980b, with permission of Biochemistry.)

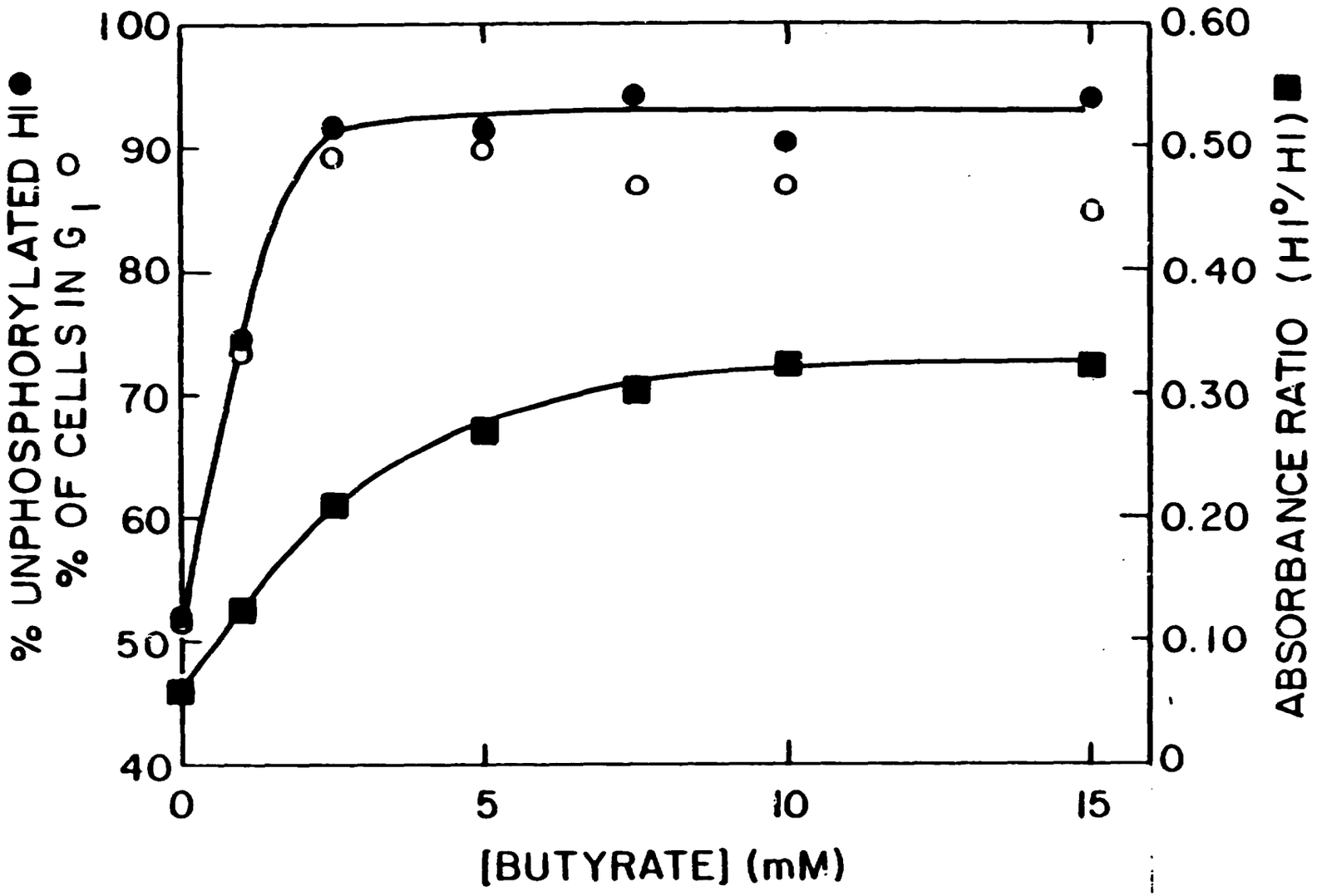
Fig. 8. Correlation of $H1^{o}$ mitotic phosphorylation (●, $H1^{o}_{M}$) with H1 mitotic phosphorylation (△, $H1_{M}$), and chromosomal condensation during entrance of cells into mitosis. The percentage of $H1_{M}$ and the percentage of cells in prophase plus metaphase (○) are taken from Fig. 6 of Gurley et al. (1978a). The line is drawn through the percentage of cells in prophase plus metaphase.

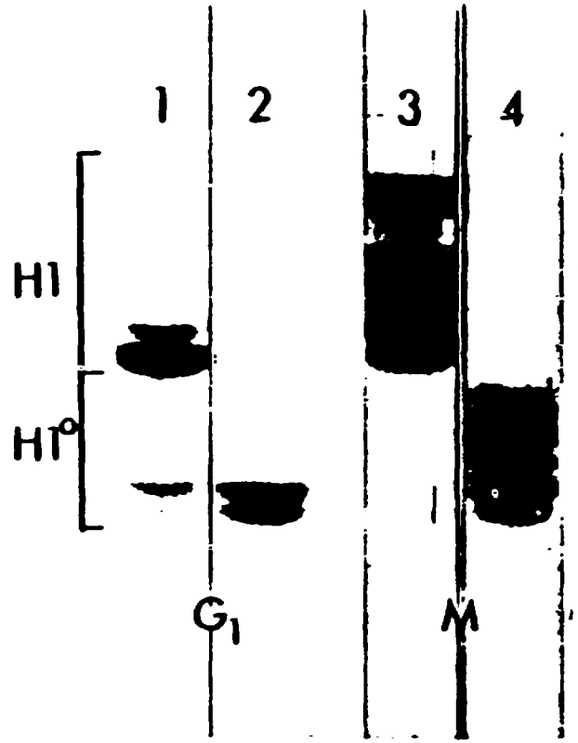
Fig. 9. Densitometer profiles from long (0.5 x 25 cm) electrophoretic gels of $H1^{o}$ isolated from cells at various times after mitotic selection. M refers to $H1^{o}$ extracted from a culture enriched (90%) in metaphase cells by mitotic selection in the presence of Colcemid. G_1 refers to $H1^{o}$ extracted from a culture arrested in G_1 by isoleucine deprivation. Direction of migration is from left to right. Numbers in the abscissa simply identify bands for comparison. (Reprinted from D'Anna et al., 1980b with permission from Biochemistry.)

Fig. 10. Correlation of the dephosphorylation of $H1^{o}_{M}$ (●) with the dephosphorylation of $H1_{M}$ (△) and the exit of cells from anaphase. The percentage of $H1_{M}$ and the percentage of cells in metaphase plus anaphase (○) are taken from Figure 12 of Gurley et al. (1978a). Since each cell in metaphase and anaphase gives rise to two cells in telophase and G_1, the number of cells in telophase and G_1 was divided by two to correct the percentage of cells to a common mass basis with metaphase and anaphase cells. The line is drawn through the percentage of cells in metaphase plus anaphase.


100
90
80
70
60
50
40
30
20
10
0
% CELLS IN EACH CELL CYCLE PHASE
G1
S
G2 / M
0
5
10
15
[BUTYRATE] (mM)


[NaBu] mM
0
1
2.5
5
7.5
10
15
H1
H1°
M

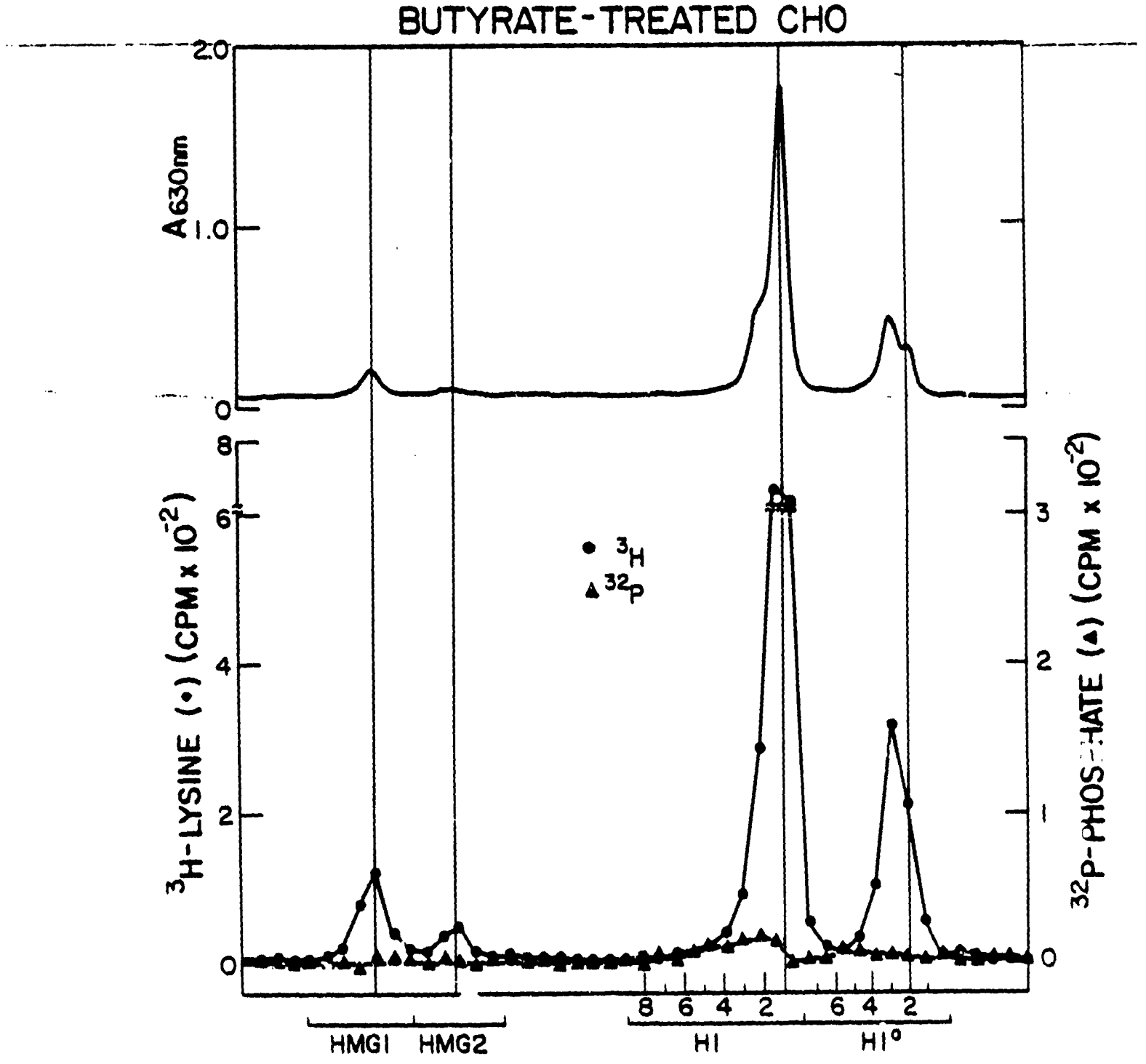

% UNPHOSPHORYLATED HI ●
% OF CELLS IN G1 ○
ABSORBANCE RATIO (HI°/HI) ■
100
90
80
70
60
50
40
0.60
0.50
0.40
0.30
0.20
0.10
0
0
5
10
15
[BUTYRATE] (mM)

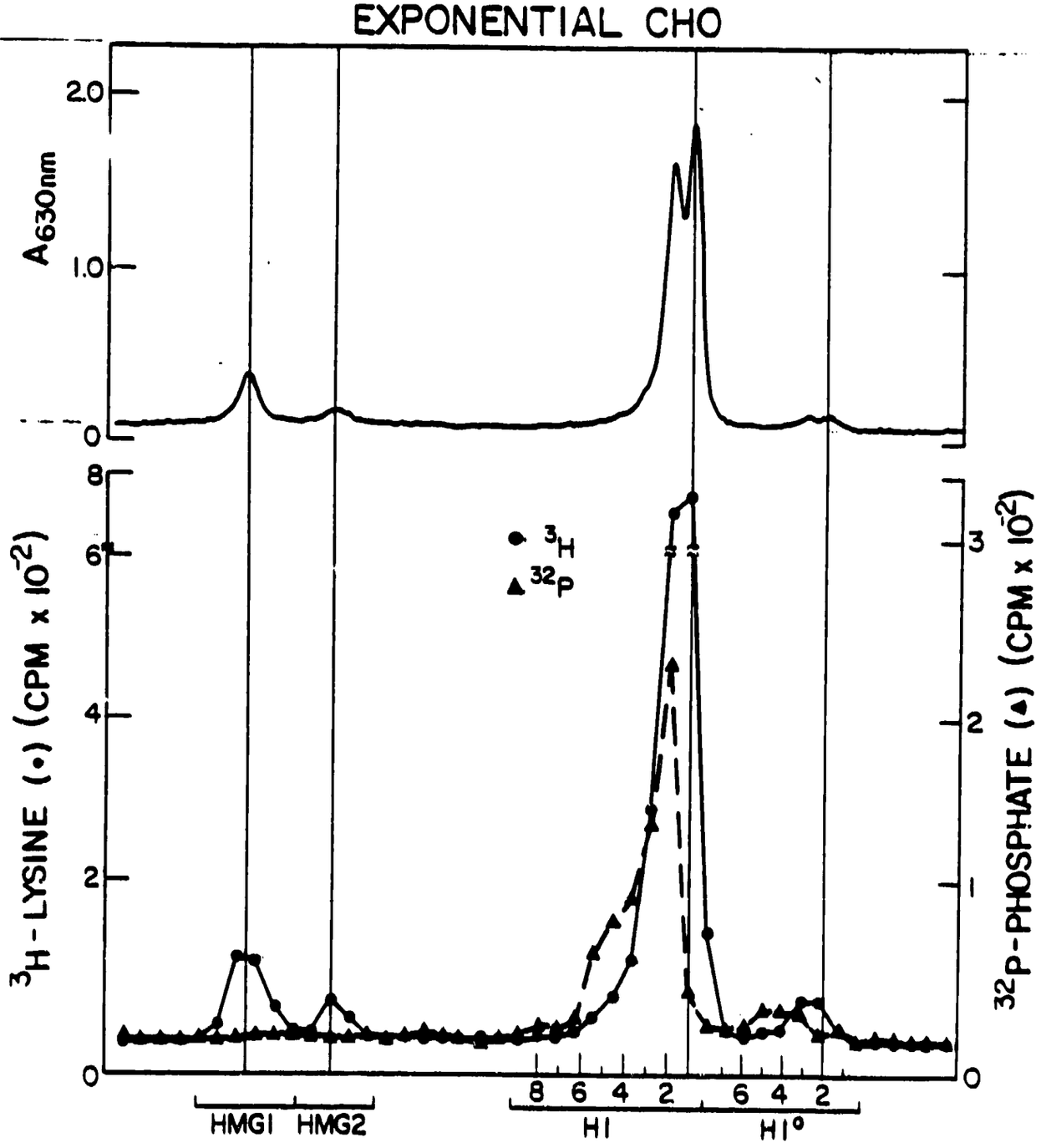

1
2
3
4
H1
H1°
G1
M

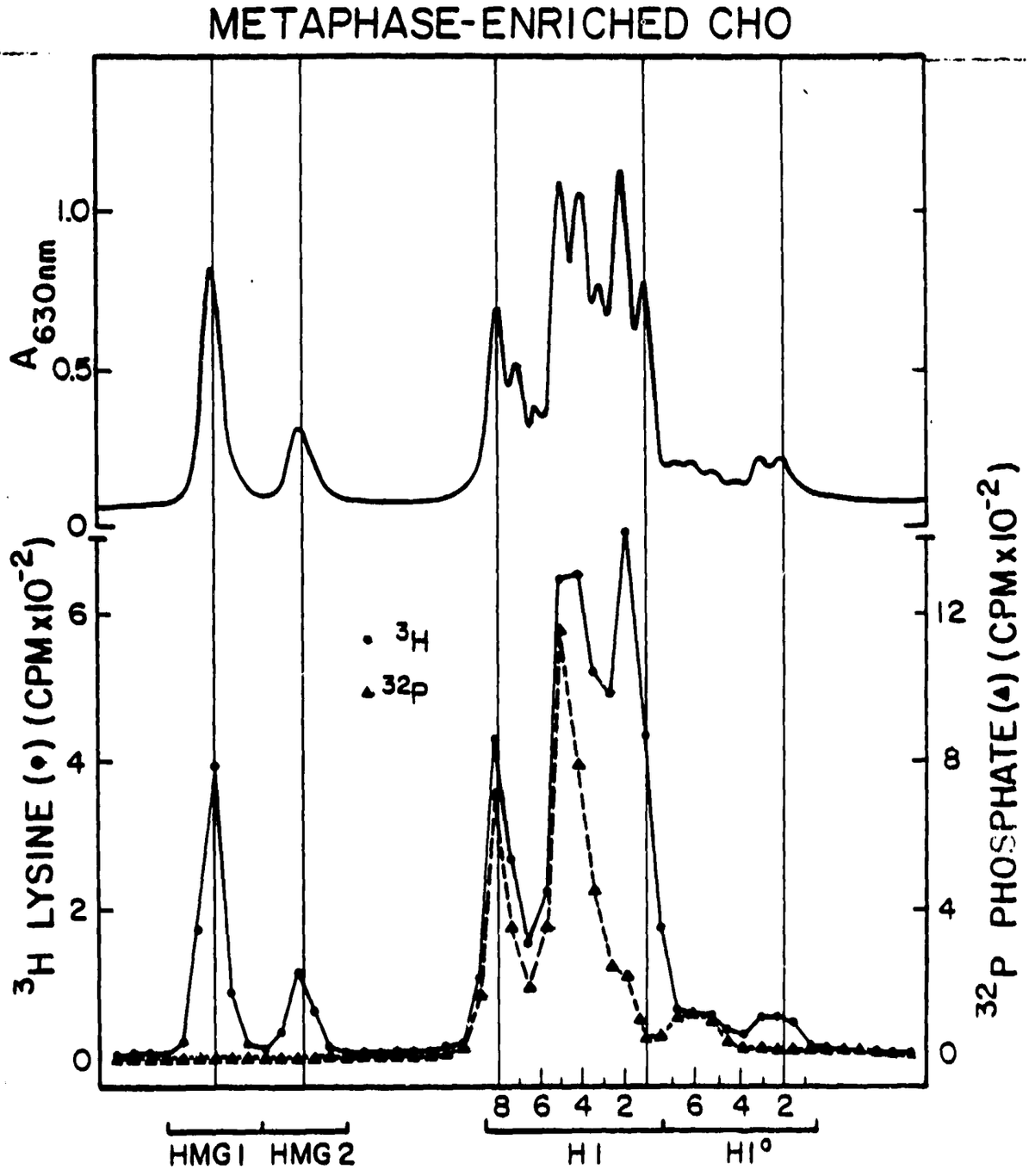
1 2

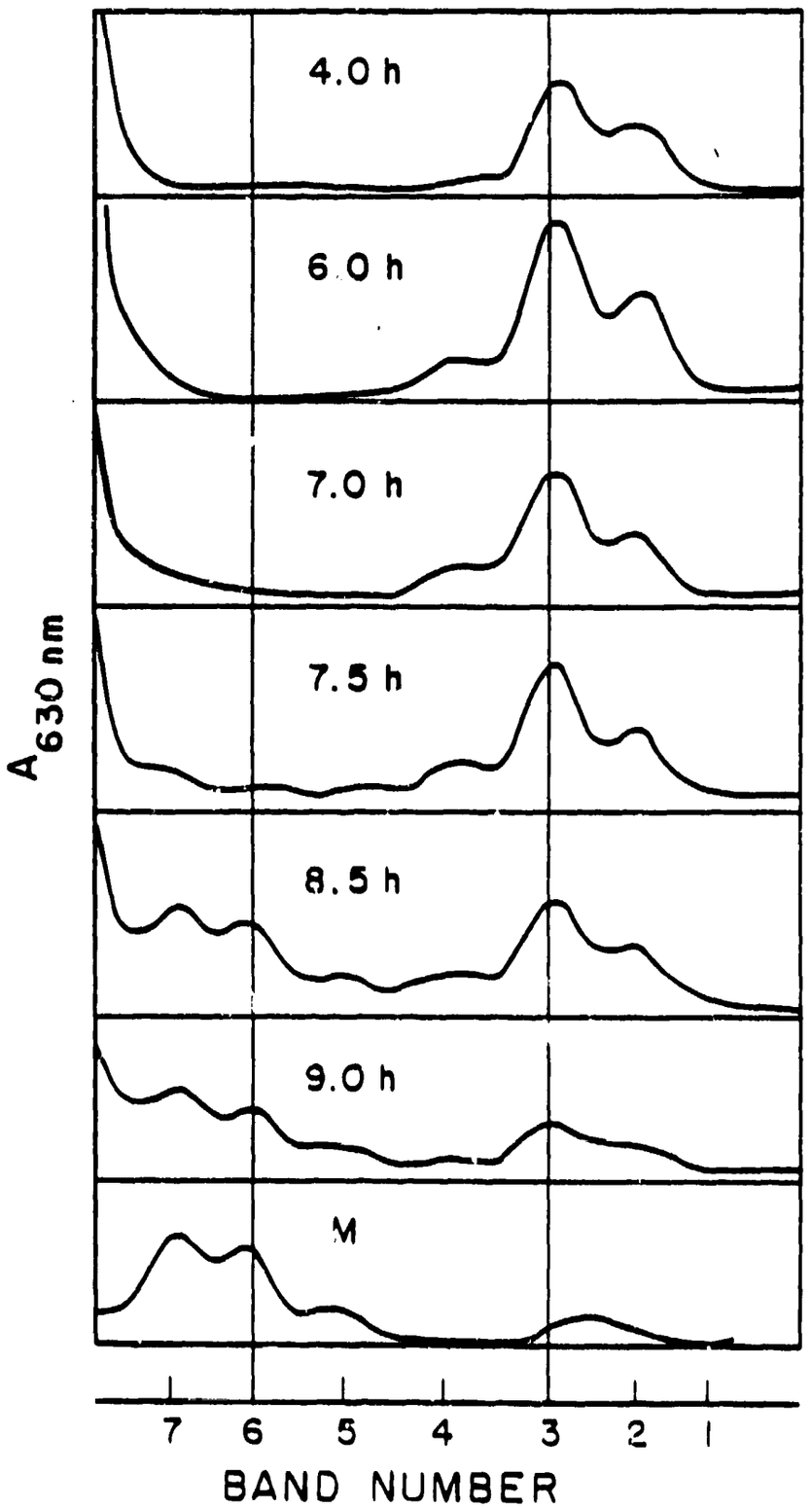
H1°

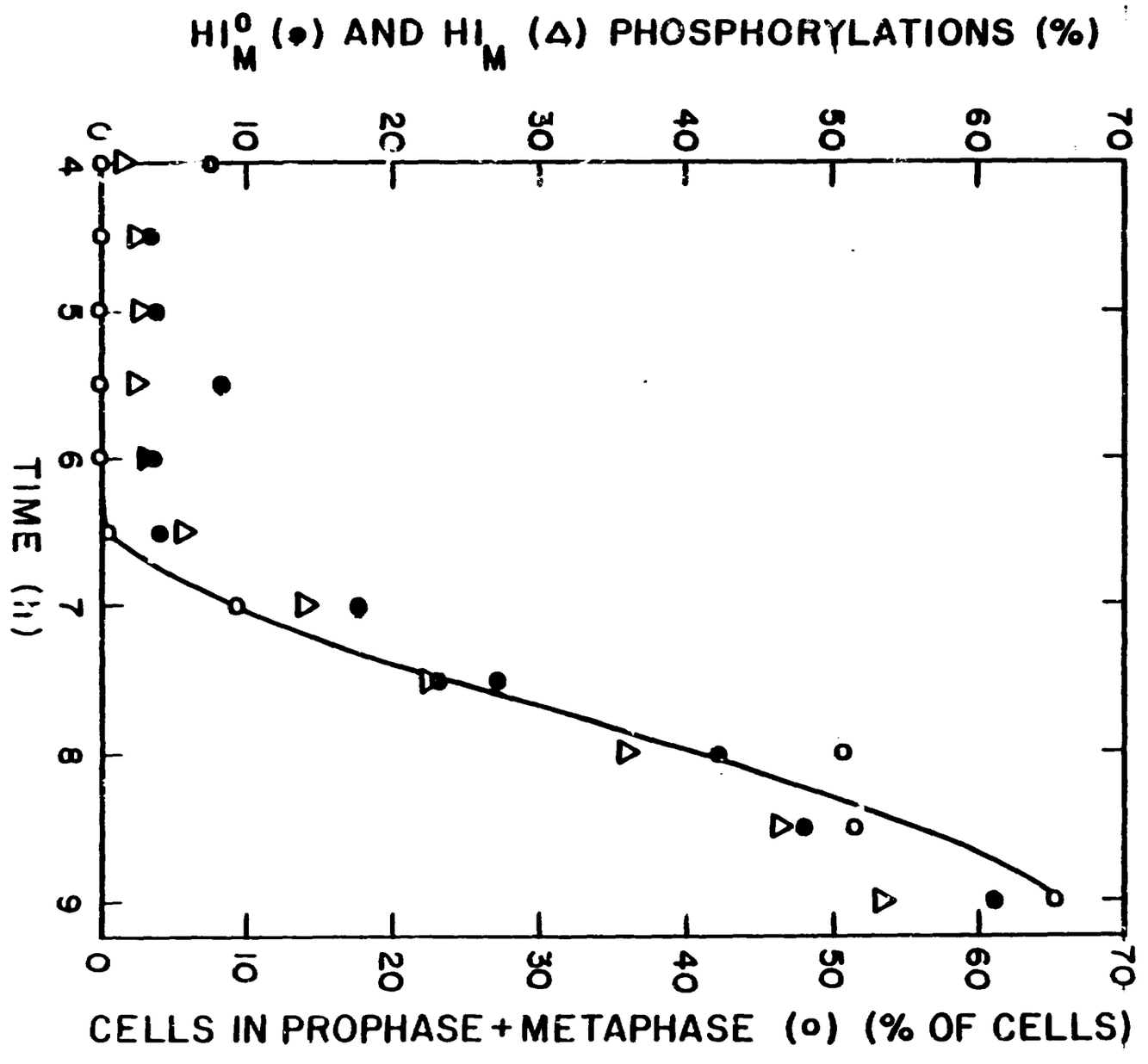
347
f 22
8.5 sec
4 filter

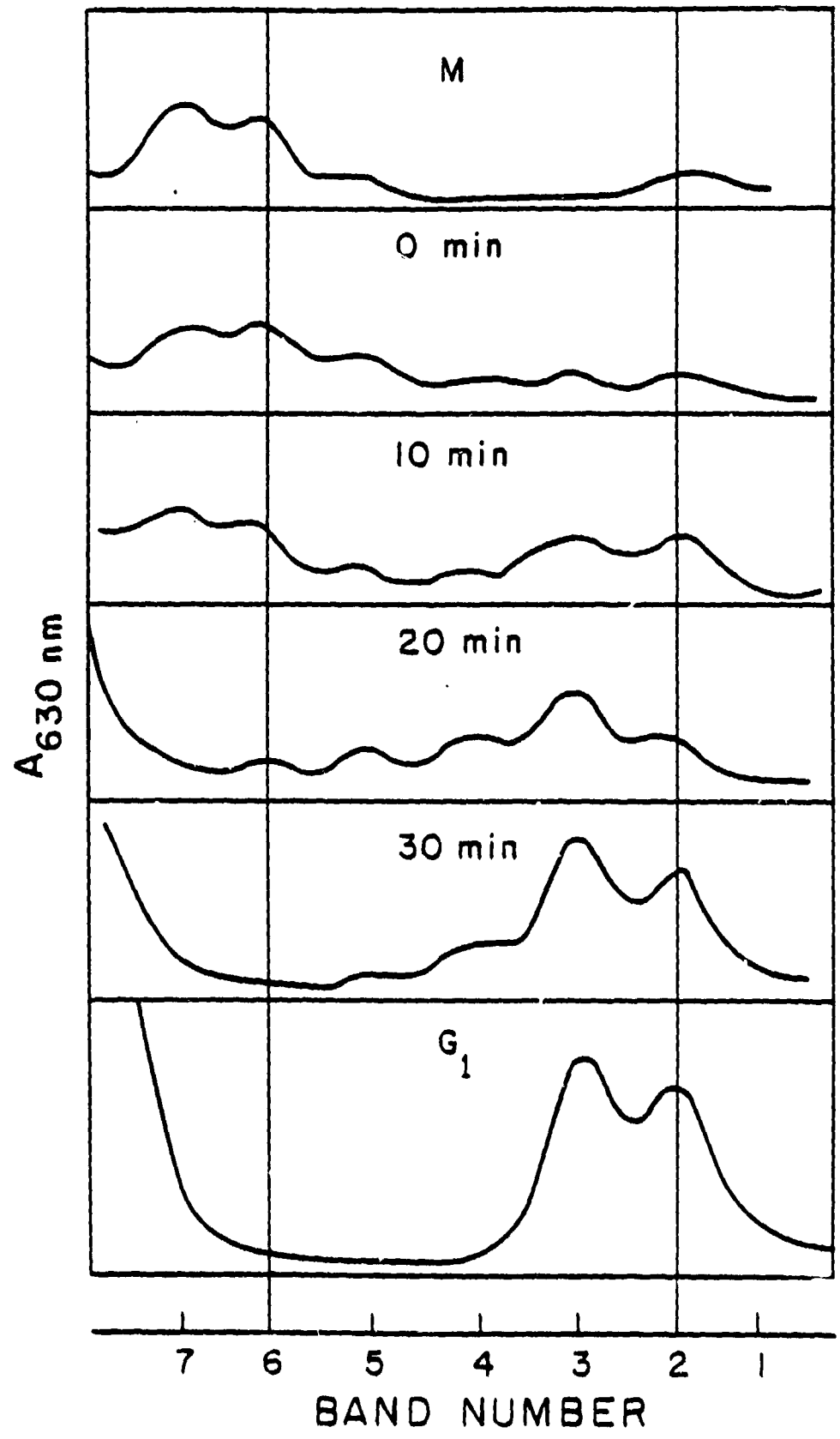

BUTYRATE-TREATED CHO
A630nm
2.0
1.0
0
^{3}H-LYSINE (•) (CPM x 10^{-2})
8
6
4
2
0
^{3}H
^{32}P
^{32}P-PHOSPHATE (▲) (CPM x 10^{-2})
3
2
1
0
8 6 4 2
6 4 2
HMG1
HMG2
H1
H1°

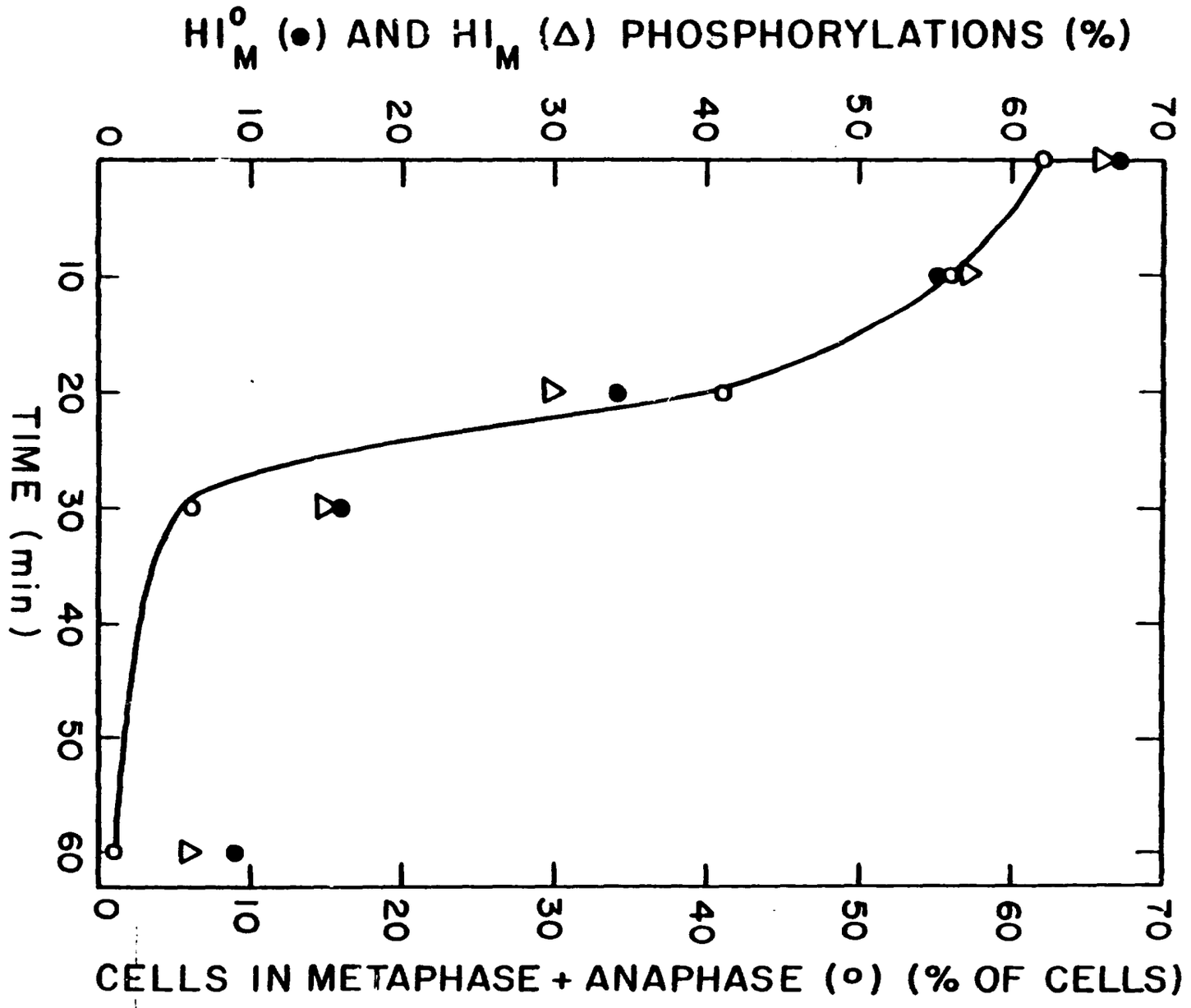

EXPONENTIAL CHO
A630nm
2.0
1.0
0
8
6
4
2
0
3H-LYSINE (•) (CPM x 10-2)
● 3H
▲ 32P
3
2
1
0
32P-PHOSPHATE (▲) (CPM x 10-2)
8 6 4 2
6 4 2
HMG1
HMG2
H1
H1°


METAPHASE-ENRICHED CHO
A_{630nm}
1.0
0.5
0
^3H LYSINE (•) (CPM x 10^{-2})
^{32}P PHOSPHATE (▲) (CPM x 10^{-2})
• ^3H
▲ ^{32}P
6
4
2
0
12
8
4
0
8 6 4 2
6 4 2
HMG1
HMG2
H1
H1°


4.0 h
6.0 h
7.0 h
7.5 h
8.5 h
9.0 h
M
$A_{630\,nm}$
7
6
5
4
3
2
1
BAND NUMBER


HI^0_M (•) AND HI$_M$ (Δ) PHOSPHORYLATIONS (%)
0
10
20
30
40
50
60
70
C
4
5
6
7
8
9
TIME (h)
CELLS IN PROPHASE + METAPHASE (o) (% OF CELLS)


M
0 min
10 min
20 min
30 min
G1
A630 nm
7
6
5
4
3
2
1
BAND NUMBER


HI^0_M (●) AND HI_M (Δ) PHOSPHORYLATIONS (%)
0
10
20
30
40
50
60
70
TIME (min)
CELLS IN METAPHASE + ANAPHASE (○) (% OF CELLS)