Histones H1°a and H1°b Are the Same as CHO Histones H1(III) and H1(IV): New Features of H1° Phosphorylation during the Cell Cycle†

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ABSTRACT: Two histone H1 fractions [H1(I) and H1(II)] and two histone H1° fractions (H1°a and H1°b) have been isolated from butyrate-treated Chinese hamster (line CHO) cells by guanidine hydrochloride gradient chromatography on Bio-Rex 70 ion-exchange resin. The fractions have been identified by electrophoresis and amino acid analyses. Electrophoretic analysis of cyanogen bromide treated H1° in long acid–urea–polyacrylamide gels suggests that H1°a and H1°b differ, at least, within the 20–30 residue fragment(s) removed by the cyanogen bromide cleavage. Shallow-gradient Bio-Rex 70 chromatography indicates that histones H1°a and H1°b are the same as the respective CHO histones, H1(III) and H1(IV), originally resolved by Gurley and co-workers [Gurley, L. R., Walters, R. A., & Tobey, R. A. (1975) *J. Biol. Chem. 250*, 3936]. This identification and the phosphate incorporation data of Gurley et al. (1975) reveal new features about H1° phosphorylation: (1) following release from G_1 arrest, H1°a and H1°b become phosphorylated in late G_1 prior to DNA synthesis; (2) H1°a and H1°b are phosphorylated at similar rates throughout the cell cycle. These and other data demonstrate that histone H1° is phosphorylated in a cell cycle dependent fashion which mimics that of histone H1.

Recently (D'Anna et al., 1980a), our laboratories reported the isolation of a histone H1 like protein (BEP) whose chromatin content becomes greatly enhanced when Chinese hamster (line CHO) cells are treated with sodium butyrate (D'Anna et al., 1980b). Amino acid analyses, cyanogen bromide cleavage, and analytical gel electrophoresis (D'Anna et al., 1980a,b) suggest that the protein is the equivalent of histone H1° from bovine lung (Panyim & Chalkley, 1969a), two fractions of H1° from bovine liver (Smith & Johns, 1980), and H1° from neuroblastoma cells (Pehrson & Cole, 1980). BEP, thus, appears to represent an H1° component (CHO H1°) of CHO cells (D'Anna et al., 1980a).

Electrophoresis of CHO H1° in short (13-cm) acid–urea–polyacrylamide gels gives one band (D'Anna et al., 1980a); however, electrophoresis in long (25-cm) acid–urea–polyacrylamide gels gives two major bands which indicates that CHO H1°, like bovine liver H1° (Smith & Johns, 1980), is composed of two major fractions. The two CHO H1° fractions are similar in several ways: (1) they both become enhanced by treatment with butyrate (D'Anna et al., 1980b); (2) they both become highly phosphorylated during chromosomal condensation at mitosis (D'Anna et al., 1980a); (3) they both appear to be cleaved by cyanogen bromide to yield fragments of similar size and charge when resolved in short (13-cm) electrophoretic gels (D'Anna et al., 1980a).

Here we report the isolation and amino acid composition of the individual CHO H1° fractions as well as the two histone H1 subfractions. Comparison of Bio-Rex 70 chromatograms of perchloric acid (PCA)[1] extracted proteins with those of Gurley et al. (1975) indicates that the respective H1° fractions, H1°a and H1°b, are the same two chromatographic fractions originally designated as H1(III) and H1(IV) by Gurley et al. (1975). This specific identification of H1(III) and H1(IV) and the data of Gurley et al. (1975) allow us to reach several new conclusions pertaining to H1° phosphorylation during the cell cycle.

Experimental Procedures

Cell Growth and Isotopic Labeling. Suspension cultures of Chinese hamster (line CHO) cells were grown in F-10 medium supplemented with 15% newborn calf serum, streptomycin, and penicillin (Tobey et al., 1966). Cultures were treated with butyrate as described previously (D'Anna et al., 1980a,b).

CHO cells to be used for the isolation of proteins for analytical Bio-Rex 70 chromatography (see below) were grown for 72 h in the presence of 60 μCi/L [^{3}H]lysine (8 Ci/mol; Schwarz/Mann). In some cases butyrate (10 mM) was added to the 1.0-L cultures 24 h before harvest.

Perchloric Acid Extraction of Histones. Histones H1 and H1° were extracted from CHO cells with 0.83 M perchloric acid (PCA) by the first method of Johns (1964), adapted to CHO cells by Gurley & Hardin (1968). Sodium bisulfite (50 mM) was added to the saline wash solution and to the PCA extraction solution to inhibit proteolysis (Bartley & Chalkley, 1970) and dephosphorylation of histone H1 (Gurley et al. 1975; D'Anna et al., 1978).

Preparative Bio-Rex 70 Chromatography. H1 and H1° fractions were separated by guanidine hydrochloride gradient chromatography of PCA-extracted proteins on Bio-Rex 70 ion-exchange resin (Kincade & Cole, 1966). PCA-extracted proteins (9.3 mg) were dissolved in 0.6 mL of 8.0% guanidine hydrochloride and 0.10 M phosphate buffer (pH 6.8), and 0.06 mL of 5 mM phenylmethanesulfonyl fluoride in isopropyl alcohol was added as a protease inhibitor. The sample was applied to a 1.5 × 22 cm Bio-Rex 70 column equilibrated with 8.0% guanidine hydrochloride in phosphate buffer (pH 6.8). Proteins were eluted from the column with an 8.0–14.0% linear gradient of guanidine hydrochloride in phosphate buffer (total volume of 354 mL). Two fractions of 1.7 mL were collected per hour, and protein was detected by its absorbance at 218 nm. Combined fractions from each chromatographic band were desalted on Sephadex G-25 and concentrated as described previoulsy (D'Anna et al., 1980a).

Analytical Bio-Rex 70 Chromatography. Analytical Bio-Rex 70 chromatography was performed as described by Gurley

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[1] Abbreviations used: PCA, perchloric acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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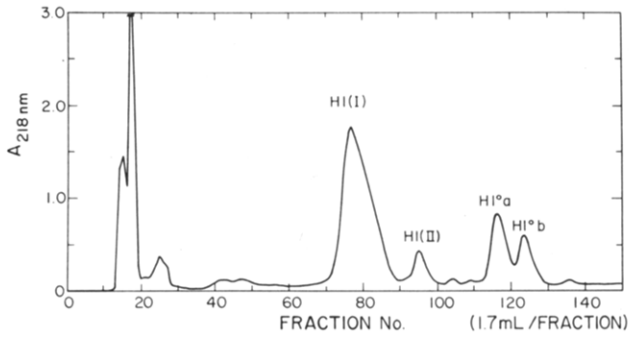

FIGURE 1: Preparative Bio-Rex 70 gradient chromatogram of PCA-extracted proteins from cultures gorwn for 25 h in the presence of 10 mM butyrate. The proteins (9.3 mg) were applied to a 1.5 × 22 cm Bio-Rex 70 column equilibrated with 8.0% guanidine hydrochloride in 0.10 M phosphate buffer, pH 6.8. A linear gradient of 8.0–14.0% was applied to the column in a total volume of 354 mL. Protein was detected from its absorbance at 218 nm.

et al. (1975). PCA-extracted proteins from exponentially growing and butyrate-treated (24-h) cultures were dissolved in 0.2 mL of 8.0% guanidine hydrochloride in 0.1 M phosphate buffer (pH 6.8), and each sample was loaded on one of two 0.50 × 17 cm Bio-Rex 70 columns equilibrated with the same buffer. Each sample was chromatographed isocratically with 65 mL of the starting column buffer. Proteins were then eluted from the columns with a linear 8.0–11.0% guanidine hydrochloride gradient (600-mL total volume; 300 mL/column) run in parallel from a single gradient maker. The gradient flow rate through each column was 1.0 mL h^{-1}. Fractions (0.50 mL) were collected directly into plastic scintillation vials, and 0.20 mL of water and 14 mL of PCS II solubilizer (Amersham Corp.) were added to each vial. Samples were mixed and then counted for 10 min in a Packard Model 3320 scintillation spectrometer.

Protein Analyses. H1 and H1° fractions were resolved by electrophoresis in 5.2% acetic acid–2.5 M urea–15% acrylamide cylindrical gels (Panyim & Chalkley, 1969b) as described previously (Gurley et al., 1978; D'Anna et al., 1980a,b). They also were subjected to electrophoresis in sodium dodecyl sulfate ($NaDodSO_4$)–12.7% acrylamide–Tris–glycine slab (0.15 × 13 × 30 cm) gels (Laemmli, 1970). Electrophoresis was performed at 26.5 mA for 6.0 h. The gels were soaked for 1 h in 20% trichloroacetic acid, and then they were stained 4 h in 0.10% Coomassie blue, 7.5% acetic acid, and 10% methanol. Gels were destained by diffusion in 7.5% acetic acid and 10% methanol.

Amino acid analyses were performed with a Beckman/Spinco 120B modified automatic analyzer (Spackman et al., 1958) with a 6-mm single column system. Proteins were hydrolyzed in constant boiling HCl at 110 °C for 22 h in sealed, evacuated tubes. Cyanogen bromide cleavage (Gross & Witkop, 1962) was performed as described previously (D'Anna et al., 1980a).

Results

Amino Acid Analyses and Electrophoretic Properties of H1 and H1° Fractions. Preparative Bio-Rex 70 gradient chromatography of PCA-extracted proteins from butyrate-teated CHO cultures separates H1 and H1° into several fractions as shown in Figure 1. Since only small portions of H1 and H1° are phosphorylated in the G_1-enriched, butyrate-treated cultures (D'Anna et al., 1980a,b), the chromatogram is simpler than those observed for non-G_1 cultures which contain bands of both phosphorylated and unphosphorylated species (Gurley et al., 1975). Starting with 9.3 mg of PCA-extracted proteins from butyrate-treated cultures, we have isolated 3.6 mg of H1(I), 0.4 mg of H1(II), 0.7 mg of H1°a, and 0.5 mg of H1°b.

Table I: Amino Acid Analyses[a] of CHO H1(I), H1(II), H1°a, and H1°b

	% composition			
	H1(I)	H1(II)	H1°a	H1°b
Asx	2.1	2.2	3.9	3.7
Thr	5.1	5.4	5.9	5.7
Ser	6.6	6.4	7.6	8.1
Glx	3.7	3.6	4.9	5.9
Pro	8.3	11.3	9.7	7.7
Gly	6.4	6.8	3.5	5.6
Ala	25.2	25.3	14.0	14.1
Val	6.1	4.8	5.5	5.5
Met	0	0	0.3	trace[b]
Ile	0.9	1.2	2.9	3.1
Leu	4.3	4.1	2.0	2.5
Tyr	0.4	0.5	1.3	1.2
Phe	0.4	0.6	0.9	1.4
Lys	28.4	26.1	32.6	29.9
His	0	0	1.5	1.0
Arg	2.1	1.9	3.4	3.8

[a] Amino acid analyses are not corrected for possible hydrolytic losses of serine and threonine. [b] Confirmed by cyanogen bromide cleavage.

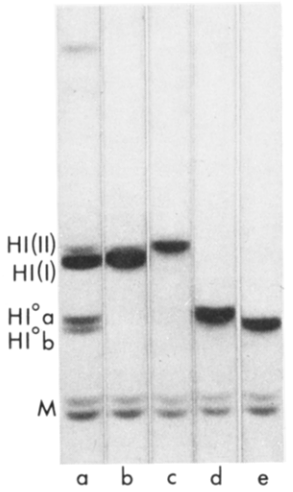

FIGURE 2: Electrophoresis of H1 and H1° chromatographic fractions in 25-cm acid–urea–polyacrylamide gels: (a) PCA-extracted proteins from a butyrate-treated culture, (b) H1(I), (c) H1(II), (d) H1°a, and (e) H1°b. The bands labeled M are purified CHO histone H1 which was loaded prior to the samples and which serves as an internal mobility reference marker (Hohmann et al., 1976).

The chromatographic fractions were identified as H1 or H1° species from their amino acid analyses (Table I) and from their electrophoretic properties in long acid–urea–polyacrylamide gels (Figure 2) and in $NaDodSO_4$ gels (Figure 3). The amino acid analyses and electrophoretic properties of the H1 fractions are typical of unfractionated CHO H1 (D'Anna et al., 1980a) and H1 proteins from other species [e.g., Bustin & Cole (1968), Smerdon & Isenberg (1976), and Seyedin & Kistler (1979)]; therefore, the fractions have been designated as histones H(I) and H1(II) in accordance with their order of elution from Bio-Rex 70 columns (Gurley et al., 1975). Although H1(II) is electrophoretically pure in acid–urea–polyacrylamide gels (Figure 2), it exhibits traces of impurities in the heavily loaded $NaDodSO_4$ gel (Figure 3).

The amino acid analyses of the two H1° fractions (Table I) are very similar to one another, to that of unfractionated H1° (D'Anna et al., 1980a), and to those of two H1° fractions from bovine liver (Smith & Johns, 1980); therefore, the CHO fractions have been designated as H1°a and H1°b in accord-

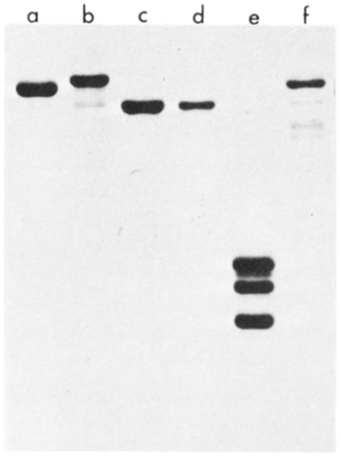

FIGURE 3: Electrophoresis of the H1 and H1° chromatographic fractions in $NaDodSO_4$ slab gels: (a) H1(I), (b) H1(II), (c) H1°a, (d) H1°b, (e) calf thymus inner histones (for reference), and (f) PCA-extracted proteins from an exponentially growing culture.

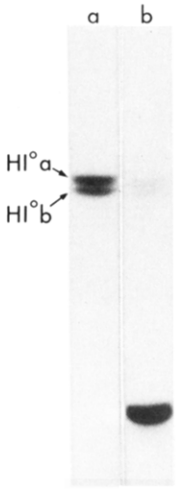

FIGURE 4: Electrophoresis of purified, unfractionated CHO H1° in 25-cm acid–urea–polyacrylamide gels (a) before and (b) after treatment with cyanogen bromide.

ance with the order of elution of bovine H1° fractions from Bio-Rex 70 columns (Smith & Johns, 1980). Electrophoretic analysis of CHO H1° fractions indicates that (1) H1°a and H1°b have the same molecular weight (Figure 3) but (2) H1°b migrates faster than H1°a in acid–urea–polyacrylamide gels (Figure 2). These electrophoretic properties correspond to those of bovine liver H1°a and H1°b (Smith & Johns, 1980).

Examination of the amino acid analyses of H1° in Table I indicates the presence of methionine in H1°a, but only a trace of methionine is detected in H1°b. On the other hand, electrophoretic analysis of cyanogen bromide treated, unfractionated CHO H1° in long acid–urea–polyacrylamide gels (Figure 4), which resolves H1°a and H1°b, shows clearly that both H1° fractions are cleaved with equal facility. This analysis confirms our previous conclusion (from short gels that did not resolve H1°a and H1°b) that both CHO H1°'s contain methionine and that cyanogen bromide treatment results in the loss of ~20 amino acid residues from the molecules (D'Anna et al., 1980a).

We note that cleavage of H1°a and H1°b leads to the appearance of a single dark band in gel b of Figure 4. This reduction in the number of bands in going from the intact molecules to the large cleavage fragments suggests that H1°a and H1°b differ in their small fragments, cleaved away by cyanogen bromide (i.e., the large cleaved fragments which

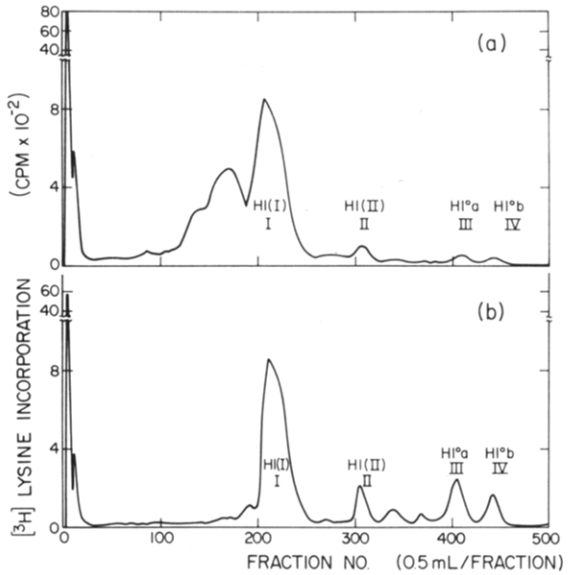

FIGURE 5: Analytical Bio-Rex 70 chromatography of PCA-extracted proteins from (a) an exponentially growing culture and (b) a butyrate-treated culture (24 h). Cultures were grown 72 h in the presence of [^{3}H]lysine. Individual Roman numerals refer to the fraction designations of Gurley et al. (1975).

migrate in Figure 4 are identical in migration properties). (1) Since H1° was extracted with acid from the chromatin of blended cells and (2) since small portions of both H1°a and H1°b remain uncleaved in gel b, it seems unlikely that an acid-labile modification was selectively hydrolyzed from one of the large cleavage fragments (single band on the gel) and not from the parent molecules during cyanogen bromide treatment. The origin of this difference between H1°a and H1°b is unknown.

Identification of H1(III) and H1(IV) as H1°'s: Phosphate Incorporation during Interphase. Previous studies (D'Anna et al., 1980a,b) have shown that histone H1° is phosphorylated in a cell cycle dependent fashion which resembles that of histone H1; however, new information about H1° interphase phosphorylation and the specific identification of "histone H1 fractions" can be obtained from reexamination of the H1 phosphorylation studies of Gurley et al. (1975). In those studies, Bio-Rex 70 gradient chromatography was used to determine phosphate incorporation into PCA-extracted proteins from synchronized CHO cells. Comparison of the closely spaced chromatographic bands of H1 and H1° in Figure 1 of this paper with the more separated chromatographic bands of Figure 1b of Gurley et al. (1975) suggests that H1°a and H1°b had been resolved previously, but they had been designated as H1 fractions III and IV. There are, however, two bands between H1(II) and H1°a in Figure 1 of this report which might be confused with H1(III) and H1(IV) of Gurley et al. (1975).

To establish the identities between H1°a and H1°b with H1(III) and H1(IV), we subjected PCA-extracted proteins from exponentially growing and butyrate-treated cultures to analytical Bio-Rex 70 chromatography according to the conditions of Gurley et al. (1975). Although our chromatograms (Figure 5) are somewhat broader than those in Figure 1 of Gurley et al. (1975), the appearance of the chromatograms from the exponentially growing cultures are the same. [The bands preceding those labeled I and II in Figure 5a correspond to the phosphorylated species of H1(I) and H1(II).] Comparison of the bands from the absorbance chromatogram of Figure 1 with the [^{3}H]lysine chromatogram of Figure 5b shows a 1:1 correspondence between the relative positions (but not

areas since we are comparing [^{3}H]lysine incorporation with absorbance) of the major bands of the two butyrate-treated cultures. Since the bands of H1°a and H1°b of Figure 5b are aligned with bands III and IV from the exponentially growing culture (Figure 5a), we conclude that H1°a and H1°b correspond to "H1 species III and IV" of Gurley et al. (1975). The two minor bands between H1(II) and H1°a in Figures 1 and 5b of this paper have not been identified; however, these minor bands also appear to be enhanced in the butyrate-treated culture (Figure 5b).

The phosphate incorporation studies of Gurley et al. (1975) confirm our results from analytical and preparative gel electrophoresis (D'Anna et al., 1980a,b), and they add new information pertaining to interphase phosphorylation. (1) Little or no phosphate is incorporated into H1°a or H1°b in cultures arrested in G_1 by isoleucine deprivation [Figure 2 of Gurley et al. (1975)]; this result is the same as we observed for cells arrested in G_1 by treatment with butyrate (D'Anna et al., 1980b). (2) Both H1°a and H1°b incorporate [^{32}P]phosphate following release from isoleucine deprivation induced G_1 arrest [Figures 3–5, Gurley et al. (1975)]; hence, it appears that the phosphorylation of H1°a and H1°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°a and H1°b are phosphorylated at similar rates during interphase, and the amounts of H1°a and H1°b which become phosphorylated are similar to one another [Figure 5, Gurley et al. (1975)]. (4) The amounts of H1°a and H1°b which become phosphorylated during interphase are somewhat less than those of H1(I) and H1(II) which become phosphorylated at the same time: (a) when synchronized cultures are released from G_1 arrest, about 10–15% of H1°a and H1°b are phosphorylated just prior to the appearance of S phase cells, while 15–18% of H1(I) and H1(II) are phosphorylated at that time; (b) by 8 h after release from G_1 arrest, when 50% of the cells are in S phase, 30–35% of H1°a and H1°b become phosphorylated, while 50–60% of H1(I) and H1(II) are phosphorylated at that time.

Discussion

Two fractions of histone H1 and two fractions of histone H1° have been isolated from CHO cells. The two CHO H1° fractions possess compositions which are very similar to those of bovine lung H1° (Panyim & Chalkley, 1969a), H1° from neuroblastoma cells (Pehrson & Cole, 1980), and two H1° fractions from bovine liver (Smith & Johns, 1980). H1°a and H1°b from CHO (this paper) and bovine liver (Smith & Johns, 1980) share many properties: (1) H1°a and H1°b from each species contain methionine so that they are cleavable by cyanogen bromide; (3) H1°a and H1°b from each species possess the same electrophoretic mobility in $NaDodSO_4$ gels; (4) the H1°a and H1°b fractions from each species are resolvable by Bio-Rex 70 chromatography; (5) H1°a from each species migrates more slowly than its corresponding H1°b in acid–urea–polyacrylamide gels. These similarities further support the suggestion that CHO H1 and bovine H1° are homologous proteins (D'Anna et al., 1980a).

Our cyanogen bromide cleavage experiments suggest that H1°a and H1°b differ in their small (20–30 amino acid) fragments which are cleaved away by cyanogen bromide [most likely the NH_2-terminal end of the molecules; see Smith et al. (1980)]; however, specific differences between H1°a and H1°b remain unresolved: (1) neither CHO H1°a nor H1°b is phosphorylated in butyrate-treated cultures from which they have been isolated (D'Anna et al., 1980a,b); (2) H1°a and H1°b do not contain diphosphoribosyl moities in bovine liver (Smith & Johns, 1980); (3) both H1°a and H1°b from bovine liver are blocked in the NH_2-terminal end (Smith & Johns, 1980); (4) tryptic peptide maps of bovine H1°a and H1°b exhibit only minor differences (Smith & Johns, 1980).

We note that methionine has been detected in all mammalian H1°'s except H1° from bovine lung (Panyim & Chalkley, 1969a). Although methionine was detected only as a trace in our amino acid analysis of H1°b, cyanogen bromide cleavage showed clearly that H1°b contained methionine. In this regard, it is possible that methionine also is present in bovine lung H1° but it was destroyed during repeated chromatography for purification or during acid hydrolysis for amino acid analysis.

Our comparison of shallow-gradient, Bio-Rex 70 chromatograms with those of Gurley et al. (1975) indicates that H1°a and H1°b are the same as the previously resolved fractions H1(III) and H1(IV), respectively. This specific identification of H1(III) and H1(IV) and the phosphorylation data of Gurley et al. (1975) allow us to reach a number of new conclusions regarding H1° phosphorylation during the cell cycle of CHO cells. (1) Little or no phosphate is incorporated into H1° in cells arrested in G_1 by isoleucine deprivation. This result is the same as we obtained previously for cells arrested in G_1 by treatment with butyrate (D'Anna et al., 1980a,b). (2) As cells are released from G_1 arrest, H1°, like histone H1 (Gurley et al., 1975; Pochron & Baserga, 1979), becomes phosphorylated in G_1 prior to the onset of DNA synthesis. (3) Previously (D'Anna et al., 1980a), we observed changes in the electrophoretic pattern of H1° in going from G_1 arrest to late interphase. We estimated that phosphorylation of 12% of one H1° fraction at two sites per molecule or the phosphorylation of 30–35% of each fraction at one site per molecule would account for the observed changes in the electrophoretic pattern. Since both H1° fractions are phosphorylated at similar rates (Gurley et al., 1975), we conclude that by late interphase, ~35% of both H1°a and H1°b become phosphorylated at approximately one site per molecule. We note that this numerical estimate is very similar to the computed values for H1°a ["H1(III)"] and H1°b ["H1(IV)"] in Figure 5 of Gurley et al. (1975).

These new data on interphase phosphorylation plus our recent data on mitotic phosphorylation (D'Anna et al., 1980a) indicate that H1° is phosphorylated in a cell cycle dependent fashion which is the same as that of histone H1. On the basis of this temporal correspondence, it seems reasonable to postulate that the cell cycle dependent phosphorylations of H1° and H1 serve similar roles (whatever they may be) in modulating chromatin structure and function.

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Phase Equilibria in Binary Mixtures of Phosphatidylcholine and Cholesterol†

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ABSTRACT: The paramagnetic resonance spectra of two spin-labels, 2,2,6,6-tetramethylpiperadinyl-1-oxy and a head-group spin-labeled phosphatidylethanolamine (L-α-dipalmitoylphosphatidyl-*N*-ethanolamine), have been used to study solid–liquid and liquid–liquid phase separations in binary mixtures of dimyristoylphosphatidylcholine and cholesterol. A quantitative analysis of these resonance spectra supports the view that at temperatures below θ_m, the chain-melting temperature of the phospholipid, and at cholesterol mole fractions $X_c < 0.2$, these mixtures consist of two phases, a solid phase of essentially pure dimyristoylphosphatidylcholine and a fluid phase having a mole fraction of cholesterol equal to 0.2. The spin-label data also provide evidence for fluid–fluid immiscibility in the bilayer membrane at temperatures above the chain-melting transition temperature of dimyristoylphosphatidylcholine.

There have been numerous studies of the physical properties of lipid bilayers containing binary mixtures of phosphatidylcholine and cholesterol. A number of early attempts to interpret these physical properties in terms of molecular structure and dynamics are flawed by failure to take into account the lateral phase separations in these mixtures. As discussed later, the cholesterol–phosphatidylcholine bilayer membrane does exhibit solid phase–fluid phase phase separations and also very probably fluid phase–fluid phase phase separations. Previous attempts to derive phase diagrams for these binary mixtures have been made by Shimshick & McConnell (1973a) and Lentz et al. (1980). In contrast to the numerous reported diagrams for mixtures of phospholipids [see, e.g., Shimshick & McConnell (1973b)], there is considerable uncertainty about many features of all of the proposed phase diagrams for binary mixtures containing cholesterol. However, in one limited temperature-composition region, the composition and some physical properties of the two coexisting phases have become clear, at least for binary mixtures of dipalmitoylphosphatidylcholine (DPPC)[1] [or dimyristoylphosphatidylcholine (DMPC)] and cholesterol (Copeland & McConnell, 1980; Owicki & McConnell, 1980; Rubenstein et al., 1979, 1980). At temperatures θ below the main chain-melting transition temperature, θ_m, of the phospholipid and for cholesterol mole fractions, X_c, less than approximately 0.2, the two coexisting phases are "solid" and "fluid". The solid phase is essentially pure DMPC (or DPPC), and the fluid phase contains ~20 mol % cholesterol. We use the terms solid and fluid for lipid phases where lipid lateral diffusion coefficients are small (10^{-10}–10^{-11} cm^2/s, characteristic of gel phase lipids, or large (10^{-9}–10^{-7} cm^2/s), characteristic of lipid mixtures at temperatures above the θ_m for all the phospholipids. This temperature-composition region is denoted by II in Figure 1. The binary mixtures in region II have a remarkable domain structure consisting of (approximately) parallel domains of solid and fluid lipid as judged by freeze–fraction electron microscopy (Copeland & McConnell, 1980). With increasing cholesterol concentration, the widths of the parallel solid domains remain nearly constant and equal to one another while their separation increases, in proportion to the increase of fluid lipid. Our earlier conclusions regarding the coexisting phases in region II are based primarily on freeze–fracture electron microscopy (Copeland & McConnell, 1980), studies of lateral diffusion of fluorescent lipid probes (Rubenstein et al., 1979; Owicki & McConnell, 1980), and scanning calorimetry (Mabrey et al., 1978). Additional discussion of the lipid bilayer structure in region II can be found in Rubenstein et al. (1980). Quite recent studies of rotational diffusion of a fluorescent lipid probe also support the above conclusions regarding the coexisting phases in region II (Smith et al., 1981).

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[1] Abbreviations used: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; EPR, electron paramagnetic resonance; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; PBS, phosphate-buffered saline.

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