Phosphorylation and Dephosphorylation of Histone V (H5): Controlled Condensation of Avian Erythrocyte Chromatin†

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Appendix: Phosphorylation and Dephosphorylation of Histone H5. II. Circular Dichroic Studies‡

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ABSTRACT: During avian erythropoiesis, the blast cells of the bone marrow mature into polychromatic erythrocytes (late stages known as reticulocytes) and then into mature red blood cells. When chickens are made anemic, the proportion of immature cells in the anemic bone marrow increases dramatically. The level of the lysine-rich histones, H1 and H5, has been found to be constant in the blood and bone marrow of normal and anemic chickens. This implies that H5 replaces H1 quantitatively. Urea–aluminum–lactate starch gel electrophoresis of H5 from these sources shows that the degree of phosphorylation of H5 is proportional to the number of immature cells. About 70% of the H5 from the most immature bone marrow is phosphorylated, while 50% of the H5 from anemic blood is phosphorylated and H5 in normal blood is almost completely devoid of phosphate. When immature cells of the anemic bone marrow are incubated in the presence of inorganic ^{32}P and [^{3}H]lysine and [^{3}H]arginine, extensive ^{32}P incorporation is found in the phospho species. A minimum of nine phosphorylated components have been demonstrated by starch gel electrophoresis. The incorporation of ^{3}H is time dependent. After 1.5 h of labeling, ^{3}H is found in H5 containing 0, 1, 2, and 3 phosphates. The combined data suggest that newly synthesized H5 becomes progressively phosphorylated and that at the terminal stage of development, the phosphorylated H5 is completely dephosphorylated. These events may be important in controlling the timing of chromatin condensation.

Erythropoiesis, the process of red blood cell formation and maturation, provides an excellent model system for studying the problems of cellular differentiation and gene regulation. The developing erythroid cells exhibit characteristic features of cytodifferentiation proceeding from genetically active early cells to inert mature erythrocytes. In mammals, this inactivation is accomplished by elimination of the nucleus during the terminal differentiation stages. In avians, however, the nucleus is retained in a highly condensed state. It is generally assumed that macromolecules in association with the erythrocyte chromatin are responsible for this metabolic inactivity. In particular, a major erythrocyte-specific histone, fraction V (H5), rich in lysine, serine, and alanine (Neelin et al., 1964, Hnilica, 1964) has been implicated (Johns, 1969).

Early studies by Purkayastha and Neelin (1966) indicated that H5 is biosynthesized during the terminal stages of erythropoiesis. This suggests a simple role for the histone in bringing about the final gene inactivation and chromatin condensation. More recently, Appels et al. (1972) and Moss et al. (1973) showed that H5 is present, though at a reduced level, in the early dividing erythroblast. This observation argues against a simple relationship between H5 biosynthesis and concomitant template inactivation. In the present paper, the metabolism of H5 is studied. We find that, after its synthesis in the cytoplasm, the protein is transported to the nucleus where it undergoes maturation. The process involves the sequential unidirectional phosphorylation of the newly synthesized H5 molecule during which nine phosphoryl groups may be introduced. At the terminal stages of red cell maturation, the phosphorylated H5 becomes dephosphorylated. The sequence of synthesis, phosphorylation, and dephosphorylation of H5 is very similar to that of the sperm-specific protein, protamine. This similarity, together with other evidence (Appels et al., 1974; Brasch et al., 1972, Kernell et al., 1971, Lurquin and Seligy, 1972), strongly suggests that the biological function of H5 in avian erythrocytes is akin to that of the sperm-specific protamine in condensing and packaging the DNA.

Materials and Methods

Materials. L-[^{3}H]Arginine (12 Ci/mmol) and L-[^{3}H]lysine (50 Ci/mmol) were obtained from Schwarz/Mann; carrier free inorganic ^{32}P from New England Nuclear; NCS reagent from Amersham Searle; hydrolyzed starch from O. Hiller Co., Madison, Wis. The avian myeloid blastosis virus (AMV) was a gift from Dr. Joseph Beard.

Chicken Blood and Bone Marrow Cells. The induction of anemia with 2-acetylphenylhydrazine and the preparation of blood and bone marrow cells from anemic and normal chickens have been fully described in another publication from this laboratory (Sung et al., 1977).

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For the induction of erythroblasts, 5–10-day-old white leghorn chickens were used. The birds were injected ip[1] or iv with 5–8 × 10^{10} particles of avian myeloid blastosis virus (AMV). The development of the disease and the proportion of erythroblast were monitored daily starting on the 8th day. Blood smears were analyzed by the method of Lucas and Jamroz (1961) using the stains of May-Grünwald-Giemsa. Near the terminal stages of the disease, the blood was obtained by cardiac puncture. The viremic blast cells were separated from matured erythrocytes by using albumin step gradients according to Leif and Vinograd (1964).

Cell Incubations. Blood and bone marrow cells were prepared as previously described (Sung et al., 1977). The incubation mixture consisted of 5 × 10^8 cells/ml, 400 μCi/ml of inorganic ^{32}P, 50 μCi/ml each of [^{3}H]lysine and [^{3}H]arginine, and modified Eagles' spinner medium (Sung et al., 1977) (minus arginine and lysine, and buffered with 10 mm Hepes instead of phosphate buffer). All incubations were carried out in a reciprocating water bath at 40 °C.

Lysine-Rich Histones. The lysine-rich histones were recovered as 5% perchloric acid soluble proteins from the 0.2 M HCl-solubilized crude histone preparations (Sung et al., 1977). H1 was separated from H5 on a Bio-Gel P-100 column (2.5 × 100) eluted with 0.01 M HCl and 0.01% azide.

Starch Gel Electrophoresis. Starch gels were prepared as previously described (Sung and Smithies, 1969). After electrophoresis, the gels were sliced horizontally and stained by the sensitive cobalt–amido black 10B procedure. For quantitation of the phospho species in H5, the stained gels were scanned in a Gilford linear-transport system. The densitometric tracing of H5 was monitored with an electronic digital integrator (Spectral Physics), and the area under each peak was automatically calculated. For analysis of radioactivity in the H5, the middle 2-mm horizontal slab was cut into 1-mm thick slices and solubilized in 0.4 ml of NCS reagent according to Louie and Dixon (1972). The samples were counted in a Packard Tricarb scintillation counter.

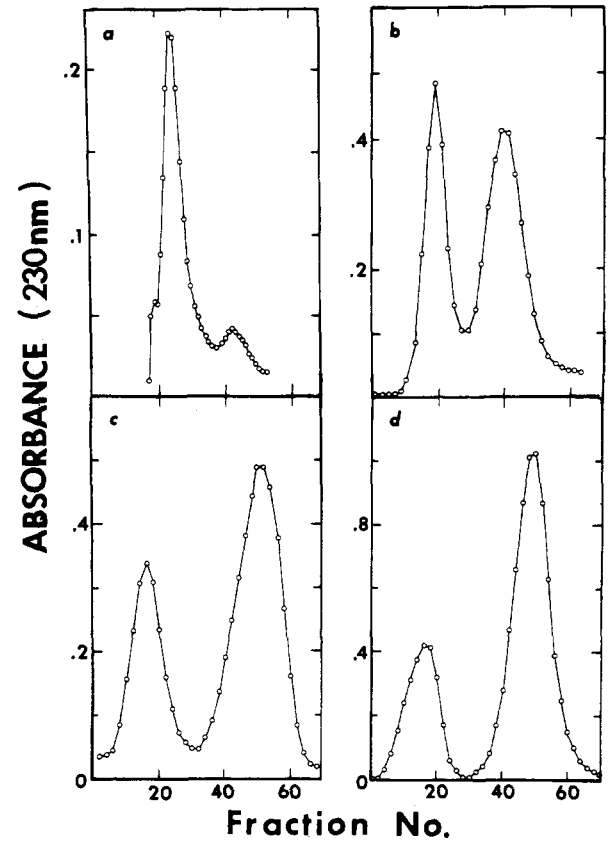

FIGURE 1: The differentiation of H5 during avian erythropoiesis. The lysine-rich histones (H1 and H5) were chromatographed on a Bio-Gel P-100 column (0.25 × 100 cm) and eluted with 0.01 M HCl containing 0.01% sodium azide, and 5-ml fractions were collected. The protein absorbance in the fractions was read at 230 nm. H1 emerged from the column first, followed by H5. The relative content of H5 was compared to H1 in (a) viremic blast cells, (b) anemic bone marrow, (c) anemic blood, and (d) normal blood.

Results

H5 Differentiation. In Figure 1 the lysine-rich histones obtained from erythropoietic tissues representing various degrees of maturation (a–d) are compared on Bio-Gel P-100 column. As expected, the relative level of H5 increases with the maturation of erythroid cells. Figure 1a shows that the viremic blast cells do contain H5, though at a reduced level. The ratio of H1 to H5 is 5:1, which is substantially lower than that obtained by Sotirov and Johns (1972) [3.4:1]. In our experiment, avian myeloid blastosis virus was used, which may have induced other myeloid blasts as well as erythroblasts. In the anemic bone marrow, 40% of the cells are erythroblasts and 38% are polychromatic erythrocytes (for composition of cell types, see Table I and also Williams 1972). The ratio of H1 to H5 is approximately 1:1 (Figure 1b). In the anemic blood, nearly all (95%) the cells are reticulocytes. The ratio of H1 to H5 is 1:2 (Figure 1c) and in the very mature red cells of the normal blood it is 1:3 (Figure 1d). The above ratios are in good agreement with the work of Billit and Hindley (1973) who analyzed in stained gels the content of the lysine-rich histones from the three tissues. The results suggest that H5 accumulates during red cell differentiation. However, if, as has been proposed, H1 is partially replaced, this could cause an overestimation of the content of H5 in the more mature cells. To overcome this difficulty, the content of H5 was next compared to H4 in these and other experiments by integrating the P-60 chromatographic peaks as monitored at 230 nm. The ratio of H4 to H5 in anemic bone marrow, anemic blood, and normal blood is approximately 1:1, 1:1.5, and 1:2. The combined ratio of (H1 + H5) is 2:1 in all three tissues. The constancy in the sum of H1 and H5 is not surprising, as others have shown that the total histone to DNA ratio appears to be constant through developmental stages (Appels et al. 1971). Our semiquantitative data indicate that H5 not only progessively accumulates but replaces H1 on a one-to-one basis. More quantitative experiments, however, are needed to establish this point.

Newly Synthesized H5 Undergoes Progressive Phosphorylation. As has been pointed out in previous work (Sung et al., 1977), H5 in the erythroblasts (anemic bone marrow) shows extensive heterogeneity (when electrophoresed in starch gel) (see also Figure 3). It is known that enzymatic phosphorylation and acetylation of histones can alter their electrophoretic mobility and contribute directly to the observed multiple bands. That this heterogeneity is due to phosphorylation alone can be substantiated by several lines of evidence. The slow migrating components disappear after treatment with alkaline phosphatase. There is no acetylation in H5 (Sung et al., 1977). There is extensive incorporation of inorganic ^{32}P in the purified H5 following the labeling of immature anemic bone marrow cells (Sung et al., 1977).

[1] Abbreviations used are: iv, intraperitoneally; iv, intravenously; CD, circular dichroism; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

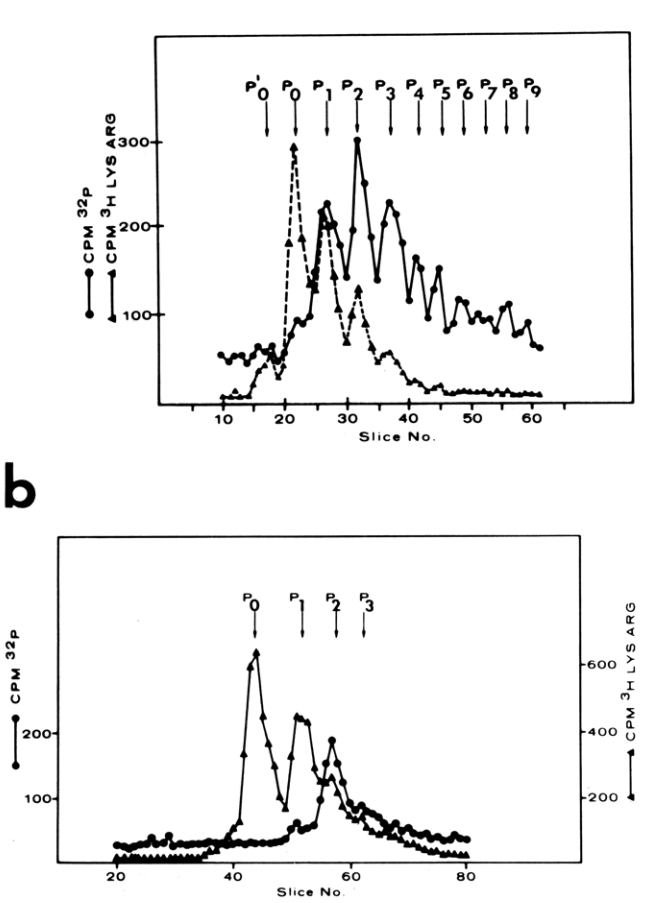

FIGURE 2: Distribution of ^{32}P and ^{3}H in the phosphospecies of labeled H5 from (a) anemic bone marrow and (b) anemic blood. The histones were electrophoresed in urea–aluminum–lactate starch gel. Following electrophoresis, the gel was trisected horizontally and the radioactivity of the histone region of the middle gel slab was determined by liquid scintillation counting of 1-mm strips.

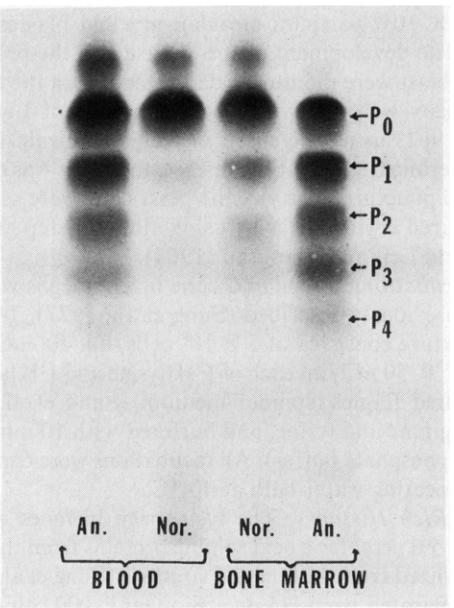

FIGURE 3: Level of phosphorylation of H5 from anemic blood, normal blood, normal bone marrow, and anemic bone marrow (the order is from left to right). The histones were electrophoresed in urea–aluminum–lactate starch gel and stained with amido-black 10B. P_0, P_1, P_2, P_3, and P_4 represent histone H5 containing 0, 1, 2, 3, and 4 phosphates.

In the cell incubation system, the incorporation of both ^{32}P and [^{3}H]lysine plus arginine is linear for at least 4.5 h at 40 °C (data not shown). This direct proportionality of incorporation of ^{32}P and ^{3}H suggests that newly synthesized histone is phosphorylated. To examine this point further, the dual labeled histone obtained after a 90-min labeling was analyzed on urea–aluminum–lactate starch gel. Figure 2a demonstrates the distribution of the two isotopes in the phospho species of H5 from anemic bone marrow. ^{32}P is incorporated into a minimum of nine electrophoretic components. The even spacing of the histone subfractions suggests that each of the phospho species differs from the preceding one by a single charge; they are appropriately labeled as P_0, unmodified H5; P_1, H5 containing a single phosphate; and P_n, H5 with n phosphates. During the 90 min of incubation with [^{3}H]lysine plus [^{3}H]arginine, the newly synthesized H5 becomes progressively phosphorylated. The ^{3}H label is found in P_0, P_1, P_2, P_3, and P_4 components. The relative specific activities of the ^{3}H label in these components are 3.6:3.0:2.2:1.3:1, respectively. This is consistent with a sequential and unidirectional phosphorylation mechanism.

Figure 2b shows the distribution of ^{3}H and ^{32}P in H5 labeled in reticulocytes (anemic blood). The distribution of ^{3}H is similar to that seen in the anemic bone marrow but much less ^{32}P has been incorporated. In contrast to the multiple peaks of ^{32}P label seen on gels of erythroblast H5, reticulocyte H5 contains only one major species labeled with ^{32}P; this corresponds to H5 containing two phosphates.

The specific activity of the ^{32}P incorporated in anemic blood is fourfold lower than in anemic bone marrow; in anemic blood 4.75×10^4 cpm/mg are incorporated while 17.2×10^4 cpm/mg are incorporated in anemic bone marrow. Since H5 continues to be synthesized in reticulocytes at a rate equal to or greater than that in erythroblasts (Sung et al., 1977), this decrease in ^{32}P incorporation must be due to other factors. There are three possible reasons for the decreased incorporation: (1) The histone kinase(s) is less active; (2) the histone phophatase(s) is more active; (3) the intrinsic phosphate pools are larger, thereby decreasing the specific activity of the radioisotope. That the latter is correct is suggested by the similar distribution of the ^{3}H label in phosphospecies of H5 from anemic blood and bone marrow.

The data of Figure 2a,b do clearly demonstrate that newly synthesized H5 becomes progressively phosphorylated shortly after its synthesis in both immature cells and reticulocytes.

The Dephosphorylation of H5. The level of phosphorylated H5 in normal blood, normal bone marrow, anemic blood, and anemic bone marrow is shown in Figure 3. These tissues differ from each other in the proportion of mature vs. immature erythroid cells (see Table I, and Sung et al., 1977). It has been established above that newly synthesized histones in the immature cells become phosphorylated and, as expected, electrophoresis of the H5 from these tissues differ only in the degree of phosphorylation. The stained protein bands were scanned and quantitative data of phosphorylated and unmodified species for each of the samples were obtained by an electronic digital integrator. From Table I, it is clear that the number of phospho species, as well as their individual levels of phosphorylation, are directly proportional to the percentage and type of immature cells. For example, there are 6 phospho species in anemic bone marrow, 5 in anemic blood, and only

TABLE I.

Cell Type				% of Total Histone H5							
Erythroblast (%)	Polychromatic Erythrocyte (%)	Mature Red Blood Cells (%)	Source of Histone H5	P_0'	P_0	P_1	P_2	P_3	P_4	P_5	P_6
0	0	99	Normal blood	24.2	57.4	9.7	8.7	0	0	0	0
4	21	53	Normal bone marrow	16.2	43.5	17.2	11.7	6.1	5.3	0	0
0.1	95	3	Anemic Blood	16.5	37.6	19.4	15.3	6.7	4.0	0.04	0
40	38	0	Anemic Bone Marrow	1.0	27.0	23.5	21.4	15.7	7.7	2.3	1.5

2 in the normal blood. In particular, the data suggest that H5 synthesized in the early cells (blast and early polychromatic erythrocytes) is highly phosphorylated and undergoes progressive dephosphorylation in the late polychromatic erythrocytes to become fully dephosphorylated in mature erythrocytes. The P_0' is the most cathodic migrating H5 species and the content increases with the maturation stages. It is not known if P_0' is a minor component of H5 or a degradation product. In any event, this minor component does not appear to affect the above conclusion.

Discussion

The appearance of a cell-specific histone H5 during the terminal differentiation of avian erythroid cells is thought to be responsible for the final repression of the erythrocyte chromatin. However, one of the strongest arguments against H5 serving such a role is its early synthesis in erythroblasts where much RNA synthesis is taking place and cell division continues. Others have tried to rationalize the problem by suggesting that the progressive inactivation is due to H5 accumulation. Yet, it is clear from our study that erythroblasts contain 20–30% of the final H5 content and are still dividing and metabolically active. Therefore, other modulations are necessary to prevent the premature inactivation of chromatin.

Appels and Wells (1972) have suggested that the chromatin-bound H5 in the immature cells is being continually renewed and that it is this dynamic state of H5 which permits template transcription. In terms of biological economy, such a control scheme is wasteful. Further, the scheme assumes a full complement of H5 in the erythroblasts and polychromatic erythrocytes, which is contradicted by our data presented in Figure 1. In early studies, we experienced difficulty in the handling of H1 and H5 from erythroid cells. Presumably, these proteins are in extended conformations making them susceptible to proteolytic attack. Whether this has bearing on the study of Appels and Wells (1972) is not known at present.

In the present study, we show conclusively that newly synthesized H5 becomes progressively phosphorylated (see also Tobin and Seligy, 1975). The phosphorylation of other basic proteins is well documented, especially during spermatogenesis in trout testis (Louie and Dixon, 1972; Sung and Dixon, 1970). In this regard, the metabolism of H5 appears to be similar to that of the sperm-specific protein, protamine; i.e., soon after synthesis, multiple sites are phosphorylated, and then dephosphorylation occurs later. These two proteins may also both play similar roles in the condensation of chromatin, although in avian erythropoiesis there is no complete replacement of histones during condensation (Appels et al., 1972; Billet and Hindley, 1972). By analogy to the protamine story and in light of the present study, a maturation pathway for the newly synthesized H5 may be explained as follows. In the first half of the process, phosphokinases catalyze sequential esterification of phosphoryl groups to strategic seryl residues in the newly synthesized H5 molecule. The conversion steps are unidirectional, from the nascent histone $P_0 \rightarrow P_1 \rightarrow P_2 \cdots \rightarrow P_n$, where P represents H5 containing 0, 1, 2, and $n \geq 9$ phosphates. The purpose of this phosphorylation may be to orient the binding of H5 to DNA such that, in the highly phosphorylated state, the strong interactions between the two macromolecules may be diminished to the point that weak, yet specific, forces could come into play. During the second half of the maturation process, the properly oriented H5 is sequentially dephosphorylated by specific phosphatases. By dephosphorylation, the histone regains its strong interactions with DNA and thereby brings about well controlled condensation of the mature erythrocyte chromatin.

On an a priori basis, the highly phosphorylated H5 will bind with reduced affinity to the erythrocyte chromatin; the reduced affinity may make the complex "functionally inactive". Consistent with this contention, we have shown in the Appendix that, in contrast to unmodified H5, the highly phosphorylated form from anemic bone marrow is not capable of mediating conformational changes in the DNA. At terminal stages of red cell maturation, the highly phosphorylated H5 becomes dephosphorylated and this event does correlate well with the cessation of macromolecular biosynthesis and chromatin condensation.

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Appendix: Phosphorylation and Dephosphorylation of Histone H5. II. Circular Dichroic Studies

The circular dichroic (CD) study described in this Appendix compares the effects of H5 samples which are phosphorylated to different degrees (see Table I) on the conformation of DNA in the in vitro reconstituted nucleohistone complexes. Details on the parameters which influence the conformational effects of H5 on DNA, such as salt concentration, DNA–histone ratio, and phosphorylation, will be reported elsewhere (Wagner et al., 1977, in preparation). Here, we describe the biological implication of this work. The purpose is to obtain preliminary physical evidence for the suggested role of H5 phosphorylation and dephosphorylation in bringing about a well-controlled condensation of avian erythrocyte chromatin.

Complexes of H5 with DNA. Chicken erythrocyte DNA was prepared by the procedure of Marmur (1961). The histone H5 samples and DNA were dissolved in separate solutions containing 2 M NaCl and 0.01 M Tris-HCl, pH 7.2 Aliquots

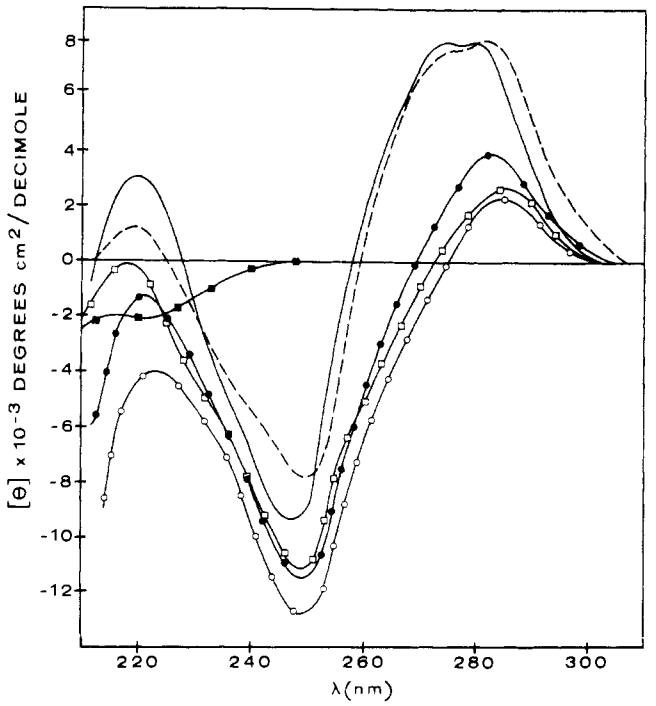

FIGURE 4: Circular dichroic spectra of H5 (■-■), DNA (—), anemic bone marrow H5-DNA complexes (- - -), anemic blood H5-DNA complexes (●-●), normal bone marrow H5-DNA complexes (□-□), and normal blood H5-DNA complexes (○-○). The histone to DNA ratio in all complexes is 0.5:1 and the spectra were measured at 0.15 M NaCl, 0.001 M Tris, pH 7.2.

of histone and DNA solutions were mixed while vortexing to obtain the histone-to-DNA input weight ratio of 0.5:1. The mixture of histone and DNA in high-salt solution was allowed to complex by the removal of salt using a gradient dialysis procedure at 4 °C (Bonner et al., 1968). A step gradient of 1.5, 1.0, 0.4, 0.3, and 0.15 M NaCl was used. Dialysis at 1.5, 1.0, 0.4, and 0.3 M with each solution containing 0.05 M Tris, pH 7.2, was for 4 h each and the final dialysis step at 0.3 M NaCl to 0.15 M NaCl was for 12 h.

Circular Dichroism. Circular dichroic spectra were obtained using a Durum Jasco J-20 circular dichrometer. The concentration of DNA in all nucleohistone H5 complexes was approximately 1×10^{-4} in DNA nucleotide residue. Mean residue ellipticity (θ) is reported in deg cm^2 dmol^{-1} on the basis of DNA nucleotide residue concentration. The degree of turbidity of complexes was measured by calculating A_{400}/A_{260} ratios (Adler et al., 1971) and these were ≤ 0.07.

Figure 4 shows the CD spectrum of chicken DNA. It is characterized by a doublet maxima at 275 and 280 nm ($\theta_{275,280} = 8100 \pm 200$), a minimum at 247 nm ($\theta_{247} = -9800$), followed by a crossover at 227 nm and with another positive maximum at 218 nm ($\theta_{218} = 3200$). The CD spectrum of histone H5, regardless of degrees of phosphorylation, is generally featureless at 250–300 nm and some secondary structure is observed at the far-UV region. On the other hand, when the unmodified H5 (indicated as normal blood in Figure 4) is complexed to DNA in 0.15 M NaCl, it causes a marked change in the spectrum which is characteristic of the DNA alone. The biphasic nature of the positive band ($\theta_{283} = 2200$) is completely removed. The crossover point and the negative maximum are both red shifted to 275 and 250 nm, respectively. At the other extreme, the CD spectrum of nucleohistone complex between DNA and the highly phosphorylated H5 from anemic bone marrow is DNA like. Comparison of the nucleohistone CD spectra for the intermediate levels of phosphorylated H5 (anemic blood and normal bone marrow) further verifies that phosphorylated H5 is less capable of altering DNA conformation. The effect of phosphorylation of H5 on DNA conformation is difficult to quantitate, since the phosphorylation-mediated effects must be due to a combination of the sites, as well as the level of phosphorylation at each site in the histones. Nevertheless, the magnitude of these effects appears to be dependent on the known level of phosphorylation in the histones (anemic bone marrow < anemic blood < normal bone marrow < normal blood).

The ability of H5 to alter DNA conformation suggests a mechanism for the genetic repression observed in nucleated erythrocytes. The decreased effectiveness of phosphorylated H5 in causing conformational change points to a means of controlling the repression.

References

Adler, A. J., Schaffhausen, B., Langan, T. A., and Fasman, G. D. (1971), *Biochemistry 10,* 909.

Appels, R., Bolund, L., and Ringertz, R. R. (1974), *J. Mol. Biol. 87,* 339.

Appels, R., and Wells, J. R. E. (1972), *J. Mol. Biol. 70,* 425.

Appels, R., Wells, J. R. E., Williams, A. F. (1971), *J. Cell Sci. 10,* 47.

Billett, M. A., and Hindley, J. (1972), *Eur. J. Biochem. 28,* 451.

Bonner, J., et al. (1968), *Methods Enzymol. 12B,* 3.

Brasch, K., Setterfield, G., Neelin, J. M. (1972), *Exp. Cell Res. 74,* 27.

Hnilica, L. S. (1964), *Experientia 20,* 12.

Johns, E. W. (1969), *Homeostatic Regul., Ciba Found. Symp., 1969,* Wolstenholm, G. E. W., and Knight, J., Ed., London, Churchill, pp 128.

Kernell, A. M., Bolund, L., and Ringertz, N. R. (1971), *Exp. Cell Res. 30,* 609.

Leif, R. C., and Vinograd, J. (1964), *Proc. Natl. Acad. Sci. U.S.A. 51,* 520.

Louie, A. J., and Dixon, G. H. (1972), *J. Biol. Chem. 247,* 5490, 7962.

Lucus, A. M., and Jamroz, C. (1961), in Atlas of Avian Hematology USDA Monograph, p 25.

Lurquin, P. F., and Seligy, V. L. (1972), *Biochem. Biophys. Res. Commun. 46,* 1399.

Marmur, J. (1961), *J. Biol. Chem. 3,* 208.

Moss, B. A., Joyce, W. G., and Ingram, V. M. (1973), *J. Biol. Chem. 248,* 1025.

Neelin, J. M., Callahan, P. L., Lamb, D. C., Murray, K. (1964), *Can. J. Biochem. 42,* 1743.

Purkayastha, R., and Neelin, J. M. (1966), *Biochim. Biophys. Acta 127,* 468.

Sotirov, N., and Johns, E. W. (1972), *Exp. Cell Res. 73,* 13.

Sung, M. T., and Dixon, G. H. (1970), *Proc. Natl. Acad. Sci. U.S.A. 67,* 1616.

Sung, M. T., Harford, J. B., Bundman, M., and Vidalakis, G. (1977), *Biochemistry 15* (preceding paper in this issue).

Sung, M. T., and Smithies, O. (1969), *Biopolymers 7,* 39.

Tobin, R. S., and Seligy, V. L. (1975), *J. Biol. Chem. 250,* 358.

Williams, A. F. (1972), *J. Cell Sci. 10,* 27.