Histones H2a, H2b, H3, and H4 Are Present in Equimolar Amounts in Chick Erythroblasts†

Jacob Joffe,‡ Michael Keene,‡ and Harold Weintraub*

ABSTRACT: A quantitative labeling method for stoichiometric analysis has been applied to an investigation of the histone composition of chick erythroblast chromatin. The necessity of long fractionation procedures and the dangers of preferential extraction and staining are eliminated through the use of this very simple procedure which involves the direct analysis of nuclear proteins on sodium dodecyl sulfate gels. Only a knowledge of the amino acid compositions of the histones is required. H2a, H2b, H3, and H4 are found to be present in nearly equimolar quantities.

Several models have been proposed to describe nucleosome or ν-body structure (Olins and Olins, 1974; Kornberg, 1974; Van Holde et al., 1974; Baldwin et al., 1975; Varshavsky and Georgiev, 1975; Li, 1975; Hyde and Walker, 1975; Weintraub et al., 1976). Many of these models require a specific molar stoichiometry (1:1:1:1) of the four inner histones, H2a, H2b, H3, and H4. Several measurements of histone stoichiometry in various organisms and tissues have been reported (Johns, 1967; Fambrough et al., 1968; Panyim and Chalkley, 1969; Bradbury et al., 1972; Moss et al., 1972; Oliver and Chalkley, 1972; Wright and Olins, 1975). With the exception of a recent report by Olins et al. (1976) most analyses have shown that the four histones are not present in equimolar concentrations. However, histones are routinely extracted with acid and then precipitated with ethanol. This procedure may lead to differential extraction and we present here a simple method of stoichiometric analysis which avoids the dangers of preferential extraction and shows that, in contrast to previous findings, the four inner histones are present in roughly equimolar quantities in chick erythroblasts.

Materials and Methods

Cells and Incubations. Developing red blood cells were isolated by vein puncture from the circulation of 4 or 5 day chick embryos as described by Weintraub et al. (1971). They were washed once and incubated for 4 h at 37 °C in minimum essential medium lacking leucine or methionine (GIBCO) with 20 μCi/mL [^{14}C]leucine (270 mCi/mM) or 100 μCi/mL [^{35}S]methionine (60 Ci/mM) (New England Nuclear). Cells were then washed twice in SSC (0.15 M NaCl–0.015 M sodium citrate) and lysed with 0.5% Nonidet P40 (Gallard Schlesinger) in 10 mM NaCl–5 mM $MgCl_2$–10 mM Tris-HCl (pH 7.4). Nuclei were pelleted by centrifugation and washed repeatedly with lysis buffer until the nuclear pellet was white.

Electrophoretic and Autoradiographic Analysis of Histone Stoichiometry. [^{35}S]Methionine or [^{14}C]leucine labeled 4-day nuclei were incubated for 30 min at 37 °C with 20 μg/mL micrococcal nuclease (Worthington) in 5 mM phosphate buffer (pH 6.8) and 1 mM $CaCl_2$ to prevent the formation of a nuclear gel upon addition of sodium dodecyl sulfate sample buffer. Samples were mixed with an equal volume of buffer containing 4% sodium dodecyl sulfate–20% glycerol–10% β-mercaptoethanol–0.001% bromphenol blue–0.125 M Tris-HCl (pH 6.8) and boiled for 1 min in preparation for electrophoresis. Samples were loaded onto 15% polyacrylamide–sodium dodecyl sulfate stacking gels made according to a modification (Weintraub and Van Lente, 1974) of the method of Laemmli (1970). The separating gel was made 15% in acrylamide and 0.2% bisacrylamide in 0.375 M Tris-HCl (pH 8.8) and 0.1% sodium dodecyl sulfate. The stacking gel was 3% acrylamide and 0.08% bisacrylamide in 0.125 M Tris-HCl (pH 6.8) and 0.01% sodium dodecyl sulfate. Electrophoresis was for 6 h at 140 V with a buffer system of 0.38 M glycine–0.05 M Tris (pH 8.3)–0.1% sodium dodecyl sulfate. After electrophoresis the gels were dried without staining and exposed to Kodak RPR-54 x-ray film for a length of time sufficient for the histone bands to attain a darkness within the linear range of the film. The developed autoradiograms were scanned with a Joyce Loebl MK IIIB microdensitometer. Peaks were quantitated by the weight of paper under the tracing. The amount of each histone present is proportional to the weight of paper under each peak divided by the number of leucine or methionine residues in that histone.

Contamination of Histones with Nonhistone Proteins. In order to monitor the possibility that a given histone comigrates with a nonhistone nuclear protein on sodium dodecyl sulfate gels, low concentrations of nuclei (<100 μg/mL) were extracted three times with acid and 8 M urea and the acid-insoluble material was run on sodium dodecyl sulfate gels. Virtually no detectable Coomasie blue staining material in the acid-insoluble nuclear fraction comigrated with H2a, H2b, H3, or H4. However, with [^{35}S]methionine (but not with [^{14}C]leucine) labeled nuclei, small amounts (1.8% of the total S-35 nuclear counts) of a nonhistone protein were seen to comigrate with H4 (which is about 8.9% of the total S-35 nuclear protein). Under identical conditions, no nonhistone protein comigrated with H2a, H2b, or H3. The data to be presented in Table I are corrected for this methionine-rich contaminant of H4.

In order to monitor the possibility that a given histone comigrates with an acid-soluble nonhistone nuclear protein, an acid-soluble extract of whole nuclei was assayed by two-dimensional electrophoresis, the first dimension being an acid–urea gel (Panyim and Chalkley, 1969) and the second dimension being a sodium dodecyl sulfate gel (Figure 1). No acid-soluble nonhistone proteins were detected that migrated

† From the Frick Chemical Laboratories, Department of Biochemical Sciences, Princeton University, Princeton, New Jersey 08540. Received June 22, 1976.

‡ In partial fulfillment of B.A. degree, Princeton University.

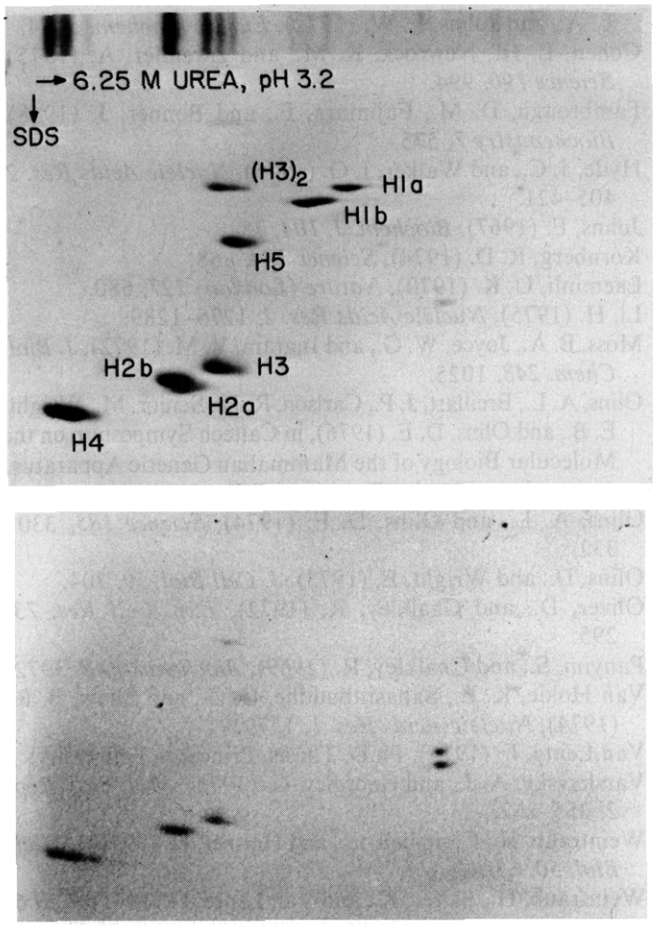

FIGURE 1: Absence of acid-soluble nonhistone proteins that comigrate with histones in sodium dodecyl sulfate gels. The first dimension is an acid–urea gel (Panyim and Chalkley, 1969); and the second dimension is a sodium dodecyl sulfate gel (Weintraub and Van Lente, 1974). The top gel is stained with Coomasie blue; the bottom gel is an S-35 methionine autoradiograph of the same gel. The one-dimensional gel on the top of Figure 1 is an acid–urea gel.

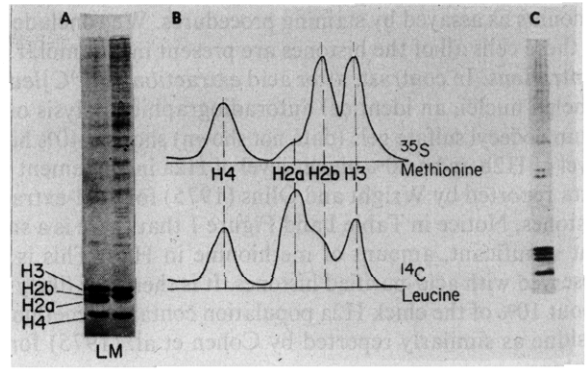

FIGURE 2: Electrophoretic and autoradiographic analysis of histone stoichiometry. Histones from 4 day chick erythroblast nuclei labeled with [^{35}S]methionine (M) or [^{14}C]leucine (L) were separated directly on sodium dodecyl sulfate gels. After autoradiography, the gels were scanned as described in Materials and Methods. (A) An autoradiogram showing H3, H2b, H2a, H4 as prominent bands; (B) densitometer tracing of the autoradiograph shown in A; (C) histones stained with Coomasie blue.

TABLE I: Equimolar Ratios of H2a, H2b, H3, and H4 in Chick Erythroblasts.

	No. of Leu/mol	Normalized Stoichiometry	No. of Met/mol	Normalized Stoichiometry
H3	12	1.00	2	1.00
H4	8	0.98 ± 0.08	1	1.05[a]
H2a	16		0	(0.09 ± 0.03)
H2b	6		2	1.03 ● 0.14
H2a + H2b	22	0.93 ± 0.13		

[a]Corrected for contamination with a nonhistone protein (see Materials and Methods). The uncorrected value is 1.26 ± 0.22. The error is calculated as the standard deviation of the mean.

in the sodium dodecyl sulfate dimension with H2a, H2b, H3, or H4 as assayed by Coomasie blue staining or [^{35}S]methionine autoradiography.

Results and Discussion

Histone Stoichiometry in Chick Erythroblasts. After the incorporation of a specific radioactive amino acid, the four major histones in chick erythroblasts were analyzed by sodium dodecyl sulfate electrophoresis, autoradiography, and quantitative densitometry. Given the known molar ratio of the radioactive amino acid in each histone, the stoichiometry of the four inner histones H2a, H2b, H3, and H4 can then be determined directly. In these studies every attempt was made to eliminate the possibility of differential staining, extraction, or quenching. Whole nuclei were mixed directly with the sodium dodecyl sulfate sample buffer and the denatured nuclear proteins loaded directly onto sodium dodecyl sulfate gels without fractionation. After electrophoresis, the resulting gels were dried and exposed to x-ray film without staining or destraining, which could conceivably cause quenching or differential loss of protein from the gel. This type of analysis is greatly facilitated by the fact that over 75% of the nuclear protein made by these cells is histones (see Figure 2). Four-day erythroblasts were cultured with [^{35}S]methionine or [^{14}C]leucine for 4 h. (Similar results are obtained for incubations as short as 15 min.) The tissue culture medium contained all amino acids with the exception of methionine or leucine, so it was expected that, if the label were converted into other forms, its specific activity would be so low that it could be ignored. A typical autoradiogram of leucine-labeled and methionine-labeled nuclear proteins is shown in Figure 2A. The results of our analysis are presented in Table I. The methionine and leucine compositions of each histone are based upon the data of Olins and Wright (1973). All values are normalized to H3 and represent averages of 20 independent experiments for each label. Inadequate electrophoretic resolution of H2a and H2b in the leucine-labeling experiments requires that these two histones be analyzed together as if they comprised one large protein.

Within the standard deviation, the histones appear to be equimolar. Our data cannot exclude significant deviations from equimolarity that are below about 10% (for example, those that may arise from a different histone stoichiometry in active regions of the genome). The leucine label shows that the ratio of H3 to H4 is 1:1, while the methionine label shows that the ratio of H3 to H2b is 1:1. Since the leucine label also shows that the ratio of H3 to (H2a and H2b) is 1:1 (treating H2a and H2b as one polypeptide), it follows that H2a must also be equimolar with H3. Similar, though less extensive, results have been obtained in four independent experiments using [^{14}C]lysine. In addition, Van Lente (1975) and Olins et al. (1976) have also shown that histones H2a, H2b, H3, and H4 separated directly on sodium dodecyl sulfate gels are present in roughly equimolar

amounts as assayed by staining procedures. We conclude that in these cells all of the histones are present in equimolar concentrations. In contrast, after acid extraction of [^{14}C]leucine labeled nuclei, an identical autoradiographic analysis on sodium dodecyl sulfate gels (data not shown) shows a 40% higher level of H2b and a 20% higher level of H2a in agreement with data reported by Wright and Olins (1975) for acid-extracted histones. Notice in Table I and Figure 1 that there is a small, but significant, amount of methionine in H2a. This is also observed with acid-purified histones. It is therefore likely that about 10% of the chick H2a population contains a methionine residue as similarly reported by Cohen et al. (1975) for sea urchins.

In summary, the great majority of our data support the conclusion that in chick erythroblasts the four inner histones, H2a, H2b, H3, and H4, are present in equimolar concentrations. Whether these conclusions can be extended to other cell types and to mature erythrocytes remains to be determined. Similarly we have not yet made a detailed study of H1 or H5. Our results support the original contention that each nucleosome contains two each of the four inner histones (Kornberg, 1974; Olins and Olins, 1974). It is also consistent with the more detailed proposal (Weintraub et al., 1976) that the eight histones in each ν body are paired as two self-complementary heterotypic tetramers with each tetramer containing one of the four inner histones and the two tetramers together defining a twofold axis of symmetry within the nucleosome.

Acknowledgments

We thank Richard Blumenthal for technical assistance and the National Institutes of Health for support (Grant 1 R01 GM 22444-01).

References

Baldwin, J. P., Boseley, P. G., Bradbury, E. M., and Ibel, K. (1975), *Nature (London) 253,* 245.

Bradbury, E. M., Molgaard, H. V., Stephens, R. M., Bolund, L. A., and Johns, E. W. (1972), *Eur. J. Biochem. 31,* 4.

Cohen, L. H., Newrock, K. M., and Zweidler, A. (1975), *Science 190,* 994.

Fambrough, D. M., Fujimura, F., and Bonner, J. (1968), *Biochemistry 7,* 575.

Hyde, J. C., and Walker, I. O. (1975), *Nucleic Acids Res. 2,* 405–421.

Johns, E. (1967), *Biochem. J. 104,* 78.

Kornberg, R. D. (1974), *Science 184,* 868.

Laemmli, U. K. (1970), *Nature (London) 227,* 680.

Li, H. (1975), *Nucleic Acids Res. 2,* 1275–1289.

Moss, B. A., Joyce, W. G., and Ingram, V. M. (1972), *J. Biol. Chem. 248,* 1025.

Olins, A. L., Breillatt, J. P., Carlson, R. D., Senior, M., Wright, E. B., and Olins, D. E. (1976), in Caltech Symposium on the Molecular Biology of the Mammalian Genetic Apparatus, Amsterdam, Elsevier-Excerpts Medica, (in press).

Olins, A. L., and Olins, D. E. (1974), *Science 183,* 330–332.

Olins, D., and Wright, E. (1973), *J. Cell Biol. 59,* 304.

Oliver, D., and Chalkley, R. (1972), *Exp. Cell Res. 73,* 295.

Panyim, S., and Chalkley, R. (1969), *Biochemistry 8,* 3972.

Van Holde, K. E., Sahasrabuddhe, C. G., and Shaw, B. R. (1974), *Nucleic Acids Res. 1,* 1579.

Van Lente, F. (1975), Ph.D. Thesis, Princeton University.

Varshavsky, A. J., and Georgiev, G. (1975), *Mol. Biol. Rep. 2,* 255–262.

Weintraub, H., Campbell, G., and Holtzer, H. (1971), *J. Cell Biol. 50,* 652.

Weintraub, H., Palter, K., and Van Lente, F. (1975), *Cell 6,* 85.

Weintraub, H., and Van Lente, F. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4249.

Weintraub, H., Worcel, A., and Alberts, B. (1976), *Cell* (in press).

Wright, E., and Olins, D. (1975), *Biochem. Biophys. Res. Commun. 63,* 642.