Altered Conformational Effects of Phosphorylated Lysine-Rich Histone (f-1) in f-1–Deoxyribonucleic Acid Complexes. Circular Dichroism and Immunological Studies*

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ABSTRACT: Reconstituted complexes of DNA with calf thymus lysine-rich (f-1) histone show significant differences in conformational properties if enzymatically phosphorylated histone is used. With phosphorylated f-1, the magnitude of the circular dichroism changes of the DNA in histone–DNA complexes is diminished, as compared to nonphosphorylated f-1. Similarly at a given f-1:DNA ratio, complexes containing phosphorylated f-1 combine with DNA antibody to a greater extent than do nonphosphorylated f-1 complexes, as measured by complement fixation. These observations indicate that phosphorylated f-1 histone is less effective than nonphosphorylated in producing conformational changes in DNA upon complex formation. This finding may be relevant for a mechanism of regulation of RNA synthesis.

The histones of eukaryotic cells may have a regulatory role in transcription from DNA, as well as a structural role in packing the DNA; the evidence is summarized elsewhere (Elgin *et al.*, 1971). Circular dichroism studies of reassociated complexes of native calf thymus DNA with lysine-rich (f-1) histone (Fasman *et al.*, 1970) have demonstrated that f-1 causes large changes in the conformation of the DNA, apparent upon specific aggregation of the f-1–DNA complexes. The present paper reports an extension of these studies to complexes formed between DNA and phosphorylated f-1 histone.

Histone preparations contain phosphate esterified to serine residues (Kleinsmith *et al.*, 1966; Ord and Stocken, 1966), and enzymes which catalyze the phosphorylation of histones by ATP are widely distributed (Langan, 1968; Corbin and Krebs, 1969; Kuo and Greengard, 1969; Jergil and Dixon, 1970). Phosphorylation of histones *in vivo* has been demonstrated (Langan, 1969a; Marushige *et al.*, 1969). In liver, histone phosphorylation occurs in response to the pancreatic hormone glucagon, *via* a cyclic AMP-dependent histone kinase (Langan, 1968, 1969b), under conditions in which the syntheses of certain liver enzymes are also induced (Holten and Kenney, 1967; Jost *et al.*, 1968; Wicks, 1968; Yeung and Oliver, 1968; Jost *et al.*, 1969). It was proposed that histone phosphorylation might provide a mechanism for the induction of RNA and protein synthesis by hormones which act *via* cyclic AMP (Langan, 1968, 1969b). According to this scheme, cyclic AMP-mediated phosphorylation of histone would cause a change in DNA–histone interaction, resulting in derepression of the template activity of the associated DNA, thereby allowing RNA synthesis.

In the present investigation, a comparison has been made between the interaction of DNA with normal preparations of f-1, which contain relatively small amounts of phosphate, and enzymatically phosphorylated lysine-rich histone, prepared by incubation with purified liver histone kinase (Langan, 1968; Meisler and Langan, 1969). This phosphorylation occurs predominantly on a single specific serine residue (Langan, 1969b), which has been shown to be Ser-37, near the amino terminus of the f-1 molecule (Langan *et al.*, 1971, in preparation), and is identical with that found *in vivo* for liver f-1 phosphorylation (Langan, 1969a,b).

The circular dichroism studies presented here show that the ability of phosphorylated histone to induce conformational changes in DNA is greatly diminished compared to nonphosphorylated histone, in keeping with the above proposal that phosphorylation can cause an alteration in the interaction of the histone with DNA. The results are confirmed by serologic experiments which show that the ability to react with native DNA antibodies is greater for phosphorylated f-1–DNA than for nonphosphorylated f-1–DNA complexes.

Materials and Methods

Histone Preparations. The lysine-rich (f-1) histone fraction from calf thymus was prepared by method I of Johns (1964). Phosphorylated f-1 was obtained from this "original" histone by incubation with purified calf liver histone kinase (Langan, 1968) as previously described (Meisler and Langan, 1969). The sample of phosphorylated f-1 which yielded the data presented in this paper was further purified by chromatography on a carboxylic acid ion-exchange resin, eluted with guanidinium chloride (Rasmussen *et al.*, 1962; Kinkade and Cole, 1966). Early fractions containing non-histone contaminants and partially degraded histone were discarded, and fractions containing the lysine-rich histone were pooled. No attempt was made to isolate individual lysine-rich histone components. Water (5.25 volumes) was added to the pooled fractions, and 100% (1 g/ml) trichloroacetic acid was added to a final concentration of 18%. The precipitated histone was collected by centrifugation, dissolved in a small volume of water, and reprecipitated with trichloroacetic acid. The histone was then washed once with acid acetone (0.5 ml of concentrated HCl/100 ml) and twice with acetone, and dried in a vacuum desiccator. The alkali-labile phosphate content (Meisler and Langan, 1969) of this sample was 41.6

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mμmoles of P/mg of histone (=0.87 P/mol wt 21,000). "Control" preparations of histone were carried through the same incubation and isolation procedures as the phosphorylated f-1, except that ATP was omitted from the incubation mixture. The sample of control f-1 utilized for the present data was chromatographed as above, and contained 6.1 mμmoles of alkali-labile P/mg.

Stock histone solutions were prepared in water and adjusted to pH 7.0 with NaOH. Concentrations of these solutions were determined by the biuret method (Zamenhof, 1957) with poly-L-lysine as standard; results were adjusted for the fact that f-1 (dried to constant weight) yields 3% more color in the reaction than does poly-L-lysine. Histone solutions had concentrations of $\sim 3 \times 10^{-3}$ M peptide residues, and were stored frozen. (Storage at 4° for long times or at low pH resulted in erratic circular dichroism data.)

DNA Preparations. The calf thymus DNA used for circular dichroism studies was prepared as previously described (by a combination of high-salt, phenol, and chloroform—isoamyl alcohol extractions; Fasman *et al.*, 1970), but with the insertion of a pronase digestion to remove protein (treatment with 30 μg/ml of pronase for 2 hr at 23° before the final chloroform–isoamyl alcohol extractions). The DNA was stored at ~3 mg/ml in 0.1 M NaF over chloroform, and was diluted to 10^{-3} M nucleotide residues before use. DNA concentrations were determined from OD_{258}, using ϵ_{258} (mole residues) = 6.8×10^3. The DNA contained <0.2% RNA, as measured by the orcinol reaction after removal of DNA (Schneider, 1957). Protein contamination, determined by the biuret method (Zamenhof, 1957), was <0.3%. The DNA showed a 46% increase in OD_{260} upon heating to 97°.

Proteus vulgaris DNA, used for complement fixation experiments, was prepared by the method of Marmur (1961), and contained <0.2% protein.

Histone–DNA Complexes. Complexes of f-1 and DNA were prepared by mixing these components, at the desired concentration and ratio, in a high-salt medium (2 M NaCl–0.002 M Tris, pH 7), of total volume 2 ml, and then performing stepwise gradient dialysis (against 0.4 M NaF for 16 hr, 0.3 M NaF for 8 hr, and final solvent for 16 hr; Huang *et al.*, 1964) to a final solution of 0.14 M NaF–0.002 M Tris, pH 7.0 (Fasman *et al.*, 1970). Dialysis tubing was boiled in 0.01 M $NaHCO_3$–0.001 M EDTA. Complexes were not centrifuged. This treatment ensures reproducible formation of complexes. High-speed (120,000*g*) centrifugation of phosphorylated and nonphosphorylated f-1–DNA complexes at $r = 1$, 10^{-3} M (nucleotide) showed that all the histone, in both cases, was found in the nucleoprotein pellet (within the accuracy of the measurement).

Concentrations of DNA in f-1–DNA complexes examined by circular dichroism were obtained by boiling 30 min in 0.5 M perchloric acid (Ogur and Rosen, 1950) and then measuring OD_{260} of the hydrolysate, for which ϵ_{260} (mole of nucleotides) = 9.9×10^3 in this solvent. Absorbance measurements were performed on a Cary 14 spectrophotometer. Protein to DNA ratios (*r*) are reported as moles of peptide residues per mole of nucleotide residues, and were determined from the input concentrations.

Circular Dichroism. Circular dichroism measurements were performed at 23° on a Cary 60 spectropolarimeter with 6001 CD attachment. The path length for all f-1–DNA complexes was 1 cm; fused quartz cells (Optical Cell Co.) were used. OD_{260} was about 0.6; the concentration range for circular dichroism measurements was $7–8 \times 10^{-5}$ M DNA nucleotide residues. The half-bandwidth was set for 1.5 nm. The time constant was usually 3 sec, sometimes 10 sec (at low λ or for increased resolution). Mean residue ellipticity $[\theta]$ is reported in (deg cm²) per dmole of nucleotide residues in the complexes. Typical average errors in $[\theta]$ are indicated in Figure 1, and are based on duplicate experiments. The precision in band wavelength is ±0.5 nm.

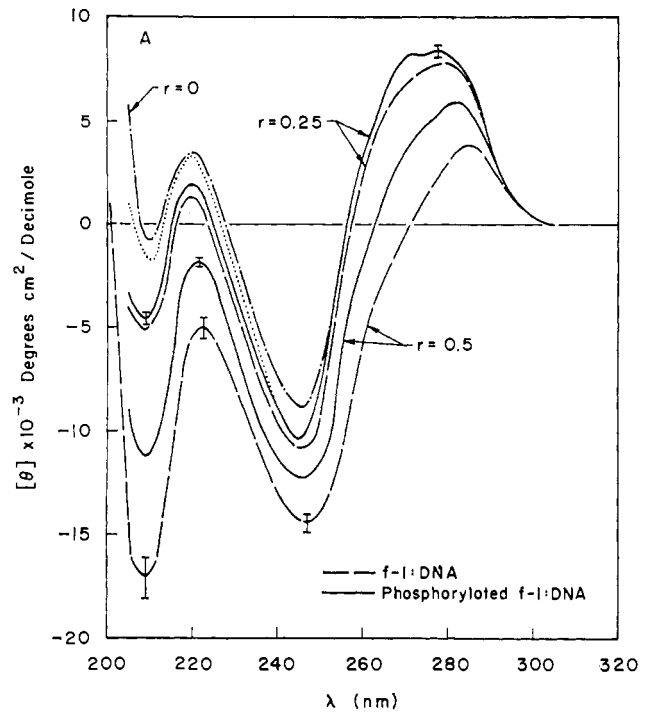
Turbidity of complexes was noticeable beginning at $r = 0.6$ and increasing with *r*. The maximum value of OD_{400}/OD_{258}, a measure of light scattering, was ~0.16. For each value of *r*, complexes displayed identical scattering, independent of phosphorylation; therefore, differences in circular dichroism spectra cannot be attributed to scattering artifacts. An apparent shift in circular dichroism base line of <0.002 deg was often noted with these turbid solutions; however, consistent data were obtained when these variable shifts were simply subtracted from the entire circular dichroism spectrum. At $r > 2$ the complexes precipitated.

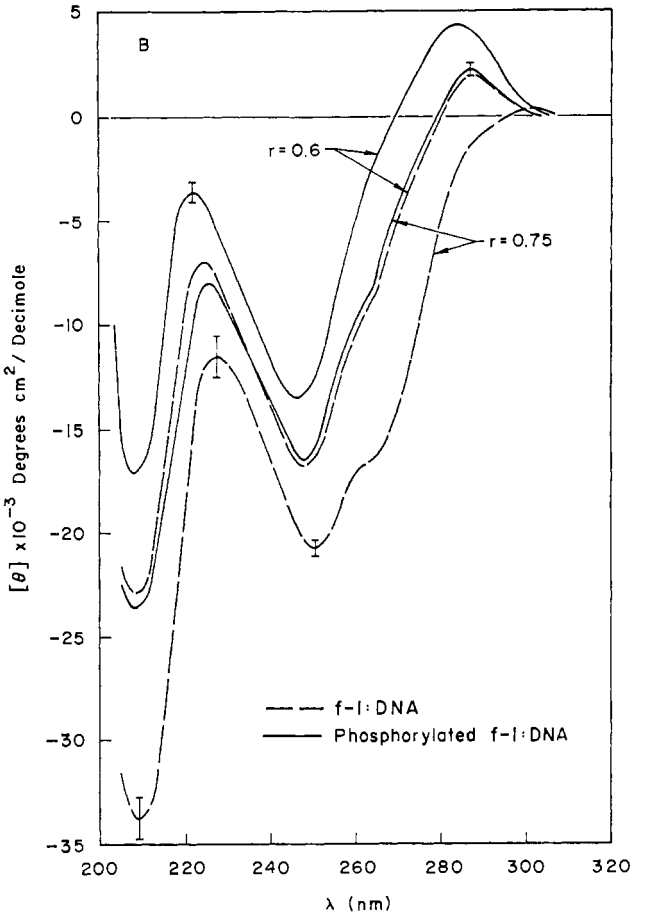
Serologic Measurements. Complement fixation (Wasserman and Levine, 1961) experiments were carried out with systemic lupus erythematosus serum T_a, which reacts with native as well as denatured DNA (Stollar, 1968). The antigens tested were native *P. vulgaris* DNA and complexes of this DNA with phosphorylated and control f-1.

Results

Phosphorylation and Histone Circular Dichroism. Circular dichroism spectra of original calf thymus f-1 histone, phosphorylated f-1, and control f-1 (ATP omitted from the enzyme system) were identical with one another and similar to an f-1 histone prepared differently (Fasman *et al.*, 1970). This indicates that phosphorylation of Ser-37 in f-1 histone does not change the nearly random coil conformation of this protein.

Effect upon Circular Dichroism of Varying the f-1-to-DNA Ratio. Formation of complexes between calf thymus DNA and increasing amounts of homologous f-1 in 0.14 M NaF causes progressive changes in the circular dichroism spectrum (Fasman *et al.*, 1970), indicating alterations in the asymmetric environment of the DNA nucleotide chromophores. Qualitatively, the same progression is depicted in Figure 1. However, the circular dichroism changes effected by a given f-1:DNA ratio (*r*) are a little more pronounced in the present work, since the f-1 samples were more highly purified. The circular dichroism alterations occurring as *r* increases can be summarized as follows. First, the DNA band at 271 nm disappears; the positive DNA band at 277 nm then decreases and becomes red shifted, while the negative DNA band at 245 nm becomes more negative and also red shifts. Starting at approximately the physiological ratio of 0.6, a negative shoulder appears at 265 nm; at higher *r* this band grows (while the 245-nm band disappears) and red shifts; by $r = 1.25$ it dominates the circular dichroism spectrum. Accompanying this entire sequence of changes, the whole circular dichroism spectrum below 240 nm becomes progressively more negative; this change is due only partially to the negative circular dichroism contribution of the protein, as can be seen in Figure 1 at $r = 0.25$ and $r = 1.0$ (dotted lines) where the appropriate amount of histone circular dichroism spectrum was subtracted from that of the f-1–DNA complexes. The sequence of circular dichroism changes upon progressive complexation with f-1 is not simply a manifestation of the growth of a new, negative circular dichroism band in the 265–270-nm region; analysis by means of a Du Pont curve resolver shows that simultaneous changes in several circular





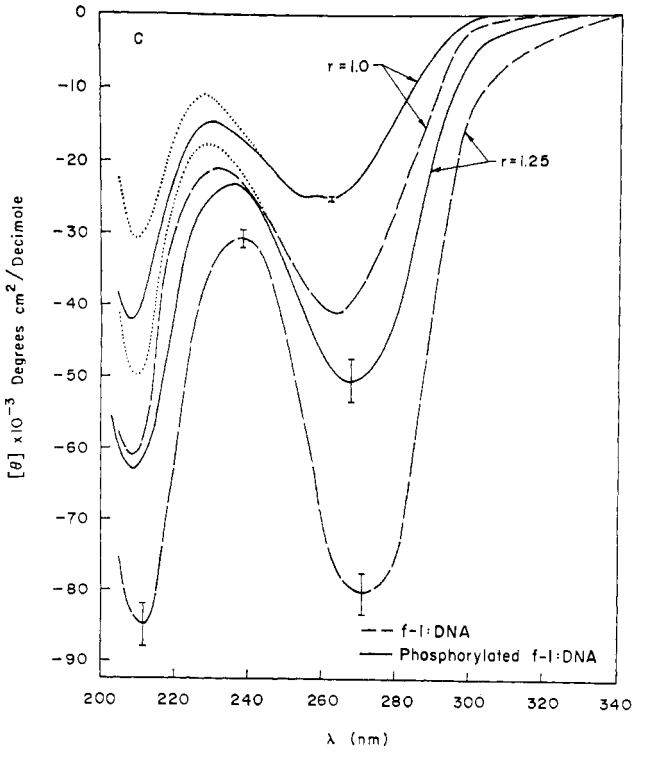


FIGURE 1: Circular dichroism spectra of calf thymus f-1–DNA complexes: effect of histone phosphorylation. The value of r, the histone (moles of peptide residues)/DNA (moles of nucleotide residues), is varied. Complexes with phosphorylated f-1, ———; complexes with control (nonphosphorylated) f-1, ----. Solvent: 0.14 M NaF–0.002 M Tris (pH 7.0). Complex concentrations ~7.5 × 10^{-5} M DNA residues. Error bars indicate reproducibility of data in duplicate runs. (A) r = 0.25 and 0.5. Native DNA (r = 0) is shown for comparison, —·—·—; this curve merges with that of phosphorylated r = 0.25 at wavelengths above 250 nm. Curve calculated by subtraction of histone contribution from data for phosphorylated r = 0.25, ·····. (B) r = 0.6 and 0.75. (C) r = 1.0 and 1.25. Note change of ordinate scale. Data for r = 1.0 (phosphorylated and control) corrected for histone contribution, ·····.

dichroism bands must be postulated. The variation in circular dichroism spectrum can be attributed to conformational changes in the DNA, dependent upon association of f-1–DNA complexes (Fasman *et al.*, 1970).

Effect of f-1 Phosphorylation upon Circular Dichroism of f-1–DNA Complexes. Series of reconstituted complexes containing lysine-rich histone and calf thymus DNA were prepared from both Ser-37 phosphorylated and control (non-phosphorylated) f-1 at values of r (ratio of peptide residues to nucleotide residues) ranging from 0.25 to 1.25. The resulting circular dichroism spectra are compared in Figure 1. At each f-1–DNA ratio, nonphosphorylated histone (dashed lines) produces much more of a distortion of the DNA circular dichroism spectrum than does phosphorylated (solid lines). For example, at r = 0.25 the phosphorylated complex retains the biphasic positive band (270–280 nm) of native DNA (r = 0) and, when the protein contribution is subtracted, is very similar to DNA throughout the circular dichroism spectrum; the nonphosphorylated complex already shows signs of a conformational change. By r = 0.75 the nonphosphorylated complex's spectrum has nearly lost the positive

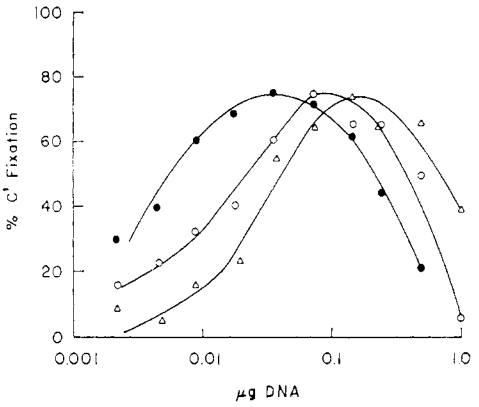


FIGURE 2: Complement fixation by lupus erythematosus serum and varying quantities of f-1 histone–DNA complexes. Native *P. vulgaris* DNA alone, ●; complex of calf thymus phosphorylated f-1 with the same DNA, ○; complex of control (nonphosphorylated) f-1 with the same DNA, Δ. The complexes were reconstituted at r = 1.22, DNA concentration 1.2 × 10^{-4} M residue.

band characteristic of DNA, and has acquired a large negative shoulder at 265 nm; the phosphorylated complex at r = 0.75 still looks like the nonphosphorylated complex at r = 0.6. At r = 1.25 (where the negative charge on the DNA is 35% neutralized by the histone) the two circular dichroism spectra are qualitatively similar but the negative circular dichroism band at ~270 nm, characteristic of high values of r (Fasman *et al.*, 1970), is much larger for the nonphosphorylated complex. Subtraction of the protein circular dichroism contribution (see r = 1.0) does not affect the nature of the results. Perhaps the clearest manner in which to view the progessive circular dichroism alterations is that the presence of the phosphate on f-1 reduces its ability to cause deviation in DNA secondary structure, as measured by circular dichroism.

The differences caused by phosphorylation are not artifacts attributable to incubation of f-1 with histone kinase, as the circular dichroism spectrum of f-1 remains invariant with this treatment, and at several values of r the circular dichroism spectra of DNA complexes with original f-1 (not incubated) were identical with those using control f-1 (incubated without ATP).

A DNA complex was prepared with phosphorylated f-1 at r = 1.0, but dialyzed to 0.01 M NaF–0.001 M Tris (pH 7.0). The circular dichroism spectrum of this material (peaks at: $[\theta]_{276}$ = +7800, $[\theta]_{245}$ = −9000) was similar to that of DNA (at λ >240 nm). Therefore, phosphorylation does not affect the sensitivity to ionic strength (Fasman *et al.*, 1970) of f-1–DNA complexes.

Effect of Phosphorylation upon Serologic Activity of f-1–DNA Complexes. Another excellent technique for distinguishing conformational changes in DNA is immunological activity, that of complement fixation (Wasserman and Levine, 1961; Levine and Van Vunakis, 1966; Stollar, 1968). As shown by Stollar (1968), combination with histones partially masks the antigenic activity of DNA. The data presented in Figure 2 illustrate the different effects of phosphorylated and nonphosphorylated f-1 histone on the serologic activity of native DNA (*P. vulgaris*) when measured with antibodies to native DNA present in a systemic lupus erythematosus serum (Stollar, 1968). The amount of complement fixed is a measure of antigen (DNA or f-1–DNA complex)–antibody lattice formation. With nonphosphorylated f-1–DNA complex (r = 1.22) about 75% of the serologic activity of the component DNA is masked (unavailable for reaction), as shown by the lateral displacement of the complement fixation curve. However, under the same conditions, only about 50% of the DNA activity is masked upon association with phosphorylated f-1 histone.

The experiments were repeated at varying histone to DNA ratios; at r = 1.1, similar results were obtained. Above r = 2 the complexes precipitated; below r = 0.5 little difference between the masking abilities of phosphorylated and nonphosphorylated histone was observed. Thus, less change in the antigenic properties of DNA is brought about by complex formation with phosphorylated than with nonphosphorylated f-1.

Discussion

The studies reported here show that phosphorylated preparations of f-1 histone (containing approximately one phosphate group per mole esterified mainly to Ser-37) form f-1–DNA complexes which retain the native structure of DNA to a markedly greater extent than do complexes formed from nonphosphorylated f-1. Many laboratories have examined differences in secondary and tertiary structure between DNA and nucleoprotein, histone–DNA complexes, or model systems (Elgin *et al.*, 1971). The alterations in the circular dichroism spectra presented here (Figure 1) have been attributed (Fasman *et al.*, 1970) to conformational changes in native DNA brought about when f-1 histone–DNA complexes associate in a specific manner; phosphorylated f-1 is much less effective in bringing about these changes. Similarly, phosphorylated f-1, upon complex formation with DNA, causes less masking of the serologic activity of the DNA than does nonphosphorylated lysine-rich histone. An increase in the antigen concentration required to reach maximum complement fixation (such as is observed in Figure 2) often indicates a general conformational change in the serologically available DNA (Levine and Van Vunakis, 1966); however, such data may merely represent the covering of DNA binding sites by the protein. In any case, phosphorylated f-1 affects DNA less than nonphosphorylated, thus reinforcing the results from circular dichroism.

Although Marushige *et al.* (1969) have observed an altered interaction of phosphorylated protamine and DNA, as indicated by its preferential dissociation at intermediate salt concentrations, these workers were unable to detect any change in salt- or detergent-induced dissociation of phosphorylated histones from nucleohistone complexes (Marushige *et al.*, 1969). This might be attributed to the relative insensitivity of these dissociation techniques to subtle changes in DNA–histone interaction.

The alteration of the DNA–histone interaction by the presence of a single phosphate group seems remarkable, since this causes only a slight change (minus two at pH 7) in the net charge of the histone ($\geq$+51 for rabbit thymus f-1, which contains 212 amino acids; Bustin *et al.*, 1969). However, the activation of phosphorylase by the introduction of one phosphate group per monomer of mol wt 125,000 (Fischer and Krebs, 1966) is a well-documented example of a significant alteration in the properties of a protein caused by phosphorylation of a specific serine residue; conformational changes are considered to be of primary importance in the activation of phosphorylase (Monod *et al.*, 1963). It seems likely that specific conformation-dependent interactions, rather than electrostatic interactions dependent only upon net charge, are responsible also for the effects of phosphorylation

upon histone–DNA complexes. Thus, although phosphorylation causes no detectable alteration in the secondary structure of f-1 histone itself, it appears to have a specific effect upon the ability of the histone to form associated complexes with DNA and to induce conformational changes in the DNA.

The major phosphorylation site (Ser-37) of f-1 histone in the liver of hormonally treated rats is the same as in the preparations studied here (Langan, 1969a,b). This suggests that the changes in DNA–histone interaction observed in this study may also occur *in vivo*. Whether such changes result in derepression of the DNA template, in accordance with the proposed mechanism for hormonal induction of RNA synthesis (Langan, 1968, 1969b), remains to be determined. However, Stevely and Stocken (1968) have reported that the ability of histone preparations to inhibit DNA-dependent RNA synthesis varies inversely with their phosphate content. Finally, it should be emphasized that DNA exists in the cell in association with the entire complement of histones, together with varying amounts of non-histone proteins (Elgin *et al.*, 1971). Therefore interpretation of the biological significance of the results obtained with simple complexes of lysine-rich histone and DNA must be made with caution.

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