Radioiodination of Chicken Erythrocyte Histones H₄ and H₃ in Chromatin*

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The conformational state of histones in isolated chicken erythrocyte chromatin was studied using procedures developed for probing surface proteins on membranes. Under controlled conditions, only exposed tyrosyl residues react with iodide radicals, generated either by the oxidant, chloramine-T (paratoluenesulfonyl chloramide), or the enzyme lactoperoxidase, giving monoiodotyrosine. Using 125-iodine, this study compared the reactive tyrosines in free and bound histones H₄ and H₃. The relative extent of iodination of these histones within (H₄) and outside (H₃) of the nucleosomes was measured after extraction and gel electrophoresis. Each of the histones was further analyzed for the extent of specific tyrosine iodination by separating the tryptic peptides by high voltage electrophoresis. The identity of the labeled peptide was determined by dansylation of the amino acids present in each hydrolyzed peptide. The results show that there is a difference in the conformational arrangement of these histones on chromatin and in the free forms, since in chromatin not all tyrosine residues are as accessible for iodination as in the denatured state. Residues 53 of histone H₃ for instance is more reactive than residues 28 and 58, indicating that the segments containing the latter residues are involved in either protein-DNA or protein-protein interactions. In histone H₄, preferential labeling of 2 of the 4 tyrosines present was also observed.

Sequence data from histones show that there is a statistically nonrandom distribution of basic amino acids in each of the histones H₄, H₂a, H₂b, H₃, H₄, and H₅ (Elgin and Weintraub, 1975). It can be calculated that such a distribution would limit the possibilities of conformational change of these proteins. Indeed, only a few definable configurations of histones can be derived (Chou and Fasman, 1974; Fasman et al., 1977). There is evidence suggesting that the configurations of histones in solution, and thus in the free state, and those in the bound state in chromatin interacting with other molecules such as DNA or other proteins, are different. The topography of these histones bound in chromatin has been studied extensively by several techniques. 125-Iodine, as a chemical probe, has been used to study histone H₃ in Xenopus laevis chromatin (Biroc and Reeder, 1976). It was concluded that 2 of the 4 tyrosyl residues in H₃ (tyrosines 72 and 98) are protected from reaction when this protein is bound to chromatin in the native form, but some of the residues may be preferentially exposed if the chromatin is exposed to media increasing in ionic strength. The reactivity of the tyrosyl residues corresponded to the degree of freedom afforded to H₃ during a stepwise extraction process. In these studies, chloramine-T was used as the oxidant. Since chloramine-T is a strong oxidizing agent, it is uncertain if the native conformation of a protein, particularly when it is involved in protein-protein or protein-DNA interactions, would not have been perturbed. Therefore, it is conceivable that the use of a milder catalyst for iodination would be desirable, to prevent disruption of the native protein conformation (Morrison and Schonbaum, 1976).

Different methods of iodination are not simply different ways of preparing the same electrophile (I⁻), there are instead fundamental differences among the mechanisms of iodination. The rate of deiodination of radioiodinated proteins varies with the method of iodination (Krohn et al., 1977). Iodine monochloride, chloramine-T, and the lactoperoxidase method yield largely 3-iodotyrosine with small amounts of other iodinated amino acids; however, the chloramine-T product spectrum varies with the chloramine-T:protein ratio. In terms of radioiodine incorporation, the efficiency of the different methods, from greatest to least, is found to be: electrolytic > chloramine-T > lactoperoxidase > iodine monochloride.

Lactoperoxidase has also been used in the probing of available tyrosines in proteins on cell membranes. It has been used successfully in the study of membrane proteins in intact cells (Phillips and Morrison, 1971; Hubbard and Cohn, 1972; Graham et al., 1975; Morrison and Schonbaum, 1976). Chloramine T and lactoperoxidase iodination techniques have also been used to probe the accessibility of envelope proteins of vesicular stomatitis virions (Moore et al., 1974). The glycoprotein and, to a lesser extent, the matrix membrane protein of intact vesicular stomatitis virions have been specifically iodinated by oxidation with chloramine T and the lactoperoxidase methods. The soluble or solid-state lactoperoxidase method of iodination has been used to probe the molecular morphology of Escherichia coli ribosomes (Michalski and Sells, 1975). From this study, it was concluded that the 30 S subunit undergoes a conformational change during its association with the 50 S subunit to form the 70 S monosome. The high molecular weight of these enzyme molecules prevents their entrance into the inner portion of the cell membrane; thus, only exposed tyrosyl residues are reactive. If chromatin also has a compacted subunit structure, then the structure of the histones should be amenable to an analogous study.

In this study, we have focused on two histones: H₃, the histone unique to nucleated erythrocyte chromatin, located outside of the nucleosome structure, and the corresponding H₄ histone, whose presence within the chromatin subunits is ubiquitous. Histone H₄ has been implicated as a major con-
Hs has been partially sequenced (Sautière et al., 1976) and shown to have only 3 tyrosine residues at positions 28, 53, and 58. The dim and diffuse fluorescent patterns in the early embryonic erythroid cells change to a bright halo distributed peripherally to the inner nuclear membrane of adult erythrocytes (Mura et al., 1978). The changing Hs staining pattern within the nucleus may well reflect the supramolecular structural organization of the chromatin during various stages of gene activity. Histone Hs has been partially sequenced (Sautière et al., 1976) and shown to have only 3 tyrosine residues at positions 28, 53, and 58.

The sequence of Hs from chick erythrocytes is yet to be determined. However, Hs from different species examined so far shows a high degree of sequence homology with that from calf thymus which contains 4 residues of tyrosine at positions 28, 53, 58, 58, and 88 (Elgin and Weintraub, 1975). The results to be presented in this communication will demonstrate that the histone interact in the chromatin with other components with sequence specificity.

### MATERIALS AND METHODS

Chicken erythrocytes were obtained from Dover Poultry Products, Inc., Baltimore. Pooled blood was collected from the production line slaughter of chickens within 1 min of decapitation. During collection of the blood, 10% sodium citrate was added to minimize clotting. Reagents used in this study were obtained from the following sources: trypsin (TPCK, 247 units/mg of protein, Lot 38B789) from Worthington, lactoperoxidase (lyophilized powder, 58 units/mg of protein, Lot 86C-9580) from Sigma, and Pronase R (Grade B, 45000 p.u./g, Lot 601137) from Calbiochem. Sodium 125 iodide (specific activity 11 to 17 mCi/g) was purchased from Amersham/Searle and stored at −20°C until use. All chemicals used for preparation of solutions, unless otherwise specified, were reagent grade purchased from Baker.

### Preparations of Chicken Erythrocyte Chromatin

All experiments were done at 4°C. Whole blood was washed in cold 0.14 M sodium chloride, 0.01 M sodium citrate (pH 7.0 to 7.6) and centrifuged at 5000 rpm for 15 min, respectively. The erythrocytes were resuspended in 0.14 M sodium chloride, 0.01 M sodium citrate, and strained through cheesecloth to remove coagulated material. Erythrocytes were then lysed with 1.2% saponin in cold 0.14 M sodium chloride, 0.01 M sodium citrate, and centrifuged at 12000 rpm for 30 min. The lysate was allowed to stand for 30 min during which time samples were examined for cell lysis under a microscope. The lysate was centrifuged to remove the saponin and washed 10 to 12 times with cold 0.14 M sodium chloride, 0.01 M sodium citrate. The preparation was then resuspended from the crude nuclear homogenate by suspending in an excess of 0.075 M sodium chloride, 0.027 M disodium ethylenediaminetetraacetic acid (Sigma), pH 8.0, and centrifuging at 6500 rpm for 15 min. This was repeated once more followed by stepwise reduction in ionic strength and swelling in ice cold distilled water, pH 8.0. Chromatin was stirred into solution at a concentration of 1 mg of histone protein/ml. The solution was checked for true solubility by attempting to pellet by centrifugation at 10000 rpm for 30 min.

### Extraction and Purification of Histones Hs and Hs

Mixtures of histones Hs and Hs were obtained by slowly adding cold 10% perchloric acid dropwise to trypsinized chromatin to a final concentration of 5%; the supernatants, after a high speed centrifugation (12000 × g, 20 min), were pooled and exhaustively dialyzed against distilled water and lyophilized. Histone Hs and Hs were separated using an Amberlite CG-50 ion exchange column (2.5 x 30 cm). Mixtures of histones Hs and Hs in 8% guanidinium chloride (Matheson, Coleman, and Bell Co.), 10 mM sodium phosphate, pH 6.8, were applied and eluted with a 60% GuCl gradient from 8 to 20%. Elution of the erythrocyte histones was monitored by trichloroacetic acid precipitation and refractile index measurement of guanidinium chloride concentration. Histone Hs eluted at 12.5% GuCl and histone Hs at 17.8% GuCl. Fractions of each histone were pooled, exhaustively dialyzed, and lyophilized. The purity of histone was tested by gel electrophoresis and the products stored at −20°C.

### Method of Protein Determination

Quantitation of histones in chromatin is given on a milligram of total histone protein/ml basis. The Lowry method of protein determination (Lowry et al., 1951) was used for quantitation. Total acid-extracted chicken erythrocyte histone was used as a standard for the assay.

### Gel Electrophoresis

15% SDS-Gel Electrophoresis: Laemmli-Weintraub SDS Slab Gel System for Histone Separation—Polyacrylamide gel electrophoresis was done using a Hoefer model 220 slab gel electrophoresis apparatus. Total histone samples were separated and analyzed using 15% polyacrylamide sodium dodecyl sulfate slab gels. The slab gels (15 x 11 cm) were prepared according to the method of Laemmli (1970). The running buffer was made with an acrylamide: methylenebisacrylamide ratio of 75:1, and the running buffer was 0.38 M glycine, 0.05 M Tris (pH 8.3) (Weintraub et al., 1975). Acrylamide, methylenebisacrylamide, and N,N,N',N'-tetramethylethylenediamine were purchased from Eastman. Tris-HCl and sodium dodecyl sulfate were purchased from Sigma.

Samples containing 10 to 50 pg of protein in a volume of 10 pl or less were boiled 1 min in 10 µl of denaturation buffer before loading onto the gel. The denaturation buffer consisted of 4% SDS (w/v), 10% glycerol, 1% mercaptoethanol (Sigma), 0.01% bromphenol blue (Sigma), 0.0125% Tris buffer, pH 6.8. The electrophoresis buffer consisted of 50 mM Tris base (Sigma), 0.18 M glycine (Sigma), pH 8.3, 0.1% SDS. The gel running time was approximately 4 h at 120 V (continuous voltage). Gels were stained in 0.1% Coomasie Brilliant Blue (Sigma), 50% methanol, 10% acetic acid, and destained in 10% acetic acid, 7% methanol solution. Gels were photographed while still wet, then dried down onto a piece of filter paper.

15% Polyacrylamide-Acetic Acid-Urea Slab Gel System—Total histones were also separated using a 15% polyacrylamide-acetic acid-urea slab gel system. The acrylamide gels (15 x 11 cm) were prepared by the method of Panyim and Chalkley (1969) with the modification that slab gels were used instead of tubular gels. Urea was purchased from Sigma.

Samples containing 10 to 80 µg of protein in a volume of 20 µl or less were mixed with 15 µl of 0.1% Pyronin Y in 0.9% acetic acid, 5% glycerol. The electrophoresis buffer consisted of 0.9% acetic acid. Gel running time was approximately 6 h at 150 V (continuous voltage).

### Quantitation of Histones in Gels—Quantitation of histones within gels was based on a linear relationship between protein and Coomassie Blue (C-250) staining intensity. Under the staining and destaining conditions described for sodium dodecyl sulfate- and acid-urea polyacrylamide slab gel electrophoresis, histone Hs exhibits a linear relationship up to 25 µg of total histone protein, and histone Hs exhibits a similar relationship up to 35 µg of total histone protein. When specific bands were cut from the gel and counted for radioactivity, the specific activity of the histone protein was determined.

### Elution of Protein Bands from SDS- and Acid-Urea-Polyacrylamide Slab Gels—Histones are eluted from acetic acid-urea polyacrylamide slab gel electrophoresis, histone Hs exhibits a linear relationship up to 25 µg of total histone protein, and histone Hs exhibits a similar relationship up to 35 µg of total histone protein. When specific bands were cut from the gel and counted for radioactivity, the specific activity of the histone protein was determined.

### Quantitation of Radioactivity—Autoradiography of dried polyacrylamide slab gels from high voltage electrophoresis paper was done using Kodak RP-54 No-screen x-ray film, and development was...
allowed to proceed for an exposure time varying from 1 h to 2 weeks. For gels or paper sheets with low radioactivity, a DuPont Cronex Hi-Plus intensifying screens (8 x 10 inches) were used to decrease the exposure time.

125-Iodine was counted in a Packard Auto-Gamma scintillation spectrometer and a Beckman Gamma 4000 counter. The counting efficiency of both units was 70 to 75%. For high voltage electrophoresis paper with exceptionally low radioactivity associated with it, strips (1 x 3 cm) were cut out and counted individually.

Chloramine-T Iodination—The procedure used for iodination by chloramine-T (paratoluenesulfonyl chloramide) was that of Biroc and Reeder (1976), except that the order of addition was altered to maximize iodine incorporation. Proteins were iodinated in 6-cm screw-top reaction bottles. The reaction mix was constantly swirled using magnetic flasks. The reaction mix, with appropriate order of addition of reagents, was as follows: 1) up to 100 µg of histone protein in 100 µl of water; 2) 100 µl of 0.1 M Tris, pH 8.0; 3) 2 µl of 125I-sodium in dilute NaOH, 100 µCi/ml; 4) 5 µl of 0.12 mM KI (0.6 nmoles); 5) 6 µl of 1 M tyrosine (6 nmoles, Mann); 6) 10 µl of 7 mM chloramine-T (70 nmol, Eastman). The reaction was terminated by the addition of 10 µl of 21 mM sodium metabisulfite (210 nmol). Acid-insoluble protein was then removed by the addition of 20 µl of 4 N H2SO4 and placing the vial in an ice bath for 30 min.

Lactoperoxidase Iodination

Coupling of Lactoperoxidase to Sepharose 4B—Sepharose 4B was activated with cyanogen bromide according to the method of Cuatrecasas. Lactoperoxidase was coupled to the activated Sepharose (Pharmacia) at a final concentration of 2.3 mg of enzyme/ml of packed beads. The mixture was exposed to 0.2 M glycine, 0.01 M phosphate, pH 7.5, at 4°C for 3 h, to saturate any remaining reactive groups. The beads were then thoroughly washed in 10 M Tris buffer and stored in distilled water/10−5 M Merthiolate, pH 7.5.

Iodination—The method of David and Reesfeld (1974) was followed. Iodinations catalyzed by lactoperoxidase were carried out in 1-ml plastic microfuge tubes (Eppendorf). The lactoperoxidase-Sepharose beads were washed three times with 10 M Tris, pH 7.0, prior to iodination to remove Merthiolate. For chromatin, the desired volume of beads (2 to 15 µl) was added to the protein solution (250 µl) in Tris buffer, pH 7.5, followed by 2.5 µl of potassium iodide solution (2 mM) to give a final concentration of iodide approximately equal to 10−5 M. Then, 2 µl of sodium 125-iodide (100 µCi/µl) was added to the reaction mix. Iodination was initiated by the addition of 15 µl of hydrogen peroxide to give a final concentration of 9 × 10−5 M. Additional increments of 5 µl of hydrogen peroxide were then added every 5 min for samples undergoing various reaction time intervals. The concentration of hydrogen peroxide in 3% commercial preparations was determined by measuring A260 and an assumed extinction coefficient of 72.4 (Phillips and Morrison, 1971). The reaction was terminated by the addition of 10 µl of 2.5 M sodium azide. After addition of 56 µl of 4 N sulfuric acid, lactoperoxidase-containing beads and acid-insoluble protein were removed by centrifugation in a Fisher analytical microfuge at 7000 rpm for 5 min.

Separation of Unreacted 125-Iodine from Iodinated Histone Proteins—For protein samples greater than 1 mg, dialysis was employed. The reaction mixture was transferred to dialysis tubing and exhaustively dialyzed for 6 h. Supernatant samples containing less than 1 mg of protein from enzymatic or chemical iodination were applied to a Bio-Gel P30 molecular sieve column (Bio-Rad) to separate iodinated histones from free 125I (Biroc and Reeder, 1976). The column, consisting of a Pasteur pipette with 1.5 ml of bed volume, was equilibrated with 30% acetic acid and, to avoid loss when desalting small amounts of protein, 1.0 ml of 0.36% Triton X-100 was passed through the column first. The elution profile of the Bio-Gel column is such that histones elute at the void volume and free 125I at the included volume. Fractions from the column were collected manually in siliconized test tubes (75 x 8 mm); 100-µl fractions were assayed for protein and free 125I peaks by removing 50-µl aliquots and counting in a Beckman γ counter.

Tryptic Digest of Histones—Both histones H1 and H2 were iodinated when bound to native chromatin, and unbound histones H1 and H2 controls were digested in the same manner; in the case of iodinated, bound histones H1 and H2, samples were dialyzed and lyophilized to remove 66% acetic acid. The residue was dissolved in 100 µl of 0.1 M ammonium bicarbonate, 1 M urea digest buffer, pH 8.5 to 9.0, and trypsin was added at an estimated ratio of either 1:100 or 1:10 (trypsin:histone). The digestion was done at 37°C for 24 h in sealed 100-µl capillary pipettes (Corning).

Tryptic Digest Peptide Separation: pH 3.5 High Voltage Paper Electrophoresis—Histones H1 and H2, from iodinated chromatin and unbound iodinated H1 and H2, were subjected to tryptic digestion, the fragments being separated by high voltage paper electrophoresis. One-dimensional high voltage paper electrophoresis was done using a Gilson model D instrument. The entire digest sample was streaked at an origin 11 cm from the end of Whatman 3MM chromatographic paper (234 x 57 cm). Aliquots of 10 µl were streaked and dried using a hot air dryer. The paper was moistened with pH 3.5 buffer (5% acetic acid, 0.5% pyridine, Eastman), blotted to remove excess buffer, and electrophoresed at 3000 V for 1½ h for histone H1, and 1½ h for histone H2.

Fig. 1 (left). The extent of iodination (125-Iodine incorporation) of chromatin by chloramine-T method. Chromatin was iodinated after various treatments: ●, free total histone extracted from native chromatin; ○, native chromatin homogenized in a Waring Blender; □, native chromatin sheared through an 18-gauge needle; △, native chromatin, untreated. All values were normalized with that obtained at 75 µg. Reaction was at room temperature for 30 min. See text for conditions.

Fig. 2 (center and right). Rate kinetics. Kinetics of 125-iodine incorporation in native chromatin and total histone extracted from native chromatin, comparing two methods of iodination: chloramine-T (A) and lactoperoxidase (B). The concentration of chromatin used for iodination (Part A, center) was: ●, 150 µg of total histone protein/ml; □, 300 µg/ml; ○, 500 µg/ml; △, 700 µg/ml. The concentration of free total histone (Part B, right) were: ●, 150 µg total histone protein/ml; □, 300 µg/ml; ○, 500 µg/ml; △, 700 µg/ml; □, 1 mg/ml.
Radioiodination of Histones H4 and H5

Identification of Monoiodotyrosine and Diiodotyrosine: pH 1.9 High Voltage Paper Electrophoresis—To determine the amount of monoiodotyrosine and diiodotyrosine in histones H4 and H5, a portion of each histone sample, using both iodination methods, was dissolved in 100 μl of distilled water, digested with 20 μg of pronase (protease R) for 5 h at 37°C, and the digest was electrophoresed at 4000 V for 45 min in pH 1.9 buffer (2.5% formic acid, 8.7% acetic acid). In this system, monoiodotyrosine and diiodotyrosine separate very well and migrate slower than tyrosine. Autoradiography compared with ninhydrin-sprayed standards of L-tyrosine (Mann Research Labs), monoiodotyrosine (gift of R. M. Herriott, The Johns Hopkins University), and diiodotyrosine (K and K Laboratories), showed that the monoiido-derivative was the major labeled product.

Tryptic Peptide Identification for Histone H5, Dansylation—For dansylation of amino acids, 5 to 50 nmol of peptide were extracted from the H5 histone maps. Spots on the chromatography paper corresponding to those identified by autoradiography were cut out, shredded, and the mixture was incubated for 12 h with saturated triethylammonium carbonate solution, pH 11, at room temperature. Similar peptides were pooled for this procedure. The slurry was then forced through a 50-ml syringe, the nozzle of which was covered with nylon mesh screen to prevent particulate matter from passing with the solution. Samples were lyophilized and hydrolyzed with 50 μl of 6 M hydrochloric acid for 18 h at 110°C in a sealed test tube.

Histones were iodinated for 30 min using chloramine-T or lactoperoxidase, and proteins were dialyzed exhaustively to remove unreacted 125-iodine. Individual histones were separated by SDS-polyacrylamide gel electrophoresis; protein bands were cut out, and quantitation of radioactivity done by γ counting. Figures represent the relative extent of 125-iodine incorporated in individual histones.

### Table I

|          | H1 | H2α | H3 | H4 | H5 | Σ |
|----------|----|-----|----|----|----|---|
| Free state |    |     |    |    |    |  
| Chloramine-T | 3.9 | 28.1 | 10.7 | 48.6 | 8.7 | 100 |
| Lactoperoxidase/4B | 3.0 | 35.0 | 13.0 | 44.0 | 5.0 | 100 |

| Bound state |    |     |    |    |    |  
| Chloramine-T | 22.2 | 40.2 | 8.2 | 45.2 | 4.2 | 100 |
| Lactoperoxidase/4B | 27.0 | 14.0 | 29.0 | 11.0 | 19.0 | 100 |

Predicted percentage iodination = 100 × (α/2(ε)) × (β × α), where ε = total histone (H1, H2α, H3, H4, H5) and α = (21,000/M, of histone) × (β × α).
were again lyophilized and counted, and 10 µl of 0.2 M sodium bicarbonate was added to each. The sample was thoroughly mixed
and lyophilized, and 10 µl of distilled water plus 10 µl of dansyl-
chloride (2.5 mg/ml acetone, Pierce) were added to the reaction mix.
The sample tube was then evacuated under a stream of nitrogen and
sealed. The reaction was allowed to proceed for 1 h at room temper-
ature.
Peptide Identification by Thin Layer Chromatography—Samples
were lyophilized over sodium hydroxide pellets and dissolved in 10
µl of acetic acid:acetic acid (3:2, v/v). Spotting was 1 cm in on both sides
of Polyamide P6 (Macherey-Nagel, Inc.) or Micropolyamide F1700
(Schleicher & Schuell) thin layer plates (7.5 × 7.5 cm). The first
dimension solvent was formic acid:water (3:200, v/v), and the second
dimension solvent was benzene:acetic acid (9:1, v/v). The plates were
air-dried and heated to 100°C for 1 min to enhance fluorescence under
ultraviolet light. Individual amino acids comprising the peptides were
treated in the same manner, as standards.

RESULTS
The extent of iodination of histones varies considerably,
depending on whether they are in the free state or bound with
other nucleoproteins in the chromatin matrix. Total histones
extracted from chicken erythrocyte chromatin were subjected
to iodination, and the extent of labeling compared with those
on the chromatin at an equivalent range of concentrations.
The results in Fig. 1 show that bound histones are much less
accessible to iodination than the free ones. At an equivalent
total histone concentration, only 15% of the iodinatable resi-
dues are available when bound in the chromatin. Homogeni-
zation and sonication of the chromatin, however, increase the
extent of iodination. The conditions used for shearing the
chromatin were those generally applied to chromatin prepa-

Fig. 5. Autoradiography. Effect of increasing trypsin concentration
in histone H, proteolytic digestion. H, was iodinated using the
chloramine-T method. Varying enzyme/substrate (E/S) ratios (w/w)
were used to empirically determine optimal digest conditions: 1, E/S
= 1:50; 2, E/S = 1:25; 3, E/S = 1:10; 4, E/S = 2:1. The ratio producing
three distinct peptide fragments was E/S of 0.10. H, digest was
separated by high voltage paper electrophoresis, pH 3.5 (5% acetic
acid, 0.5% pyridine).

The extent of iodination is described by the following formulas:

\begin{align}
R_{\text{bound}} &= \frac{[\text{bound}]_{\text{total}}}{[\text{total}]_{\text{bound}}} \\
R_{\text{free}} &= \frac{[\text{free}]_{\text{total}}}{[\text{total}]_{\text{free}}} 
\end{align}

where [bound] and [free] represent the concentrations of iodinated
histones in bound and free states, respectively, and [total] refers to
the total amount of histones.

Kinetics of Iodination—The extent of iodination as mea-
ured by radioactive iodine covalently bound to the free and
bound histones was studied kinetically by two procedures
using chloramine-T and lactoperoxidase. The results (Figs. 2,
A and B) show that a rapid incorporation is observed within
30 min following either procedure, after which the lacto-
peroxidase-catalyzed reaction diminishes to a plateau. In terms
of specific activities of iodinated total histone, the chloramine-
T method is approximately 100-fold more efficient than the
lactoperoxidase method. While the lactoperoxidase-catalyzed
reaction is less effective in the total extent of iodination, it is
affected prominently by the enzyme/substrate ratio in the
reaction. The kinetic experiments involving optimal iodination
time, using lactoperoxidase coupled to Sepharose 4B, are
consistent with the results of David and Reisfeld (1974).

Nature of the Iodination Product—In iodination, one or
more iodide radicals may be added to tyrosine generally
resulting in mono- and diiodotyrosine. If an even distribution
of these two forms exists on different histones and their
residues, an erroneous estimation would be made in the inter-
pretation of the results. Care was taken to check the nature of
labeling under the reaction conditions specified. The results
obtained by high voltage electrophoresis of the protease-R
digests of labeled histone H, indicated that monoiodotyrosine
was indeed the major product. Histidine, another possible site
of iodination, was not significantly reactive under the condi-
tions used. An example of the paper electrophoretogram of an
analysis is depicted by its corresponding autoradiograph in
Fig. 3.

Extraction of Histones from Native Chromatin—Total his-
tones were selectively extracted for further analysis to deter-
mine the extent of labeling of individual histones. These histones
remain intact after iodination as demonstrated by gel electro-
phoresis and autoradiography (Figs. 4, A and B).

Labeling Profile of Individual Histones when Bound to
Chromatin—The relative iodination efficiency of various histo-
tones was of particular importance to this study. The propor-
tion of histones recovered from these iodination procedures
was measured after separation of the extracted histones by
polyacrylamide gel electrophoresis. The bands were stained,
photographed, and densitometric tracings were made on posi-

tive transparencies from the photographic negatives for quan-
tification of the proteins. Afterwards, the gels were dried and
autoradiographed. Autoradiography, representing the radio-
activity of each band, was also quantitated by densitometric
tracing. The autoradiograph densitometry was confirmed by
cutting out the bands and counting directly for radioactivity.

The results are summarized in Table I. It can be seen that not
all histones on the chromatin are iodinated to the same
specific activity as those extracted from chromatin prior to
iodination. In chromatin, internucleosomal histones H2 and
H3 appear to be preferentially iodinated, along with intranu-

Iodinated histones H1, H2, and H3 were extracted from gels for
further analysis to determine the extent of labeling of their tyrosine residues at specific sites of their sequences. After 66% acetic acid extraction from gels at 40°C, the histones remained intact, as demonstrated by rerunning extracted samples on SDS-gels, sectioning the gels, and checking radioactivity for co-migration with standards of H4 and H5.

Tryptic Analysis of Iodinated Histones (E/S) Ratio Determination—Histones H4 and H5, free or bound to chromatin, were recovered after iodination for tryptic analysis. The optimal enzyme to substrate ratio for the hydrolysis was empirically determined by examining the digestion products by high voltage paper electrophoresis. As shown in Fig. 5, an E/S ratio of 1:10 yields three peptides carrying the radiolabeled tyrosines as expected from the H4 sequence. At a high E/S (of 2:1) or lower E/S (of 1:50) an anomalous peptide appeared. Within the range of E/S ratios tested, however, H5 yielded the same four major peptides and one additional minor one. For quantitation of the radioactivity in the peptides, an E/S ratio of 1:10 was thus chosen, but for the identification of the peptides an E/S ratio of 1:100 was chosen to reduce possible contamination of histone peptides by trypsin peptides resulting from autodigestion.

Identification of Labeled Peptides from Trypsin Digestion—There are three tryptic peptides from histone H5, predicted from previous sequence data which carry tyrosine (Sautiere et al. 1976). Their sequences are:

\[
\begin{align*}
&\text{Ser-Ala-Ser-Pro-Thr-Tyr-Ser-Glu-Ala-Ala-Ala-Ile-Arg} & (1) \\
&\text{Tyr-Ile-Lys} & (2) \\
&\text{Ser-His-Tyr-Lys} & (3)
\end{align*}
\]

In order to assign the proper identity to the three bands shown on the high voltage paper electrophoretogram for H5 (Fig. 5), the bands were excised and the peptides were eluted and subjected to complete acid hydrolysis. The resulting amino acids were dansylated and analyzed by two-dimensional thin layer chromatography along with standards containing a mixture of the respective dansylated amino acids.

By this procedure, the slowest migrating band was assigned to the peptide containing tyrosine residue 28 (peptide digest 1, top), the middle one (peptide digest 2) containing tyrosine 53, and the fastest migrating one (peptide digest 3), tyrosine 58 (bottom). This is evidenced by the unique presence of the

![Fig. 6. Histone H3 tryptic peptide identification. Individual histone tryptic peptides (E/S of 0.01) from chloramine-T experiments were eluted and pooled for hydrolysis. Polyamide thin layer chromatography (first dimension, formic acid:water, 300:2; second dimension, benzene:acetic acid, 9:1) was used to identify characteristic dansyl-amino acids present in the hydrolysate. Peptide digest 1 (top) contains the characteristic amino acids of the peptide associated with Tyr 28: Ala, Pro, Ser, His, and Ile. Similarly, peptide 2 (middle) contains characteristic amino acids Ile and Lys and peptide 3 (bottom) contains His, Ser, and Lys that identify peptides 2 and 3 as those peptides associated with Tyr 53 and Tyr 58, respectively. Autoradiography of the three peptide digests shows one major spot (arrows) corresponding to monodansyl-monoiiodotyrosine. In peptide digest 2, a second spot, believed to be a didansyl product, is observed. Amino acid spots have been labeled with numbers for identification: 1, isoleucine; 2, proline; 3, alanine; 4, serine; 5, histidine; 6, lysine.](http://www.jbc.org/)}
Radioiodination of Histones H4 and H5

FIG. 7. Electrophoretic elution of chicken erythrocyte H4 histone by acetic acid-urea polyacrylamide gels. Total histone, acid-extracted from native chromatin, was separated and eluted from the gel, the fractions being collected and assayed by SDS-gel to locate those which contained histone H4. Samples on the extreme left and right are standard total histone markers. The fraction above the arrow was identified as H4 and used for further studies.

FIG. 8. Calf thymus (CT) and chicken erythrocyte (CE) histone H4 tryptic peptide identification by dansylation. Individual histone tryptic peptides (E/S of 0.01) from chloramine-T experiments were eluted and pooled for dansylation. Polyamide thin layer chromatography (first dimension, formic acid:water, 30:70; second dimension, benzene:acetic acid, 9:1) was used to identify the characteristic dansylated NH2-terminal amino acid present (circle). Peptide 1 (left) alanine residues in: the peptide containing Tyr 28 (Fig. 6, peptide 1); both Ile and Lys in the middle band (Fig. 6, peptide 2); and Ser, His, and Lys, but not Ala and Ile, in the fastest migrating band (Fig. 6, peptide 3). The autoradiograms (Fig. 6, 1A, 2A, and 3A) also show that a single radioactive spot (arrow) corresponding to dansylated monooiodotyrosine exists in all three peptides, further supporting the earlier analysis (Fig. 3) that iodoxyrosine was the major iodination product. The extra radioactive spot in the peptide containing tyrosine residue 53 at the NH2 terminus may be a didansylation product of monooiodotyrosine according to its mobility in the two solvent systems used here.

Identification of Histone H4 Tryptic Peptides from Calf Thymus and Chick Erythrocytes—Other studies have suggested a high degree of homology in the amino acid sequences of H4s from various species of plants and animals (DeLange et al., 1969). Histone H4s from Xenopus laevis and calf thymus both show identical iodinated tryptic peptides by their mobil-
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Chicken erythrocyte H4 was purified by electrophoretic elution (Fig. 7) and its tryptic peptides identified along with those from calf thymus H4. In this case, the dansylated NH2 termini were analyzed. The NH2-terminal amino acids of the four radiolabeled calf thymus (CT) tryptic peptides are Ile (peptide 1), Thr (peptides 2 and 3), and Asp (peptide 4). The NH2-terminal dansyl amino acids for chicken erythrocyte (CE) H4 are shown to be Ile (peptide 1) and Thr (peptides 2 and 3), and Asp for peptide 4 inferred. The results are shown in Fig. 8. With this information, the four major iodinated peptides for chicken erythrocyte histone H4 were assigned (migration towards cathode):

1. -Ile-Ser-Gly-Leu-Ile-Tyr-Glu-Thr-Arg- (1)
2. -Thr-Val-Thr-Ala-Met-Asp-Val-Tyr-Ala-Leu-Lys- (2)
3. -Thr-Leu-Tyr-Gly-Phe-Gly-Gly-COOH (3)
4. -Asp-Ala-Val-Thr-Tyr-Thr-Glu-His-Ala-Lys- (4)

However, the mobilities of the chicken erythrocyte H4 peptides containing tyrosine differ considerably from purified calf thymus H4 digest peptides under identical electrophoresis conditions (Fig. 9). Since the nature of this difference is not known, it could be due to a genuine heterogeneity, or, more likely, a difference in modification. The sequence assignment is thus tentative.

Quantitation of Monoiodotyrosine in Labeled Peptides—
With the tryptic peptides from H4 and H5 identified and the iodination conditions set forth, the ratios between radioactivity in these peptides after various iodination reaction times were compared (Figs. 10 and 11) and the quantitative results of all comparisons are summarized in Tables II and III.

Fig. 10A shows that the H4 peptide containing Tyr 53 was preferentially iodinated when this histone was bound to native chromatin. This is in contrast to the same histone labeled in

![Fig. 10](http://www.jbc.org/)

**Fig. 9.** Tryptic peptides from free histone H4 from chicken erythrocytes (CE) and calf thymus (CT) labeled identically with 125I by the chloramine-T method were separated on the same electrophoresis run. (See Fig. 5 and text for conditions.) The paper electrophoregram was cut and counted after the separation. The results are shown in histograms.

![Fig. 10](http://www.jbc.org/)

**Fig. 10** (left and center). Autoradiography of tryptic digest (E/S of 0.01) of histone H4 iodinated by chloramine-T method when free and bound to native chromatin. Tryptic peptides of samples iodinated for different reaction times were separated by high voltage paper electrophoresis, pH 3.5 (0.5% pyridine, 5% acetic acid). The reaction times for H4 bound to native chromatin were: 0 min; 15 min; 30 min; 60 min; and 120 min. The relative radioactivity of the three tyrosine-containing peptides is shown here in Part A, (left) for the 15-, 30-, and 60-minute samples in histogram form. Peptides 1, 2, and 3 contain tyrosines 28, 53, and 58, respectively (see Fig. 6). Those for free histone H4 (Part B, center) were shown autoradiographically: 1, 15 min and 2, 120 min. 3, a carrier-free 125-iodine marker and (I-TYR, box) monoiodotyrosine marker are also shown.

![Fig. 11](http://www.jbc.org/)

**Fig. 11** (right). Autoradiography of tryptic digest (E/S of 0.01) of histone H4 iodinated by chloramine-T method, when bound to native chromatin (NC). Tryptic peptides of samples iodinated for different reaction times were separated by high voltage paper electrophoresis, pH 3.5 (pyridine/aceticate). The reaction times were: 2, 15 min; 3, 30 min; 4, 60 min; 5, 120 min. The relative radioactivity is summarized in Table III.
the free state shown in the autoradiogram in Fig. 10B. The extent of iodination of the tyrosine residues in each of the three peptides was approximately equal for unbound Hs, but there is a two fold difference between Tyr 53 and the 2 residues Tyr 28 and 58. With increasing time of reaction, Tyr 58 became more accessible to iodination than Tyr 28 when lactoperoxidase was used as the catalyst in the iodination reaction (Method B, Table II). In contrast, the chloramine-T reaction, while favoring the labeling of Tyr 53, did not give the same results for the other residues (Method A, Table II). The changes in profile of label with time are noted in Table II.

Fig. 11 shows a distinct change in labeling profile in the major tryptic peptides for Hs bound to chromatin. This is in contrast to the relatively uniform labeling of all four peptides, through the respective tyrosines they carry, in free Hs (Fig. 9 and Table III). Peptide 2 specifically can be seen to increase in labeling with reaction time, although iodinated only to about one-tenth of peptides 1 and 4 (Table III). Similarly, the tyrosine residue in peptide 3 was also iodinated to a lesser extent throughout the reaction.

**TABLE II**

*Extent of iodination of tyrosine residues in chick erythrocyte histone Hs, in native chromatin and unbound states*

| Tyrosine number | Reaction time (min) | A | B | A | B | A | B | A | B |
|-----------------|---------------------|---|---|---|---|---|---|---|---|
|                 | 15                  | 30 | 60 | 120 | 15 | 30 | 60 | 120 |
| Unbound         |                     |    |    |    |    |    |    |    |    |
| 28              | 1.1                 | 1.1 | 1.3 | 1.3 | 1.6 | 1.6 | 1.6 | 1.6 |
| 53              | 1.1                 | 1.1 | 1.1 | 1.1 | 1.2 | 1.2 | 1.2 | 1.2 |
| 58              | 1.0                 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Bound           |                     |    |    |    |    |    |    |    |    |
| 28              | 0.94                | 1.5 | 1.7 | 0.50 | 2.1 | 0.34 | 1.2 | 0.61 |
| 53              | 1.6                 | 2.9 | 1.8 | 2.0 | 2.0 | 1.4 | 1.2 | 1.9 |
| 58              | 1.0                 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |

**TABLE III**

*Extent of iodination of tyrosine residues in chick erythrocyte histone Hs, in native chromatin and unbound states*

| Assumed tyrosine number | Peptide | Reaction time (min) | A | B | A | B | A | B | A | B |
|-------------------------|---------|--------------------|---|---|---|---|---|---|---|---|
|                         |         | 15 | 30 | 60 | 120 | 15 | 30 | 60 | 120 |
| Unbound                 |         |    |    |    |    |    |    |    |    |
| 51                      | 1       | 1.0 | 0.95 | 0.85 | 0.95 | 0.85 | 0.95 | 0.85 | 0.95 |
| 88                      | 2       | 0.82 | 0.90 | 0.90 | 0.95 | 0.90 | 0.95 | 0.90 | 0.95 |
| 98                      | 3       | 1.1 | 0.95 | 0.95 | 1.1 | 0.95 | 1.1 | 0.95 | 1.1 |
| 72                      | 4       | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Bound                   |         |    |    |    |    |    |    |    |    |
| 51                      | 1       | 0.80 | 1.4 | 0.74 | 1.3 | 0.71 | 1.3 | 0.95 | 1.2 |
| 88                      | 2       | 0.12 | 0.70 | 0.11 | 0.63 | 0.15 | 0.68 | 0.57 | 0.71 |
| 98                      | 3       | 0.33 | 0.69 | 0.46 | 0.40 | 0.48 | 0.51 | 0.56 | 0.59 |
| 72                      | 4       | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |

**DISCUSSION**

In this study, the accessibility of tyrosine residues in histones from chicken erythrocytes was analyzed. The major finding is that differences in the accessibility of various tyrosine residues in different regions of a histone sequence on the chromatin may be assessed by either chloramine-T or lactoperoxidase iodination. Purified histones are more susceptible to iodination, whether using chloramine-T (Fig. 1) or lactoperoxidase as a catalyst, than histones bound to chromatin. Chromatin in the native form showed fewer reactive tyrosine residues than sheared chromatin (Fig. 1). These results suggest that some tyrosine residues are either buried or involved in interaction with other proteins and that shearing of chromatin changes their native configuration.

The extent of iodination for histones free in solution is not only greater than that for chromatin-bound histones, but it differs qualitatively as well (Table I). With use of the lactoperoxidase method, the tyrosine residues in Hs, Hs, and Ht are labeled more than those of Hs, Hs, and Hs when bound to chromatin. If Hs and Hs are located outside the nucleosomal core (i.e. in the spacer region), one would expect them to be more accessible to iodination than the histone core. However, since Ht is within the core, its high reactivity was surprising. It is possible that portions of Hs containing the reactive tyrosines extend outside of the nucleosomes and serve as reactive sites for iodination.

The size of the oblate nucleosome structure has previously been estimated at 100 × 50 Å. For this size, it would seem a priori that a large molecule like lactoperoxidase (~60 Å diameter) would have difficulty penetrating the chromatin matrix. Lactoperoxidase-catalyzed iodination, while superior to chloramine-T in terms of mildness, must be carefully controlled as I2 generated from iodide may nevertheless penetrate a compact structure. We have used an excess substrate to minimize the ratio as recommended by Morrison and coworkers (1975) to avoid this problem. Whether lactoperoxidase will react with a nucleosome and induce change in its conformation is not known; however, no detectable changes have been observed with membrane proteins.

The use of 125-iodine to probe protein conformation has been used to study histone Hs in Xenopus laevis chromatin (Biroc and Reeder, 1976) and the present results extend the applicability to both histones Hs and Hs in another chromatin system. The data obtained in this laboratory with chicken erythrocyte Hs, although comparable, can not be directly compared with those of earlier works since a complete sequence of neither X. laevis nor chicken Hs has been determined. It is significant that in both systems only 2 of the 4 tyrosine residues of Hs on chromatin are relatively available for iodination. While many differences exist between the chromatin of Xenopus cells and chicken erythrocytes, notably the lack of transcriptional activity, longer nucleosomal DNA, and the presence of Hs in nucleated chicken erythrocytes, the same basic nucleosomal structure in which a specific Hs conformation plays a role must be there. Our studies imply that those tyrosine residues not readily accessible for iodination in the chromatin are involved in interaction with DNA and with other chromosomal proteins.

It can be pointed out that this is the first study showing a conformational difference between free and bound histone Hs by radioiodination. Hs, being internucleosomal, is expected to be more available for iodination than the intranucleosomal histones such as Hs. This is generally confirmed (Table I). The meaning of this general quantitation, however, could be...
obscured unless the exact profile of iodination of the individual tyrosine residue is known. In this study, tyrosine 53 of histone H4 was apparently more accessible than 28 and 58 (Fig. 1A). Unfortunately, the relative availability between tyrosine 28 and 58 can not be more accurately determined due to the inconsistent results obtained by two procedures of iodination.

The present study shows that procedures involving either chloramine-T or lactoperoxidase are comparable in the initial labeling profile, although the efficiency of the two differs 100-fold in order of magnitude. The fact that only tyrosine residues were monitored in this study was considered. It has been shown that tryptophan, methionine, and cysteine residues may also be iodinated at high pH (Bayse et al., 1972). The reaction conditions used here preclude such a possibility. Furthermore, the iodination of tryptophan may involve breakage of its aromatic ring; however, tryptophan is notoriously absent in histones.

The differences in labeling profiles for H5 using the chloramine-T and lactoperoxidase methods, and uniformity of profiles for H4 histone, are highly suggestive of the following model: since H5 is an internucleosomal histone, it is more susceptible to iodination as suggested by our kinetic data. The chloramine-T method may disrupt H5 histone structure, particularly in the region containing tyrosine 28, making it more accessible to iodination; however, H4, by virtue of its interaction with histones in the nucleosomal matrix or by being deeply buried, assumes a more stable configuration and is not readily disrupted by the oxidant.

A systematic monitoring of titratable or reactive amino acid residues of various histones in the chromatin, in its native or reconstituted states, should add more knowledge to the understanding of chromatin structure.

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