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Amino Acid Analysis and Cell Cycle Dependent Phosphorylation of an H1-like, Butyrate-Enhanced Protein (BEP; H1°; IP_{25}) from Chinese Hamster Cells†

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ABSTRACT: A fraction enriched in the butyrate-enhanced protein (BEP) has been isolated from Chinese hamster (line CHO) cells by perchloric acid extraction and Bio-Rex 70 chromatography. Amino acid analyses indicate that the composition of BEP resembles that of CHO H1; however, BEP contains 11% less alanine than H1, and, in contrast to H1, BEP contains methionine. Treatment of BEP with cyanogen bromide results in the cleavage of a small fragment of ~20 amino acids so that the large fragment seen in sodium dodecyl sulfate–acrylamide gels has a molecular weight of ~20 000. Radiolabeling and electrophoresis indicate that BEP is phosphorylated in a cell cycle dependent fashion. In G_1-arrested cells, little or no phosphate is incorporated into BEP. As cells progress through interphase, BEP becomes phosphorylated so that 12–35% of the BEP molecules are phosphorylated at one to two sites by late interphase. During mitosis, all BEP molecules become phosphorylated at approximately four sites per molecule (BEP_M). Electrophoresis and the analysis of cell populations by electron microscopy indicate that the appearance of BEP_M is temporally correlated with the mitotic phosphorylation of histone H1 ($H1_M$) and with chromosomal condensation during prophase, metaphase, and anaphase. During exit from mitosis, BEP_M undergoes dephosphorylation. The dephosphorylation of BEP_M is temporally correlated with dephosphorylation of $H1_M$ and with the unraveling of fully condensed chromosomes near the anaphase–telophase transition. These data suggest that (1) BEP is a specialized histone of the H1 class and (2) BEP is the species equivalent of calf lung histone H1° [Panyim, S., & Chalkley, R. (1969) *Biochem. Biophys. Res. Commun. 37*, 1042], rat H1° [Medvedev, Zh. A., Medvedeva, M. N., & Huschtscha, L. I. (1977) *Gerontology (Basel) 23*, 334], and IP_{25}, a protein enhanced in differentiated Friend erythroleukemia cells [Keppel, F., Allet, B., & Eisen, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 653]. The data also indicate that putative HMG1 and HMG2 proteins do not undergo the extensive cell cycle dependent phosphorylations measured for histone H1 and BEP.

Recently, this laboratory has observed an enhancement in the cellular content of an H1-like protein in Chinese hamster (line CHO) cells grown in the presence of sodium butyrate (D'Anna et al., 1980). The relative amount of that butyrate-enhanced protein (BEP) increases from 6–8% of H1 in control cultures to 32% of H1 when cultures are grown in the presence of 10–15 mM butyrate for 24 h. BEP can be extracted by 5% $HClO_4$ and by 0.20 M H_2SO_4 from the chromatin of blended whole cells and from the chromatin of isolated nuclei. BEP migrates slightly in front of H1 in both the urea–acrylamide–acetic acid electrophoresis gel system of Panyim & Chalkley (1969a) and in the sodium dodecyl sulfate ($NaDodSO_4$)[1]–acrylamide–Tris–glycine gel electrophoresis system of Laemmli (1970).

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[1] Abbreviations used: BEP, butyrate-enhanced protein; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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The extraction properties of BEP and its electrophoretic properties in urea–acrylamide–acetic acid gels (D'Anna et al., 1980) suggest that it is related to calf lung H1°[2] reported by Panyim & Chalkley (1969b). The electrophoretic mobility of BEP in urea–acrylamide–acetic acid gels, its electrophoretic mobility in $NaDodSO_4$ gels, and its enhancement in butyrate-treated cultures suggest that it also is related to IP_{25}, a protein enhanced in Friend erythroleukemia cells by treatment with dimethyl sulfoxide, hexamethylenebis(acetamide), or sodium butyrate (Keppel et al., 1977, 1979; Candido et al., 1978).

In this communication we report the amino acid composition of BEP and histone H1 from CHO cells. It is shown that BEP, like CHO histone H1 [see Gurley et al. (1978a,b) for a review], is phosphorylated in interphase, and it is highly phosphorylated ("superphosphorylated") during mitosis. During mitosis it appears that all BEP molecules become superphosphorylated at approximately four sites per molecule, and the occurrence of the BEP superphosphorylation is temporally correlated with both chromosomal condensation and the occurrence of H1 superphosphorylation (Gurley et al., 1978a). These data indicate that BEP is related to both histone H1 and calf lung histone H1°. Furthermore, they suggest that BEP, H1°, and IP_{25} are species equivalents of the same protein and should be regarded as a special class of H1 histone.

Experimental Procedures

Cell Growth and Cell Synchrony. Suspension cultures of Chinese hamster cells (line CHO) were grown in F-10 medium supplemented with 15% newborn calf serum, streptomycin, and penicillin (Tobey et al., 1966). Cultures were maintained free of *Mycoplasma* contamination determined by periodic assay (Walters et al., 1974). Cell concentrations in cultures were measured with an electronic particle counter (Tobey et al., 1967a).

CHO cells for preparative isolation of BEP and histone H1 were grown in 4.0-L suspension cultures. Cultures used for isolation of BEP for amino acid analysis were treated with sodium butyrate to enhance the cellular content of BEP (D'Anna et al., 1980). Sodium butyrate (sterile 1.0 M solution in isotonic saline, pH 7.3) was added to exponentially growing cultures [$(2.0–2.4) \times 10^5$ cells/mL] to make the solutions 10 mM in butyrate. After 24 h the cultures [$(3.0–3.6) \times 10^5$ cells/mL] were harvested by centrifugation.

Phosphorylated BEP was obtained from 4-L suspension cultures synchronously enriched in metaphase-arrested cells (Gurley et al., 1975). Growth and synchrony of these cultures have been described (D'Anna et al., 1979). The mitotic fraction determined by phase-contrast microscopy was typically 67%.

The experiments used to examine BEP phosphorylation during entry and exit of cells from mitosis were reported previously (Gurley et al., 1978a) for the determination of H1 phosphosphorylation. Briefly, to examine changes in BEP phosphorylation during entry of cells into mitosis, we synchronized cells near the G_1/S boundary by isoleucine deprivation, followed by hydroxyurea blockade (Tobey, 1973). The cultures were then released into fresh F-10 medium to resume synchronous cell-cycle traverse (t = 0 h). Four hours later Colcemid (0.06 μg/mL) was added to the culture. In this way as cells entered mitosis, they became blocked in metaphase and could not enter G_1 of the next cell cycle. Starting at the time of addition of Colcemid (t = 4.0 h), samples were withdrawn for electrophoretic analysis of histone phosphorylation and for the determination of cells in interphase, prophase, prophase, and metaphase by electron microscopy. These procedures have been described in detail by Gurley et al. (1978a).

To examine BEP phosphorylation during exit from mitosis, we synchronized cells by mitotic selection in the absence of Colcemid (Tobey et al., 1967b). This procedure yielded a series of 125-mL suspension cultures containing 99% mitotic cells. At various times after mitotic selection, each culture was harvested for analysis of histone phosphorylation and for cell-cycle analysis by electron microscopy. Cells were scored by electron microscopy as being in metaphase/anaphase, early telophase, late telophase, or G_1 (Gurley et al., 1978a).

Isotopic Labeling. To determine if phosphate was being incorporated into BEP in exponentially growing cells, we labeled cultures for 76 h (5.4 generations) with [^{3}H]lysine (8 Ci/mM; Schwarz/Mann) at an isotope concentration of 50 μCi/L. They were then labeled for 1 h with $H_3{}^{32}PO_4$ (New England Nuclear) at an isotope concentration of 20 mCi/L.

Phosphate incorporation into BEP also was determined in cultures synchronously enriched in metaphase cells. Cultures were labeled with [^{3}H]lysine (50 μCi/L) throughout the entire synchronization procedure. Exponentially growing cultures were first labeled 48 h with [^{3}H]lysine, and then they were synchronized in G_1 by the isoleucine deprivation method of Tobey & Ley (1971). Following 36 h of growth in isoleucine-deficient medium, the cells were resuspended in fresh F-10 containing 1 mM hydroxyurea which resynchronized the cells near the G_1/S boundary. After release from hydroxyurea blockade, Colcemid (0.06 μg/mL) was added to the culture to block cells in metaphase. $H_3{}^{32}PO_4$ (20 mCi/L) was added to the culture 8 h after release from hydroxyurea, and the culture was harvested 1 h later by centrifugation. These cultures typically contain 65–70% metaphase cells.

Isolation of BEP and Histone H1. Histone H1 and BEP were extracted from CHO cells with 5% perchloric acid by the first method of Johns (1964) as adapted to CHO cells by Gurley & Hardin (1968). Sodium bisulfite was added to the saline wash solution and to the 5% perchloric acid to inhibit proteolysis (Bartley & Chalkley, 1970) and the dephosphorylation of histone H1 (Gurley et al., 1975; D'Anna et al., 1978). The acetone-washed proteins were dissolved in water, lyophilized, and stored in desiccated containers at −16 °C for further purification or for analysis.

Partially purified histone H_1 and a fraction enriched in BEP were obtained by chromatography of the $HClO_4$-extracted proteins on Bio-Rex 70 (Kincade & Cole, 1966). Bio-Rex 70 columns of 2.0 × 17 cm and 1.5 × 20 cm were used for the isolations. Approximately 3.0–4.5 mg/cm^2 (cross-sectional area) were dissolved in 8.0% guanidine hydrochloride (Schwarz/Mann Ultrapure) in 0.10 M phosphate buffer (pH 6.8) and applied to a Bio-Rex 70 column equilibrated with the same buffer. After the loaded column was washed with 3 column volumes of starting buffer, H1 was eluted from the column with 10.27% guanidine hydrochloride in 0.10 M phosphate buffer (pH 6.8). Protein was detected by absorbance at 218 nm (Hohmann et al., 1976). After H1 was eluted from the column (see Figure 1), the column buffer was changed to 14.0% guanidine hydrochloride in 0.10 M phosphate buffer (pH 6.8) to elute BEP. Those fractions containing H1 or BEP were desalted on Sephadex G-25 equilibrated with 10 mM HCl and then lyophilized. H1 was purified further as described previously (D'Anna et al., 1979). [Note that in D'Anna et al. (1979) the HCl concentrations used for the

[2] Histone H1° refers to the H1-like protein first reported by Panyim & Chalkley (1969b). H1° has also been called $H1_o$, F1°, and $F1_o$.

Sephadex columns in the H1 preparation were actually 10 mM rather than the 1 mM mistakenly listed in that paper.]

Treatment with Alkaline Phosphatase. Perchloric acid extracted proteins at a concentration of 0.5 mg/mL were treated with alkaline phosphatase (EC 3.1.3.1; Worthington Biochemical Corp.) in 0.10 M Tris-HCl (pH 8.0) as described by Lake et al. (1972). After incubation at 37 °C for 6 or 12 h, the samples were made 20% in trichloroacetic acid and allowed to precipitate overnight at 4 °C. The pellets were washed with acidified acetone and acetone (Johns, 1964), and they were dissolved in water and lyophilized.

Protein Analyses. 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 h in sealed evacuated tubes.

To verify the amino acid analyses regarding the presence or absence of methionine, we subjected CHO H1 and BEP to treatment with cyanogen bromide (Gross & Witkop, 1962). Protein (30–140 μg) was dissolved in 25–50 μL of 70% formic acid (Sautiere et al., 1968) containing 10 mg/mL cyanogen bromide. The tubes were sealed with Parafilm and left in the dark at room temperature, with occasional shaking, for 26 h. Four volumes of water were added to the samples which were then lyophilized to remove solvent and excess cyanogen bromide.

Electrophoresis. Electrophoresis of BEP and histone H1 was performed with a number of different gel systems. Na-DodSO$_4$–15% acrylamide–Tris–glycine gel electrophoresis was performed in 0.5 × 10 cm cylindrical gels according to the method of Laemmli (1970) as described previously (D'Anna et al., 1979). Charge-dependent analytical gel electrophoresis was performed in short (0.5 × 13 cm) 6 M urea–12% acrylamide–5% acetic acid gels containing 0.38% Triton DF-16 (Alfageme et al., 1974) and in long (0.6 × 25 cm) 2.5 M urea–15% acrylamide–5% acetic acid gels (Panyim & Chalkley, 1969a). With the latter two methods, proteins were dissolved at an estimated 0.5 mg/mL per major protein in a sample buffer of 6 M urea, 5% 2-mercaptoethanol, and 5% acetic acid. A sample volume of 25 μL was applied to the gels. Electrophoresis in the short gels was performed at 180 V for 4 h. Electrophoresis in the long polyacrylamide gels was performed at 240 V for 27 h as described previously (Gurley et al., 1978a). Gels were stained overnight in 0.2% amido black–30% methanol–9% acetic acid and destained by diffusion. No difference in the electrophoretic patterns of the perchloric acid extracted proteins was observed if DF-16 was omitted or if the percentage of acrylamide was changed from 12 to 15%.

Densitometer profiles of analytical gels were measured with a Gilford Model 240 spectrophotometer equipped with a gel linear transport device. Different BEP bands were resolved and quantified electronically with a Du Pont Model 310 curve resolver (Panyim & Chalkley, 1969a).

Radiolabeled proteins separated by analytical electrophoresis were analyzed first by densitometry of the stained gels and then by scintillation spectrometry (Enger et al., 1979). After densitometry, the cylindrical gels were sliced into 2.2-mm pieces and placed in glass scintillation vials containing 1.0 mL of 30% hydrogen peroxide. The vials were closed tightly, and they were incubated for 16–18 h at 55–60 °C. Fifteen milliliters of Aquasol II (New England Nuclear) or Hydrofluor (National Diagnostics) was added to the vials. The samples were counted for 10 min each in a Packard Model 3320 scintillation spectrometer.

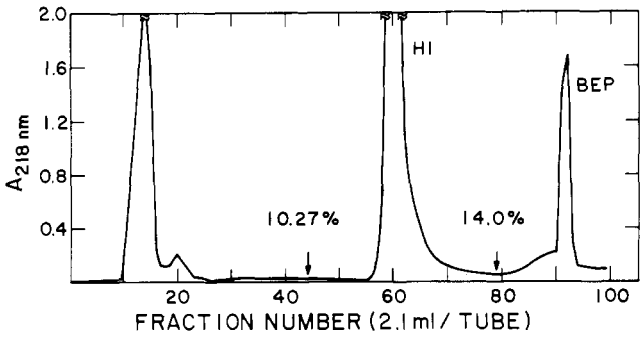

FIGURE 1: Bio-Rex 70 chromatogram of the perchloric acid extracted proteins from cultures grown 24 h in the presence of 10 mM butyrate. The proteins (8.3 mg) were applied to a 1.5 × 20 cm Bio-Rex 70 column equilibrated with 8.0% guanidine hydrochloride in 0.10 M phosphate buffer, pH 6.8. The arrows indicate a change in the concentration of guanidine hydrochloride applied to the column.

Preparative electrophoresis of the perchloric acid extracted proteins was performed with a Canalco Prep-Disc apparatus in which protein is continuously removed from the bottom of the gel with a cross flow of buffer (Gurley & Walters, 1971). A 4.5 cm long gel (6 M urea–15% acylmide–0.9 M acetic acid containing 0.38% DF-16) was used for the separations. Fractions (2.0 mL) from the cross flow buffer were collected in scintillation vials, mixed with 12 mL of Aquasol II, and counted by scintillation spectrometry.

Results

Isolation of BEP. BEP has been isolated from cells grown in the presence of sodium butyrate for 24 h (BEP_B) and from cultures synchronously enriched (no butyrate) in metaphase cells (BEP_{ME}). Figure 1 shows the typical elution profile when the proteins extracted by 5% perchloric acid (Johns, 1964) are separated by chromatography on Bio-Rex 70 ion-exchange resin. Impure proteins containing HMG1 and HMG2 are eluted in the guanidine hydrochloride wash (fractions 10–17); partially purified H1 is eluted by 10.27% guanidine hydrochloride (fractions 56-70); and the BEP fraction is eluted as a band by 14.0% guanidine hydrochloride.

If elution with 10.27% guanidine hydrochloride is continued after H1 is removed from the column, BEP appears to be eluted as a broad band; however, changing the buffer to 14% guanidine hydrochloride effectively strips the rest of the BEP from the column so that it elutes sharply. Tubes 86–92 in Figure 1 were pooled as the BEP_B fraction. A yield of 1.0 mg of BEP_B was obtained from 8.3 mg of the perchloric acid extracted proteins from butyrate-treated cultures.

Prior to finding that butyrate enhances the cellular content of BEP, BEP had been isolated from the perchloric acid extracted proteins of cultures synchronously enriched (67%) in metaphase cells. A yield of 0.17 mg of BEP_{ME} was isolated from 10 mg of the perchloric acid extracted proteins.

Electrophoresis of BEP. Electrophoresis of BEP_B (from butyrate-treated cultures) and BEP_{ME} (from cultures enriched in metaphase cells) in NaDodSO$_4$ gels (Figure 2) shows that both preparations yield single bands which have the same electrophoretic mobility. By use of a molecular weight of 23 600 for CHO H1 (D'Anna et al., 1979) and published molecular weights for calf thymus histones (Elgin & Weintraub, 1975) as standards, plots of electrophoretic mobilities from Figure 2 indicate that BEP has a molecular weight of ~22 000 (or ~1500 less than H1).

When BEP_B and BEP_{ME} are subjected to analytical gel electrophoresis on short Triton DF-16 gels (Figure 3), BEP_B migrates as a major band preceded by a minor (presumed)

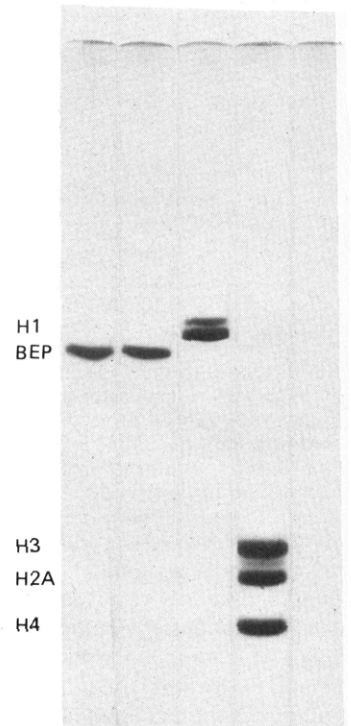

FIGURE 2: $NaDodSO_4$ gel electrophoresis of BEP_B and BEP_{ME}. From left to right: BEP_B from butyrate-treated cultures, BEP_{ME} from cultures enriched in metaphase cells, purified CHO H1 from exponentially growing cells, calf thymus inner histones extracted with ethanol by the first method of Johns (1964), and a blank gel.

impurity (≤6%). In contrast, BEP_{ME} migrates as two major bands of similar intensity preceded by a minor (presumed) impurity (≤4%). Comparison of the mobilities of the major bands of BEP_B and BEP_{ME} indicates that the faster major band of BEP_{ME} coincides with the major band of BEP_B; however, the other major band of BEP_{ME} migrates slightly slower. These results suggest either that BEP_{ME} is made up of two major proteins or that BEP is modified in metaphase cells so that the modified BEP possesses a retarded electrophoretic mobility. As we shall see, other evidence indicates that BEP_B and BEP_{ME} are the same protein and that BEP is phosphorylated in a cell cycle dependent fashion.

The identity of the presumed impurity in the BEP preparations is not known; however, its electrophoretic mobility suggests it may be similar to a band labeled $H1^{oo}$ by Medvedev et al. (1977).

Amino Acid Analysis of CHO H1 and BEP. Amino acid analyses of BEP_B and purified CHO H1 are presented in Table I for comparison with those of calf thymus H1 (Sherod et al., 1974) and calf lung $H1^o$ (Panyim & Chalkley, 1969b). We note that because only limited quantities of protein were available, samples were not examined for the presence of tryptophan nor were the analyses corrected for possible hydrolytic losses of serine and threonine; nevertheless, the analyses allow us to reach a number of important conclusions about the relationships between CHO H1, BEP, calf H1, and calf $H1^o$.

(1) Comparison of CHO H1 and calf thymus H1 in Table I shows that (a) both proteins are very basic, (b) they possess large quantities of alanine, and (c) neither contains cysteine or methionine. A quantitative comparison (Marchalonis & Weltman, 1971) of the difference in the amino acid composition between the two H1's yields an $S\Delta Q$ value of only 4.5 (see below). Since CHO H1 and calf thymus H1 undergo similar conformational changes in solution (D'Anna et al., 1979), there is no doubt that they are the respective H1 proteins of each organism.

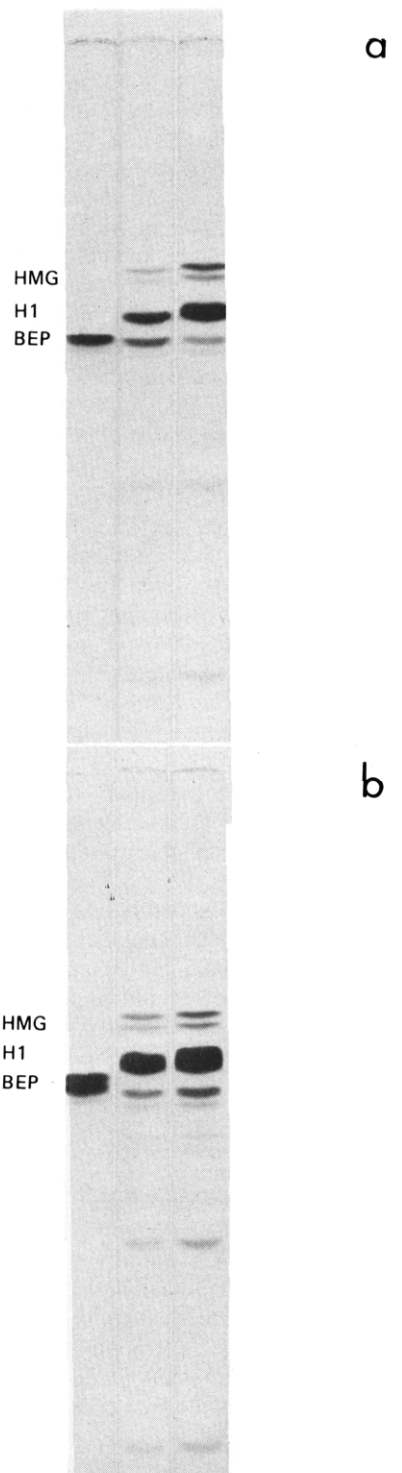

FIGURE 3: Electrophoresis of BEP_B and BEP_{ME} in Triton DF-16 gels. (a) Comparison of BEP_B with the perchloric acid extracted proteins from a butyrate-treated culture and from an exponentially growing culture. From left to right: BEP_B, perchloric acid extracted proteins from a butyrate-treated culture, and proteins from an exponentially growing culture. (b) Comparison of BEP_{ME} with two preparations of perchloric acid extracted proteins from exponentially growing cultures. BEP_{ME} is on the left.

(2) Comparison of the amino acid analyses of BEP and calf lung $H1^o$ (Table I) indicates strong similarities. Both proteins, like H1, are highly basic, and they possess similar quantities of all of their amino acids. A major feature that distinguishes BEP from $H1^o$ is that BEP appears to contain one methionine

Table I: Amino Acid Analyses of CHO H1 and CHO BEP: Comparison with Calf Thymus H1 and Calf Lung H1°

	composition			
	CHO H1[a]	calf thymus[b] H1	CHO BEP[a]	calf lung[c] $H1_o$
Asx	2.5	2.7	3.7	3.3
Thr	4.6	5.4	6.0	7.7
Ser	7.0	6.1	7.2	8.5
Glx	3.9	4.3	5.0	4.2
Pro	8.3	8.3	7.9	9.4
Gly	6.1	7.2	3.7	4.3
Ala	25.7	25.4	14.3	16.8
Cys	0.0	0.0	0.0	0.0
Val	5.3	4.1	5.7	5.2
Met	0.0	0.0	0.5	0.0
Ile	0.8	0.8	3.2	1.9
Leu	4.1	4.2	2.2	2.1
Tyr	0.5	0.5	1.3	1.1
Phe	0.6	0.5	1.0	0.9
Trp	0.0[d]	0.0		0.0
Lys	28.3	28.1	32.8	31.3
His	0.3	0.0	2.0	0.6
Arg	2.1	2.2	3.6	2.6

[a] The analysis is taken from a 22-h hydrolysate which is not corrected for possible hydrolytic losses of serine and threonine. [b] Amino acid analysis taken from Table I of Sherod et al. (1974). The analysis was not corrected for possible hydrolytic losses of serine and threonine. [c] Amino acid analysis taken from Table II of Panyim & Chalkley (1969b). It is not stated if the analysis was corrected for possible hydrolytic losses of serine and threonine. [d] Determined from the absorbance spectrum (D'Anna et al., 1979).

per molecule, but calf lung H1° does not contain any. Despite the difference in methionine, quantitative comparison of amino acid composition yields an $S\Delta Q$ value[3] of 22. This result is similar to the median value of 20 computed between cytochrome *c* from 14 species (Marchalonis & Weltman, 1971); it is similar to the value of 24 computed from the composition (Mardian & Isenberg, 1978) of calf thymus H4 and yeast H4; and it is less than the median value of 80 computed between hemoglobins of 14 vertebrate species (Marchalonis & Weltman, 1971). Therefore, while similar composition alone does not prove that proteins have related sequences (Cornish-Bowden, 1977, 1978, 1979; Black & Harkins, 1977), the $S\Delta Q$ value and other index values [e.g., see Cornish-Bowden (1979)] between BEP and calf lung H1° are within the relm expected for homologous proteins from different species.

(3) BEP and calf lung H1° are basic proteins like histone H1; however, BEP and H1° differ significantly from H1 in a number of ways: (a) they contain 8–11% less alanine than H1; (b) they differ from H1 in the percentages of leucine and isoleucine; (c) they appear to contain two to three tyrosines per molecule rather than one as in H1; (d) they contain even higher percentages of lysine than H1. Additionally, BEP appears to contain methionine, and CHO H1 does not. Verification of this difference (see below) would prove that BEP cannot be an H1 degradation product.

Cyanogen Bromide Cleavage of BEP. If BEP does contain methionine as indicated in the amino acid analysis, then BEP should be cleavable with cyanogen bromide (Gross & Witkop, 1962). The demonstrated cleavage of a large percentage of BEP molecules would prove that methionine is truly present in BEP and that it is not simply a contaminant.

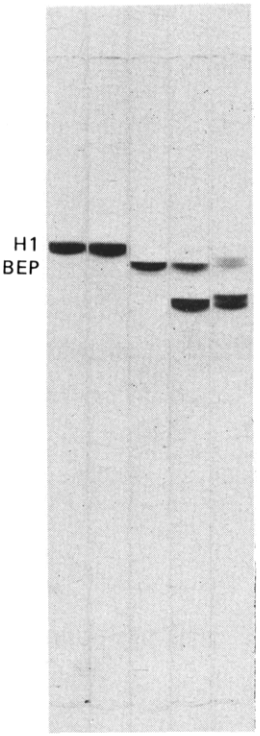

FIGURE 4: Electrophoresis (Triton DF-16 gels) of CHO H1 and BEP before and after treatment with cyanogen bromide. From left to right: untreated H1, treated H1, untreated BEP_B, treated BEP_B, and treated BEP_{ME}.

The results of cyanogen bromide treatment of CHO H1, BEP_B, and BEP_{ME} can be seen from the urea–acrylamide–acetic acid gels of Figure 4. CHO H1 is not attacked by cyanogen bromide, but both BEP samples give rise to faster moving bands. Therefore, we conclude the following: (1) Methionine is present in BEP but not H1; hence, BEP cannot be an H1 degradation product. (2) BEP_B and BEP_{ME} are very likely the same protein. Since the separation between the two BEP_{ME} cleavage products in the gels of Figure 4 is similar to the separation between the parent species, it appears that all (or most) of the putative phosphorylations of BEP_{ME} are located in the large cleavage product seen on the gel.

Examination of cleaved BEP in $NaDodSO_4$ gels (Figure 5) indicates that the molecular weight of the cleaved molecule is only ~2000 less than that of the parent BEP. Therefore, cyanogen bromide treatment results in the cleavage of a small fragment (or fragments) containing a total of ~20 amino acids.

[^{32}P]Phosphate Incorporation into BEP. Recently this laboratory has shown that BEP, like histone H1, is unphosphorylated or only slightly phosphorylated when cells are arrested in G_1 by treatment with sodium butyrate (D'Anna et al., 1980). To determine (1) if BEP becomes phosphorylated in other phases of the cell cycle and (2) if the slower migrating major band of BEP_{ME} in Figure 3 arises from phosphorylation during mitosis, we labeled exponentially growing cultures and cultures synchronously enriched in metaphase cells for 1 h with $H_3{}^{32}PO_4$. Their perchloric acid extracts were then separated by preparative electrophoresis in Triton DF-16 gels. Since urea–acrylamide–acetic acid gels were also used for analytical electrophoresis (Figure 3), the data of Figures 3 and 6 can be compared.

[3] $S\Delta Q$ (Marchalonis & Weltman, 1971) is defined as

$$S\Delta Q = \sum_{i=1}^{N}(X_i - Y_i)^2$$

in which X_i and Y_i refer to the mole percent of the amino acid residue of the *i*th type in proteins X and Y. *N* is the number of different types of amino acids determined in the analysis.

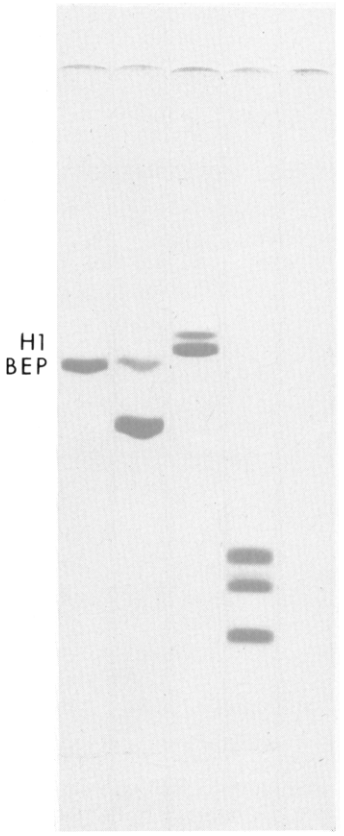

FIGURE 5: $NaDodSO_4$ electrophoresis of BEP cleaved with cyanogen bromide. From left to right: untreated BEP, cyanogen bromide treated BEP_B, CHO H1 (for reference), calf thymus inner histones (for reference), and a blank gel.

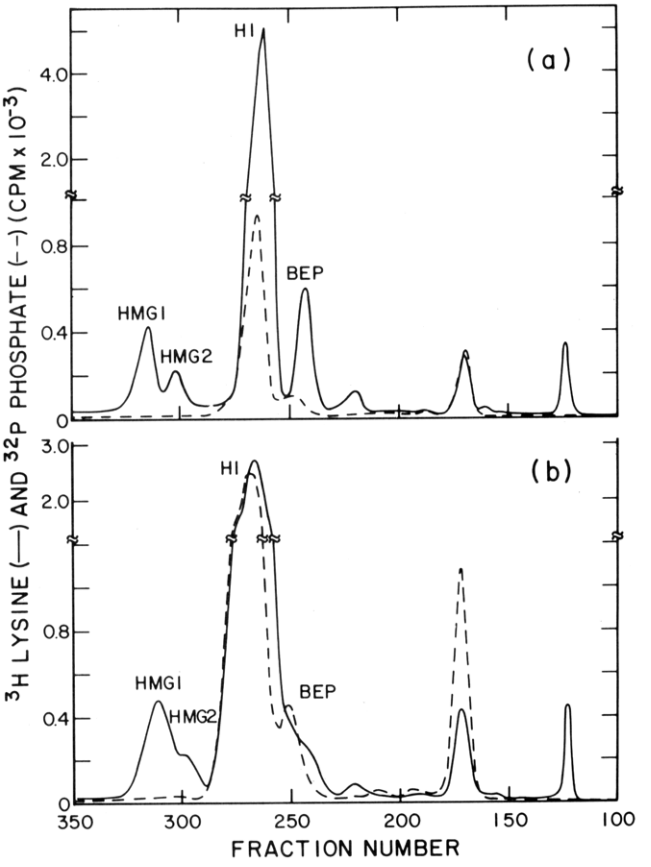

FIGURE 6: [^{32}P]Phosphate incorporation (- - -) into BEP, determined from Triton DF-16 preparative electrophoresis: (a) perchloric acid extracted proteins from exponentially growing cultures; (b) perchloric acid extracted proteins from cultures synchronously enriched in metaphase cells. Both cultures were labeled with [^{3}H]lysine (—) for several generations and then labeled with $H_3{}^{32}PO_4$ for 1.0 h before harvest.

The labeling profiles in Figure 6 indicate that, like histone H1, (1) BEP is phosphorylated in both exponentially growing and in metaphase cultures, and (2) phosphate incorporation into BEP is much higher (about fourfold) in metaphase cells. In Figure 6a (exponentially growing cultures) the ^{3}H and ^{32}P profiles of BEP are well resolved; however, in Figure 6b (metaphase-enriched cultures) the well-resolved ^{3}H peak of BEP is replaced by a broad shoulder. Additionally, there is a prominent increase in the ^{32}P peak on the BEP shoulder which corresponds to the slower migrating band of BEP_{ME} in Figure 3b. These data therefore support the assignment that in metaphase cells BEP, like CHO H1 (Gurley et al., 1975, 1978a; Hohmann et al., 1976), becomes highly phosphorylated so that its electrophoretic mobility is retarded in urea–acrylamide–acetic acid gels.

Additional similarities between BEP and H1 can be seen when the phosphate incorporation profiles are examined from long (0.5 × 25 cm) 2.5 M urea–15% acrylamide–acetic acid gels (Figure 7). The labeling and absorbance profiles of both BEP and H1 from metaphase cultures (Figure 7b) are sharply contrasted to those seen from exponentially growing cultures (Figure 7a). In exponentially growing cultures ~85% of BEP is found in bands 2 and 3, and the small amount of incorporated ^{32}P has a maximum at band 4. With H1, most of the histone is found in bands 1 and 2, and the maximum ^{32}P incorporation is found in band 2. In the metaphase-enriched culture, a large portion of BEP is shifted to bands 5–7, and the highest $^{32}P/^3H$ ratios are seen in this region. Similar shifts are seen for histone H1 in that a large portion of H1 is shifted from bands 1 and 2 to bands 4–8. Since (1) the $^{32}P/^3H$ ratio of BEP in bands 5–7 is similar to that seen in H1 for bands 4–8, and (2) since H1 in bands 4–8 is superphosphorylated at an estimated four to six sites per molecule (Hohmann et al., 1976), the labeling data suggest that BEP in bands 5–7 also is superphosphorylated at an estimated four to six sites per molecule. (This assumes that BEP and H1 undergo similar rates of phosphate incorporation and turnover during the 1.0-h labeling period.)

We mention in passing that little or no [^{32}P]phosphate is incorporated into regions of putative HMG1 and HMG2 (Goodwin et al., 1973, 1978) in the gels of Figures 6 and 7. For HMG1 and HMG2 from exponentially growing cultures (Figures 6a and 7a), no phosphate incorporation above base-line levels is detected. For HMG1 and HMG2 from metaphase-enriched cultures, there is a slight ^{32}P shoulder at HMG_2 at Figure 6b, but no detectable phosphorylation is measured in the analytical gels in Figure 7b. Therefore, while (1) HMG1 phosphorylation has been detected in vitro (Sun et al., 1980) and (2) it has been suggested that HMG1 and HMG2 may replace H1 in some regions of chromatin (Jackson et al., 1979), HMG1 and HMG2 do not appear to undergo the extensive cell cycle dependent phosphorylations observed for histone H1 and for BEP. In contrast to putative HMG1 and HMG2, the protein eluting at fraction 175 in Figure 6 is highly phosphorylated. Since this protein is extracted with 5% perchloric acid, it may be another member of the high mobility group proteins reported by Goodwin et al. (1973, 1978). This remains to be determined, however.

BEP Phosphorylation during Entry into and Exit from Mitosis. Because of the similarities between H1 and BEP phosphate incorporation, we wondered if the changes in BEP phosphorylation (as indicated by changes in electrophoretic

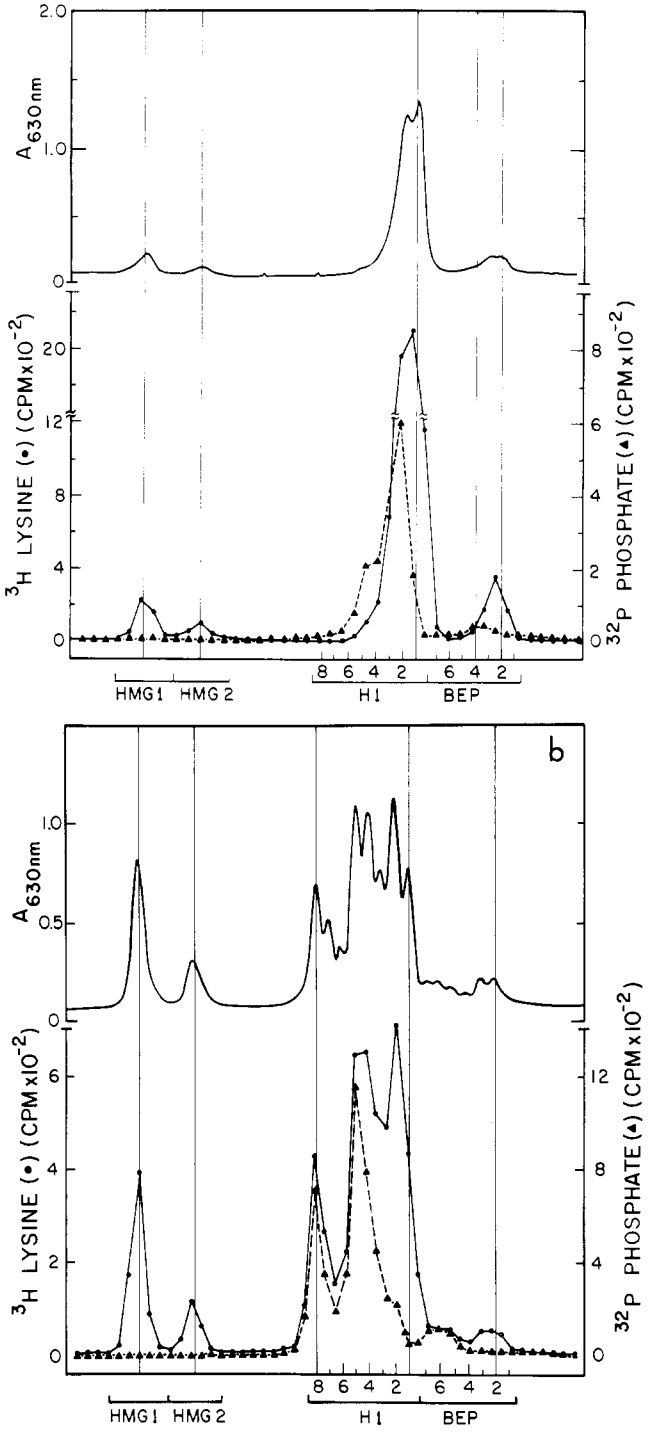

FIGURE 7: [^{32}P]Phosphate incorporation (▲) into BEP and H1, determined from electrophoresis in long (0.5 × 25 cm) 2.5 M urea–15% acrylamide–acetic acid gels: (a) the major perchloric acid extracted proteins from exponentially growing cultures; (b) the major perchloric acid extracted proteins from cultures synchronously enriched in metaphase cells. Both cultures were labeled with [^{3}H]lysine (●) for several generations and then labeled for 1 h 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.

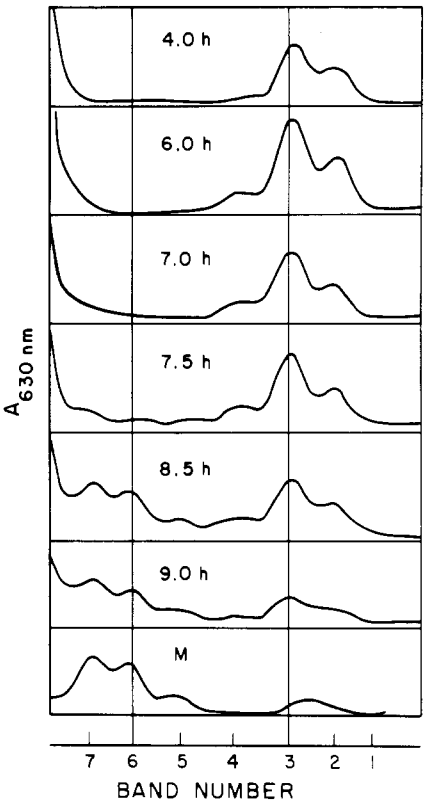

FIGURE 8: Densitometer profiles from long (0.5 × 25 cm) 2.5 M urea–15% acrylamide–acetic acid electrophoretic gels of BEP isolated from cells at various times after release from hydroxyurea blockade. M refers to BEP extracted from a culture enriched (90%) in metaphase cells by mitotic selection in the presence of Colcemid. Direction of migration is from left to right. Numbers on the abscissa simply identify bands for comparison.

pattern in long analytical gels) would be similar to those of H1 during late interphase and during entry into and exit from mitosis. Previous studies (Gurley et al., 1978a) have shown that changes in the electrophoretic pattern of H1 can be correlated with changes in chromatin structure as visualized by electron microscopy. During interphase, CHO H1 becomes phosphorylated so that by late interphase 55–60% of the H1 molecules contain one to three phosphates per molecule (Hohmann et al., 1976; Gurley et al., 1978a). During mitosis, however, all H1 molecules become superphosphorylated at four to six sites ($H1_M$). Additionally, the superphosphorylation of H1 is restricted to those stages of mitosis (prophase, metaphase, and anaphase) when chromosomes are maximally condensed.

In order to compare changes in BEP phosphorylation with changes in chromatin structure, we have examined the unpublished region of the electrophoretograms which were used for the analysis of H1 phosphorylation during entry into and exit from mitosis (Gurley et al., 1978a). For studies involving entry into mitosis, a culture was synchronized near the G_1/S boundary with hydroxyurea and then released to resume cell-cycle traverse. Four hours after release from hydroxyurea blockade, when most cells were in S phase, Colcemid was added to the cultures. In this way as cells entered mitosis, they become arrested in metaphase and could not enter G_1 of the next cell cycle. Starting at the time of addition of Colcemid (t = 4 h after release from hydroxyurea blockade) and at 0.5 h intervals thereafter (to t = 9 h), cells were withdrawn for analysis by electron microscopy and for the isolation and analysis of histones.

Figure 8 shows electrophoretograms of BEP extracted with 5% perchloric acid at various times after release from hydroxyurea blockade. Since BEP comprises only 6–8% of H1 in cultures not treated with butyrate (D'Anna et al., 1980), the resolution of BEP and the signal/background are inferior to those of H1 reported by Gurley et al. (1978a); nevertheless, BEP can be compared qualitatively and quantitatively with similar data for histone H1 [see Figure 3; Gurley et al., 1978a)].

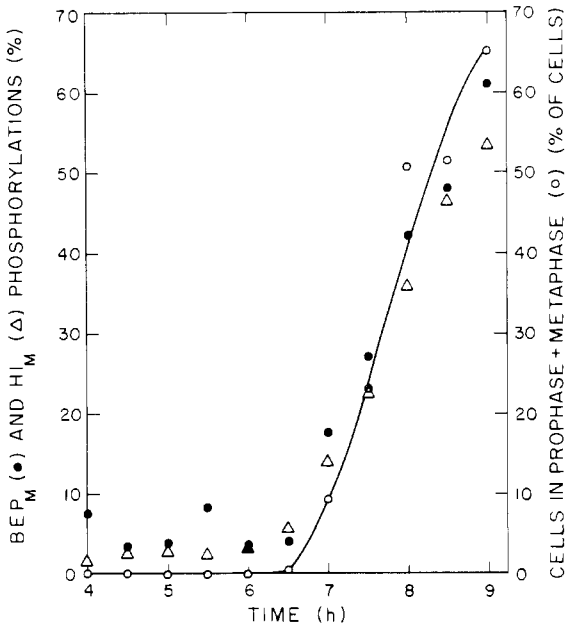

FIGURE 9: Correlation of BEP mitotic phosphorylation [(●) BEP_M] with H1 mitotic phosphorylation [(△) $H1_M$] and chromosomal condensation during entrance of cells into mitosis. The percentages of $H1_M$ and the percentages of cells in prophase plus metaphase (○) are taken from Figure 6 of Gurley et al. (1978a). The line is drawn through the percentage of cells in prophase plus metaphase.

Qualitatively there are strong similarities between changes in the electrophoretic patterns of BEP (Figure 8) and histone H1 (Gurley et al., 1978a) during entry into mitosis. (1) Between t = 4.0 h and t = 6.0 h after release from hydroxyurea blockade, there is little change in the electrophoretic pattern of either protein. [Note, however, that the band distributions of H1 and BEP during this period are different from those of G_1-arrested cells (Figure 10) in which BEP (D'Anna et al., 1980) and H1 are essentially unphosphorylated.] Although the percentage of phosphorylated BEP and the number of phosphorylations per BEP molecule in late interphase have not been determined, limits for these quantities can be estimated, if it is assumed that acquisition of n phosphate groups will retard the electrophoretic mobility of BEP by n equally spaced band positions.[4] Using this assumption, we estimate that (a) phosphorylation of 30–35% of each BEP species (unphosphorylated BEP from G_1-arrested cells, Figure 10) at one site or (b) the phosphorylation of 30% of BEP in band 2 (12% of total BEP) at two sites would account for the shift in the pattern between G_1 arrest and late interphase. Therefore, it appears that during late interphase 12–35% of BEP becomes phorphorylated at one to two sites.

(2) At 7.0 h a small amount of H1 is shifted to bands 5–8 (Gurley et al., 1978a), and there is a corresponding absorbance increase in the region of bands 5–7 for BEP in Figure 8. (These changes in BEP are seen more easily at 7.5 h.) (3) As the proportion of H1 in bands 5–8 increases during the 7.0–9.0-h period (Figure 3; Gurley et al., 1978a), so does the proportion of BEP in bands 5–7 (Figure 8).

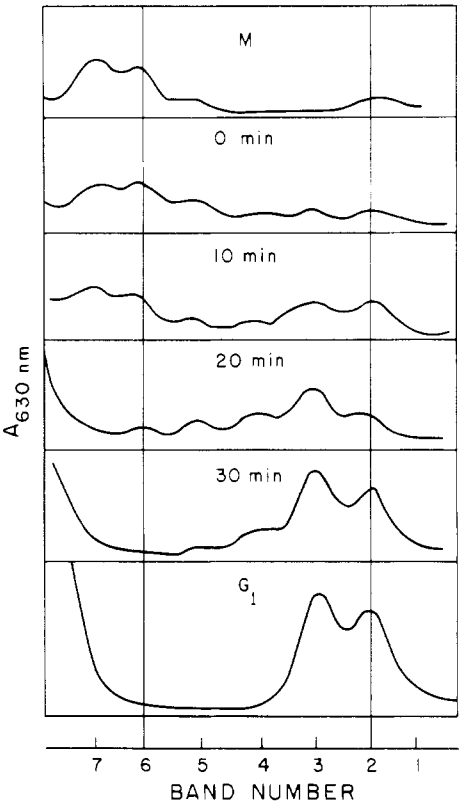

FIGURE 10: Densitometer profiles from long (0.5 × 25 cm) electrophoretic gels of BEP isolated from cells at various times after mitotic selection. M refers to BEP extracted from a culture enriched (90%) in metaphase cells by mitotic selection in the presence of Colcemid. G_1 refers to BEP extracted from 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.

Since BEP is seen in bands 5–7 only at $t \geq 7.0$ h (Figure 8) when some cells begin entering into prophase and metaphase (Figure 2; Gurley et al., 1978a), it appears that the increased phosphorylation of BEP, like "superphosphorylation" of H1 (Gurley et al., 1978a), may be temporally correlated with chromosomal condensation. To test this hypothesis, we compared the percentage of total BEP found in bands 5–7 (BEP_M) with the percentage of superphosphorylated H1 ($H1_M$) and the percentage of cells in prophase and metaphase (Figure 9). The data show a striking numerical correspondence between all three functions. Therefore, during the G_2/M transition, the appearance of BEP_m, like $H1_M$, is correlated with chromosomal condensation.

To examine H1 (and BEP) phosphorylation during exit from mitosis, we synchronized cultures by mitotic selection in the absence of Colcemid (Tobey et al., 1967b). Cell-cycle traverse was then monitored at various times after mitotic selection by electron microscopy, and BEP phosphorylation was determined by electrophoresis. Some electrophoretograms of BEP are shown in Figure 10, and plots of BEP_M, $H1_M$, and the percentage of cells in metaphase and anaphase are shown in Figure 11. These data show that the dephosphorylation of BEP_M is temporally correlated with both the dephosphorylation of $H1_M$ and the decondensation of chromatin at the anaphase–telophase transition (Gurley et al., 1978a).

Comparison of the electrophoretic patterns of BEP from cultures arrested in G_1 by isoleucine deprivation and cells obtained by mitotic selection in the presence of Colcemid (Figure 10) indicates that most BEP is shifted from bands 2 and 3 in G_1 cells to bands 6 and 7 in metaphase cells. Again,

[4] This assumption is consistent [see Chalkley et al. (1973)] with our experimental results. (1) The incorporation of phosphate gives rise to slower migrating BEP bands which are equidistant in electrophoretic gels. (2) The slower migrating bands contain phosphate, and their electrophoretic mobility decreases with increased phosphate incorporation. (3) The basicity of BEP, its molecular weight, and its reduced electrophoretic mobility (~1.0%) in 2.5 M urea–15% acrylamide–acetic acid gels are similar to those of histone H1 for which a 1.1% reduction in electrophoretic mobility has been associated with the acquisition of one phosphate group per molecule (Chalkley et al., 1973; Gurley et al., 1978a).

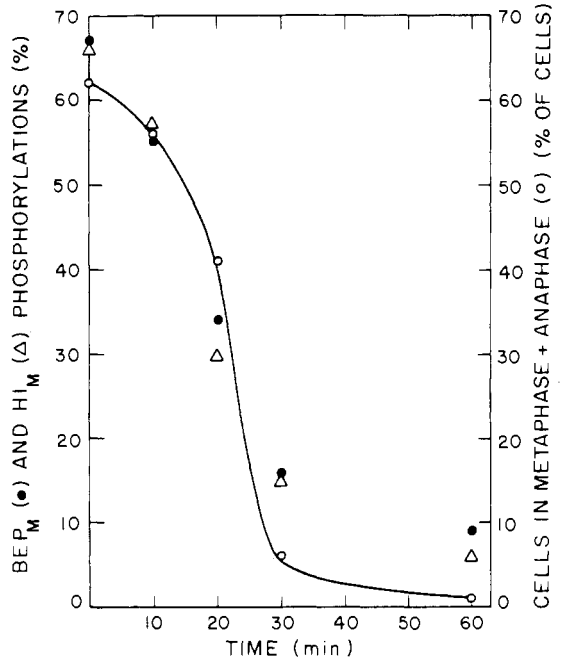

FIGURE 11: Correlation of the dephosphorylation of BEP_M (●) with the dephosphorylation of $H1_M$ (△) and the exit of cells from anaphase. The percentages of $H1_M$ and the percentages of cells in metaphase plus anaphase (○) are taken from Figure 12 of Gurley et al. (1978a). Since each cell in metaphase 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.

if it is assumed that a shift of n band positions arises from the acquistition of n phosphate groups, then shifting BEP from bands 2 and 3 to bands 6 and 7 requires an average of four phosphates per molecule. This estimate compares favorably with the estimate of four to six phosphates per molecule obtained from [^{32}P]phosphate incorporation in Figure 7b. We note that alkaline phosphatase treatment of BEP from G_1-arrested cells (15 mM butyrate for 24 h) has no effect upon the electrophoretic mobilities or distribution of BEP bands in long urea–acrylamide–acetic acid gels. Therefore, (1) BEP bands 2 and 3 represent two different unphosphorylated fractions in G_1 cells and (2) the incorporation of an estimated 4 phosphates per molecule during mitosis represents the total number of phosphates rather than the number of phosphates relative to G_1.

Discussion

These data show that BEP is an H1-like protein which has a molecular weight of ~22 000. Although BEP is related to histone H1, it contains 11 mol % less alanine than H1, it contains methionine (CHO H1 does not), and it appears that it contains two or three tryosines rather than the one found in H1. Treatment of BEP with cyanogen bromide results in the removal of ~20 residues so that the large fragment of cleaved BEP has a molecular weight of ~20 000. Since H1 does not contain methionine and is not attacked by cyanogen bromide, the cleavage of BEP proves that BEP cannot possibly be an H1 degradation product.

BEP is phosphorylated in a cell cycle dependent fashion. [^{32}P]Phosphate labeling and alkaline phosphatase treatment indicate that BEP is essentially unphosphorylated in cultures enriched in G_1 cells by treatment with sodium butyrate or by growth in isoleucine-deficient medium (unpublished results); nevertheless, electrophoresis in long urea–acrylamide–acetic acid gels indicates that BEP is composed of two major bands (which comprise ~85% of BEP) and two minor bands. Both short gels and two-dimensional electrophoresis (D'Anna et al., 1980) indicate that these bands have the same electrophoretic mobility in $NaDodSO_4$ systems. Hence, it appears that BEP is composed of at least two major unphosphorylated fractions which differ by one positive charge in 5% acetic acid. Presently, it is not known if this charge difference arises from a difference in amino acid composition (true subfractions) or if the difference arises from modifications other than phosphorylation.

During interphase, BEP is phosphorylated so that by late interphase 12–35% of BEP is phosphorylated at one to two sites per molecule. These estimated percentages for BEP phosphorylation are smaller than those for histone H1 in which 55–60% of the H1 molecules become phosphorylated at one to three sites by late interphase (Gurley et al., 1978a). Therefore, while the interphase phosphorylations of H1 and BEP appear to be temporally related, they differ quantitatively.

As cells enter into mitosis, BEP becomes "superphosphorylated" (BEP_M) at an average of approximately four sites per molecule. The appearance of the more highly phosphorylated BEP_M is temporally correlated with superphosphorylation of histone H1 ($H1_M$) and with chromosomal condensation at prophase and metaphase. As cells progress through mitosis, BEP_M becomes dephosphorylated. The dephosphorylation of BEP_M is temporally correlated with dephosphorylation of $H1_M$ and with the unraveling of fully condensed chromosomes near the anaphase–telophase transition. These cell cycle dependent phosphorylations of BEP are remarkably similar to those of histone H1. To our knowledge, BEP is the only protein whose cell cycle dependent phosphorylations mimic those of H1; therefore, with respect to phosphorylation, BEP appears to be a specialized histone of the H1 class.

Besides being related to histone H1, the extraction properties of BEP, its electrophoretic mobility in urea–acrylamide–acetic acid gels, and its amino acid composition suggest that BEP is the species equivalent of calf lung H1° (Panyim & Chalkley, 1969b). Additionally, the data suggest that BEP also is the species equivalent of (1) rat H1° (Medvedeva et al., 1975; Medvedev et al., 1977), (2) IP_{25}, the H1-like protein induced in Friend erythroleukemia cells by treatment with dimethyl sulfoxide, hexamethylenebis(acetamide), or sodium butyrate (Keppel et al., 1977, 1979; Candido et al., 1978), and (3) ox liver H1° (Smith & Johns, 1980).

Rat H1° is extracted from rat liver and rat spleen chromatin by 5% perchloric acid; it migrates slightly in front of H1 in urea–acrylamide–acetic acid gels; it is cleaved by cyanogen bromide; and the large fragment from cyanogen bromide cleavage migrates in front of the parent H1° in urea–acrylamide–acetic acid gels (Medvedeva et al., 1975; Medvedev et al., 1977).

IP_{25} is extracted from chromatin by 5% perchloric acid (Keppel et al., 1979); it migrates slightly in front of H1 in both urea–acrylamide–acetic acid and $NaDodSO_4$–acrylamide–Tris–glycine gels (Keppel et al., 1979); it is enhanced in erythroleukemia cells by treatment with sodium butyrate (Candido et al., 1978); and it contains methionine (Keppel et al., 1977).

Very recently, Smith & Johns (1980) reported the isolation of two H1° fractions from ox liver. Comparison of the amino acid composition of these two fractions with BEP reveals even greater similarity than between BEP and calf lung H1° (Panyim & Chalkley, 1969b). The $S\Delta Q$ values between BEP and

the two ox liver H1° fractions are 10.3 and 8.0, and both ox liver fractions, like BEP, contain methionine.

While comparative peptide mapping or sequence studies will be required to prove the equivalence of BEP, IP_{25}, and H1° from different species, the circumstantial evidence is strong. If it is assumed that BEP, H1°, and IP_{25} are indeed the same protein, then there is a growing body of information regarding the composition, metabolism, and location of this protein in chromatin. (1) Originally (Panyim & Chalkley, 1969b), H1° was found in tissues which replicate slowly or not at all. (2) The amounts of H1° isolated from rat liver and spleen (Medvedev et al., 1977) and from bovine liver (Piha & Valkonen, 1979) have been observed to increase with the age of the animal. (3) IP_{25} increases in Friend erythroleukemia cells treated with a number of agents which induce differentiation and inhibit cell proliferation (Keppel et al., 1977; Candido et al., 1978). (4) CHO cultures treated with butyrate become enriched in G_1 cells, and the increased quantity of BEP appears to be related to the increased proportion of cells in G_1 arrest (D'Anna et al., 1980). (5) BEP, like IP_{25} (Keppel et al., 1977), is found in small amounts in rapidly proliferating cells as well as slower replicating tissues. (6) In proliferating cells, BEP is phosphorylated in a cell cycle dependent fashion, and, during mitosis, BEP superphosphorylation is temporally correlated with chromosomal condensation. (7) IP_{25}, like histone H1, appears to be located outside of the nucleosome, and it does not appear to be located in active regions of chromatin (Keppel et al., 1979). (8) Peptide maps indicate that neither IP_{25} (Keppel et al., 1979) nor ox liver H1° (Smith & Johns, 1980) shares large peptide fragments with histone H1.

It appears that the common theme in all of the studies is that H1° (BEP, IP_{25}) is different from H1, and it is found in increasing amounts in cells and tissues which are not proliferating or which have a limited proliferative capacity. On the other hand, (1) the protein is not found exclusively in nonproliferating tissues (Keppel et al., 1977; Candido, 1978; this report) nor (2) is it appreciably enhanced in CHO cells arrested in G_1 by isoleucine deprivation [see D'Anna et al. (1978)] or in cultures grown in the absence of serum (Keppel et al., 1977). Keppel et al. (1979) have suggested that IP_{25} (BEP, H1°) plays a role either in the higher order structure of chromatin or in the turn off of genes not required for the final postreplicative stages of erythroid differentiation. The cumulative data for H1° (BEP, IP_{25}) are consistent with those hypotheses; however, the proposed role in turning off genes may be more general than simply during erythroid differentiation. Whether BEP (H1°, IP_{25}) molecular structure is similar to that of H1 or if BEP plays a special role in the higher order structure of chromatin remains to be determined.

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Activation of Polysaccharides with 2-Iminothiolane and Its Uses†

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ABSTRACT: 2-Iminothiolane, a cyclic thioimidate, is known to react readily with amino groups of proteins to give amidinated derivatives containing reactive sulfhydryl groups. In this report it will be shown that 2-iminothiolane also reacts with hydroxyl and sulfhydryl groups. Such a reaction with hydroxyl groups can be used to introduce sulfhydryl groups into polysaccharides. When this reaction is carried out in the presence of 4,4′-dithiodipyridine, polysaccharides containing 4-dithiopyridyl groups can be prepared. These activated polysaccharides couple easily via intermolecular disulfide bond formation with proteins containing thiol or 4-dithiopyridyl groups. The resulting polysaccharide–protein conjugates have good stability, and they are useful reagents for different biochemical applications such as the purification of proteins containing thiol groups and affinity chromatography.

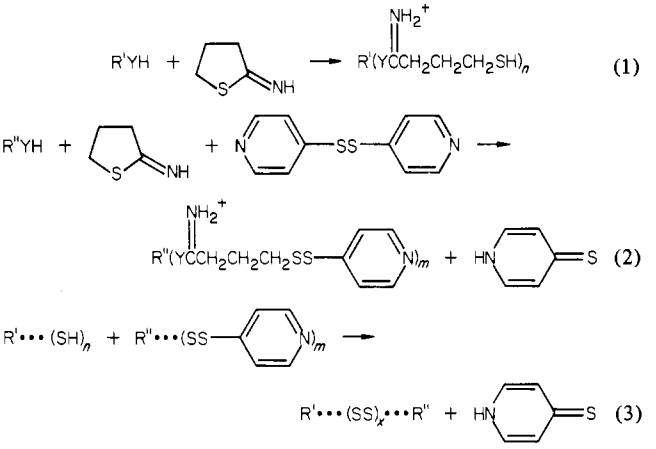
2-Iminothiolane is a useful reagent for chemical modification of proteins to yield amidinated derivatives which contain sulfhydryl groups (Traut et al., 1973; Schramm & Dülfer, 1977; Jue et al., 1978). When modification of proteins with 2-iminothiolane is carried out in the presence of 4,4′-dithiodipyridine, the thiol groups of the amidinated derivatives are converted in situ into 4-dithiopyridyl groups, as shown in Scheme I (King et al., 1978).

When amidinated proteins were separated from an excess of 2-iminothiolane by passage through Sephadex G-25, we frequently obtained variable recoveries of proteins. The losses were higher after repeated use of the same gel. We soon realized that the 2-iminothiolane in the reaction mixture was activating the gel to which the activated proteins could establish a disulfide bond. Under certain conditions, 2-iminothiolane was in fact found to be fairly reactive with a number of polysaccharides and model compounds. In this paper will be reported the findings on the reaction of 2-iminothiolane with hydroxyl-containing compounds and some of their biochemical applications.

Materials and Methods

2-Iminothiolane was synthesized as described before (King et al., 1978) and purified by vacuum distillation (bp 71–72 °C at 6 torr). The thiol content of a fresh solution of 2-iminothiolane at room temperature and pH 7.2 (0.1 M sodium phosphate buffer) was ≤0.003 residue/mol as determined by spectrophotometric titration with 4,4′-dithiodipyridine (Grassetti & Murray, 1967). 2-Iminothiolane (neat) is stable to storage, but it is hydrolyzed slowly in aqueous solution. The estimated $t_{1/2}$ values for 2-iminothiolane at 25 °C are 390, 210, and 1.8 h, respectively, at pH 9.1, 10, and 11. 4,4′-Dithiodipyridine and DL-*N*-acetylhomocysteine thiolactone were from Aldrich Chemical Co. and Nutritional Biochemical Corp.,

Scheme I



Y = NH, O, or S

respectively. The latter was recrystallized from toluene before use (mp 110–112 °C) and had a thiol content of <0.0015 residue/mol. Methyl acetimidate hydrochloride was prepared as described by Ludwig & Hunter (1967), and it had correct microanalytical data and a mp of 105–107 °C. Sephadex G-25 (medium), Sephadex G-200, Sepharose 4B, and Sepharose 6B were from Pharmacia. Paper disks (6-mm diameter, ~3 mg dry weight/disk) were punched out from Whatman No. 50 filter paper.

Ragweed antigen E (AgE)[1] was prepared as described (King, 1972). Rabbit anti-AgE serum was prepared as previously described (King et al., 1977). Mouse serum albumin was purified from pooled normal mouse serum (Miles Laboratories) by ammonium sulfate precipitation at 50–65% saturation, followed by ion-exchange chromatography on a column (25 × 0.9 cm) of Whatman DE-32 cellulose in 0.05 M

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[1] Abbreviations used: AgE, antigen E; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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