

GLUTARALDEHYDE FIXATION OF ISOLATED EUKARYOTIC NUCLEI

Evidence for Histone-Histone Proximity

DONALD E. OLINS and EVERLINE B. WRIGHT

From the University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences and the
Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

ABSTRACT

Isolated chicken erythrocyte nuclei have been incubated with dilute concentrations of the bifunctional cross-linking agent glutaraldehyde (0–20 mM) in order to stabilize histone-histone interactions within the native nucleus. The kinetics of the disappearance of acid-soluble histones, free amino groups, and of individual histones have been observed to be pseudo first-order. Apparent first-order rate constants for the disappearance of individual histones correlate with the lysine mole percent of that fraction and follow the ranking, $k_{app} : F1 > F2C > F2B \geq F2A2, F2A1, F3$. Histone polymers were observed to form very rapidly during the fixation reaction. Partial fractionation and amino acid analyses of these polymers support the view that they are composed principally of cross-linked $(F2C)_n$ molecules (where $n = 2$ to ~ 8). The rate of glutaraldehyde reaction with free amino groups in histones is drastically reduced in solvents that promote chromatin decondensation (i.e., low ionic strengths in the absence of divalent cations) whereas the formation of cross-linked F2C polymers is less severely reduced. It is proposed that some F2C histones exist in close proximity within the isolated erythrocyte nucleus.

INTRODUCTION

The use of bifunctional reagents (Wold, 1967) to study the spatial arrangements of protein subunits in oligomeric proteins and in supramolecular structure (e.g., ribosomes and cell membranes) has increased rapidly over the past few years. As an example, ribosomal structure is stabilized by glutaraldehyde fixation (Subramanian, 1972) and it is believed that the most reactive proteins expose lysyl groups to the outside of the ribosome (Kahan and Kaltschmidt, 1972). The purpose of the present study is to extend this type of chemical approach to isolated eukaryotic nuclei, in an effort to demonstrate spatial arrangements among chromosomal proteins.

MATERIALS AND METHODS

Reagents

Glutaraldehyde was purchased from Polysciences, Inc., Warrington, Pa., as a neutralized aqueous 8% solution in glass ampules sealed under nitrogen. The glutaraldehyde solution was stored at 4°C and, after opening, was transferred to screw-cap culture tubes, gassed with nitrogen, and kept at 4°C for periods of up to several months. Such solutions appear to be quite stable in storage. The aldehyde content of several ampules was measured by titration with 2,4-dinitrophenylhydrazine (Mitchell, 1953); butyraldehyde was used as a standard. A freshly opened sample, and one stored for approximately 2 mo after opening,

deviated by less than 3% from the expected aldehyde concentration (i.e., expected concentration equal to 0.799 M, assuming a molecular weight of 100.12). Therefore all glutaraldehyde stock solutions were denoted as 8% or 0.8 M, and dilutions were made accordingly. All other chemicals were reagent grade (or better). Guanidine HCl (6 M) was prepared from guanidine carbonate as described previously (Olins and Olins, 1971). Iodoacetamide was obtained from K & K Laboratories, Inc., (Plainview, N. Y.). 2,4,6-Trinitrobenzene sulfonic acid was purchased from Eastman Organic Chemicals Div. (Eastman Kodak Co., Rochester, N. Y.). All buffers and solutions were made with glass-distilled water. Solvent conditions were similar to those employed in an earlier study (Olins and Olins, 1972), except that cacodylate buffer systems were substituted for tris(hydroxymethyl)-aminomethane buffers.

Preparation of Nuclei

Chicken erythrocyte nuclei were prepared as described previously (Olins and Olins, 1972) based upon the method of Zentgraf et al. (1969), except that the supersaturated sucrose solution was not employed. Instead, after three or four cycles of blending and centrifugation in the homogenization buffer, the nuclei were suspended in a mixture of 1 part homogenizing buffer and 2 parts 2.3 M sucrose buffer (0.01 M Tris [pH 7.0], 1 mM CaCl₂, 4 mM *n*-octanol) blended as described previously, and layered over 2.3 M sucrose buffer. The density interphase was broken several times with a glass rod. This procedure allowed the nuclei to pellet more reproducibly during the ultracentrifugation step than they did in the method previously described.

Nuclear pellets were washed and centrifuged twice in 40 ml of "CKM" (0.05 M cacodylate [pH 7.5], 0.025 M KCl, 5 mM MgCl₂, 0.25 M sucrose) or in "MgCl₂ buffer" (0.02 M cacodylate [pH 7.5], 5 mM MgCl₂, 0.25 M sucrose), resuspended in the washing buffer, and counted in a hemacytometer. DNA concentrations were calculated as previously described (Olins and Olins, 1972).

Glutaraldehyde Fixation and Extraction of Histones

Most experiments were performed in 5 mM MgCl₂ buffer. 1 ml samples containing nuclei equivalent to 2 mg DNA and 0–20 mM glutaraldehyde were fixed at 0°C, at times varying from 0 to 2 h. Glutaraldehyde was added at different times, and all tubes were centrifuged simultaneously for 5 min at top speed in a clinical centrifuge. Nuclear pellets were drained and 2 ml of ice-cold 0.25 M H₂SO₄ was added to each tube. Pellets were vigorously blended with a close-fitting spatula, allowed to sit in ice about 15 min, and

centrifuged as before. Three extractions with 0.25 M H₂SO₄ (total 6 ml) were pooled, 18 ml 95% ethanol was added, and the tubes were stored overnight in a freezer to precipitate histones. After centrifugation, histone pellets were dissolved in 2 ml of 0.01 N HCl and dialyzed extensively against 0.01 N HCl. After dialysis, all samples were brought to a constant volume and absorbancies were measured at 230 nm. Histone concentrations were calculated as milligrams per milliliter, assuming that 1 mg/ml whole histone possesses an A₂₃₀ of 3.5 (Bonner et al., 1967).

In several experiments, fixed nuclear pellets were extracted with 2 ml of 0.01 M Tris (pH 8.5), 2.0 M NaCl, 5.0 M urea, 1 mM NaEDTA, and 1 mM sodium bisulfite for 3 h at 0°C and centrifuged for 18 h at 50,000 rpm in a Spinco type 65 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Solvents of this composition have been shown to produce almost total extraction of nuclear histones and nonhistones (O'Malley et al., 1972). A portion of the supernate was dialyzed against 0.01 N HCl after centrifugation. No precipitation was observed at this point. Volumes of different samples were kept comparable throughout. After dialysis, samples were measured at A₂₃₀ nm.

The influence of solvent composition (and nuclear morphology) upon the rates and products of glutaraldehyde fixation was measured in two types of experiments. By one method, nuclei (equivalent to 2 mg DNA) were diluted into 10 ml of the chosen solvent, centrifuged, washed, recentrifuged, and resuspended in 1 ml of the final solvent. Nuclei in solvents of low ionic strength formed a gelatinous mass which resisted dispersion; therefore the nuclei were fixed as a gel pellet. By the other method, devised to prevent gelatinous pellets from forming, nuclei were diluted from a very concentrated stock solution (e.g., ~20 mg DNA/ml) into 10 ml of the chosen buffer, gently shaken, and fixed in this larger volume of solvent. Nuclear morphology was checked, and showed the expected changes with solvent composition (Olins and Olins, 1972).

Besides those mentioned, the following solvents were also employed: (a) 0.02 M cacodylate (pH 7.5), 0.2 M KCl; (b) 0.001 M cacodylate (pH 7.5), 0.02 M KCl; (c) 0.001 M cacodylate (pH 7.5), 0.002 M KCl; (d) 0.001 M cacodylate (pH 7.5). All solvents contained 0.25 M sucrose. In addition, several experiments utilized solvents containing 5 mM NaEDTA.

Gel Electrophoresis

Electrophoretic separation of histones was performed in 15% polyacrylamide gels (0.9 N acetic acid, pH 2.8, 2.5 M urea) as described by Panyim and Chalkley (1969 a, b). Gel dimensions were 9.0 × 0.5 cm. Electrophoresis was performed at ~6 mA/gel, 130 V, with voltage regulation. Methyl green was

used as tracking dye, and electrophoresis was terminated when the second dye band had reached the end of the gel tube. Gels were stained overnight in 0.1% naphthol blue black, 7% acetic acid, 20% ethanol, and destained in a diffusion destainer (Hoefer Scientific Instruments, San Francisco, Calif.).

Examination of glutaraldehyde cross-linked histones was performed in neutral 10% polyacrylamide gels containing sodium dodecyl sulfate (SDS) as described by Weber and Osborn (1969). Histone extracts in 0.01 N HCl or 2.0 M NaCl-5.0 M urea were dialyzed several hours against 0.01 M sodium phosphate buffer (pH 7.0) containing 1% SDS and 1% β -mercaptoethanol, then heated at 37°C for 2 h and dialyzed overnight against 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1% SDS and 0.1% β -mercaptoethanol. Samples were brought to constant volumes before electrophoresis. Gels were stained with either 0.1% naphthol blue black as described above, or for 1 h with 0.25% Coomassie blue (Weber and Osborn, 1969) followed by diffusion destaining. Molecular weights of the cross-linked histones were estimated by their relative electrophoretic mobility in alkaline (pH 10) SDS, 15% polyacrylamide gels, as described by Panyim and Chalkley (1971). These gels were stained as described for neutral SDS gels.

Gels were scanned at 570 nm with a Gilford 2000 automatic recording spectrophotometer equipped with gel scanner (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Bands were measured by xeroxing the gel scans, and cutting and weighing the separate peaks.

Isolation of Glutaraldehyde

Cross-Linked Histones

Gel filtration in 6 M guanidine hydrochloride (Fish et al., 1969) was employed in the separation of the acid-soluble glutaraldehyde, cross-linked histones. Nuclei (equivalent to 300 mg DNA) were fixed for 30 min at 0°C, at 2 mg DNA/ml, in 5 mM MgCl₂ buffer containing 4 mM glutaraldehyde; extracted 3 times with 0.25 M H₂SO₄; and histones were precipitated by the addition of ethanol. The resulting histone pellet was washed four times with 50 ml 100% ethanol, dried in a vacuum desiccator, and dissolved in 6 M guanidine HCl (pH 8.6) at a concentration of ~130 mg protein/ml. The solution was made 0.14 M β -mercaptoethanol, incubated 4 h at 37°C, made 0.3 M iodoacetamide, and dialyzed overnight against 6 M guanidine HCl (pH 6.5). 1 ml volumes of the reduced and alkylated histones, made 10% sucrose, were loaded onto a 90 × 1.5 cm column of Sepharose 6B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). 1- to 2-ml fractions were collected. The column flow rate varied from 3 to 5 ml/h. In several column runs, blue dextran and *ε*-2,4-dinitrophenyl (DNP)-lysine HCl were added as dye markers

for column void volume and total solvent volume, respectively. Columns were monitored by recording the absorbances for each fraction at 630 nm (for blue dextran), 360 nm (for DNP-lysine), and at 280 nm for protein. The presence of blue dextran interfered with measurements at 280 nm. A protein assay, in which fractions were spotted on filter paper squares and then stained with xylene brilliant cyanin G (ESBE Laboratory Supplies, Toronto, Canada), was utilized (Bramhall et al., 1969). The guanidine HCl is washed away by this procedure and does not interfere with the assay. Selected column fractions were pooled, diluted to 3 M guanidine HCl with water, and concentrated in Amicon ultrafiltration cells (Amicon Corp., Lexington, Mass.) with type UM-2 and UM-10 ultrafilters. Concentrated fractions were dialyzed against 6 M guanidine HCl (pH 6.5) and rechromatographed.

Assay of Free Amino Groups

The number of free amino groups in acid extracts of histones from control and glutaraldehyde-fixed nuclei was measured by titration with trinitrobenzene sulfonic acid as described by Habeeb (1966).

Amino Acid Analyses

Protein was hydrolyzed with 6 N HCl at 110°C for 21 h in sealed, evacuated (<50 μ m of Hg) tubes. The hydrolysates were concentrated to dryness on a rotary evaporator and analyzed with a Beckman 120C amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.) according to the method of Spackman et al. (1958).

RESULTS

Kinetics of Glutaraldehyde Fixation

When isolated chicken erythrocyte nuclei are incubated in 5 mM MgCl₂ buffer with low concentrations of glutaraldehyde (e.g., 3-7 mM) at 0°C, there is a gradual decrease in the amount of acid-extractable histones. If the reaction is stopped at various times over a 2 h period, and the acid-soluble histones are examined by gel electrophoresis, the differential rates of disappearance of the major histone classes can be readily visualized. Densitometric scans of several of these gels (Fig. 1 *a*) reveal a rapid disappearance of the F1 and F2C histone bands, and slower rates of disappearance of F2A1, F2A2, F2B, and F3 monomer and dimer. In addition, a new band (GLU-X) appears, migrating just ahead of the F3 dimer. Other weak-staining bands with

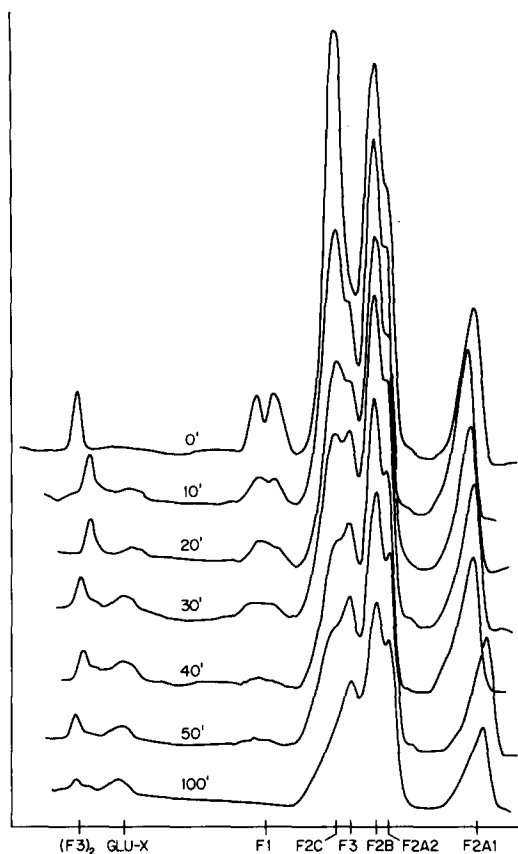


FIGURE 1a Densitometric scans of acid-urea gel electrophoresis of acid-soluble proteins from glutaraldehyde-fixed chicken erythrocyte nuclei. Solvent conditions during fixation: 3.3 mM glutaraldehyde, 0.2 M KCl, 0.25 M sucrose, 0.02 M cacodylate (pH 7.5). Origin is at left. Time, in minutes of total fixation time. Scans at 570 nm.

mobilities slower than $(F3)_2$ also appear during the fixation reaction.

The decrease in acid-extractable histones (as measured by A_{230nm}), and the disappearance of the different histone classes appeared to follow first-order kinetics (Fig. 1b). Numerous such studies, involving the use of short and long histone gels, revealed the following ranking of apparent first-order rate constants: $k(F1) > k(F2C) > k(F2B) \geq k(F2A1), k(F2A2), k(F3)$ monomer + dimer; $k(F1)$ was found to be 1.2–2.2 greater than $k(F2C)$; $k(F2C)$, 2.2–2.6 greater than $k(F2B)$; and $k(F2B)$, 1.1–1.2 greater than the rate constants for the other histones. The apparent rate constant for the disappearance of total histones, measured by the decrease in absorbance at 230 nm, was usually about equal

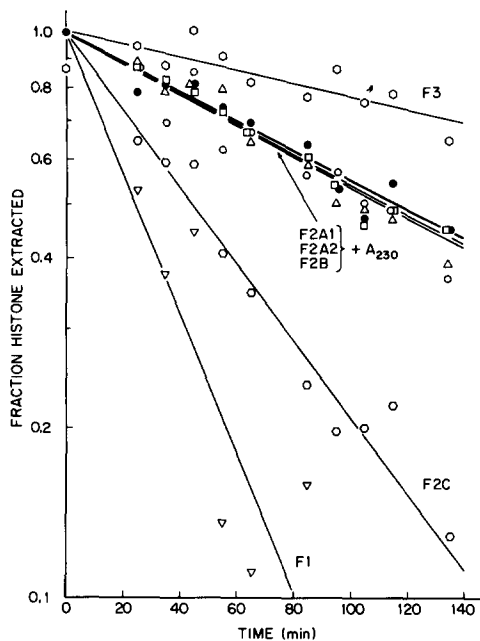


FIGURE 1b Semilog plots of disappearance of total acid-soluble proteins (i.e., A_{230nm}) and of individual histone fractions (i.e., peak areas from densitometric scans, Fig. 1a).

to the rate constants for F2A1, F2A2, and F3, histones. Some experiments indicated a relative order of disappearance, $F2A2 > F2A1 > F3$, but this could not be reliably reproduced. Free amino groups, as measured by reaction with 2,4,6-trinitrobenzene sulfonic acid (Habeeb, 1966), also disappeared with apparent first-order kinetics, generally faster than the disappearance of acid-soluble histones. Table I represents the results of a single kinetic study, where all parameters, mentioned above, were measured.

That the reaction described here actually follows pseudo first-order kinetics was demonstrated by examining the reaction rates in different concentrations of glutaraldehyde. At each glutaraldehyde concentration, an apparent first-order rate constant could be readily calculated for the overall disappearance of acid-extractable histones. A plot of these apparent first-order rate constants against glutaraldehyde concentration would be expected to yield a straight line, with slope equivalent to the second-order rate constant and ordinate-intercept equal to the backward rate constant (see von Hippel and Wong, 1971). In fact, such a plot (Fig. 2) suggests that the second-rate constant is itself a function of

TABLE I
Apparent First-Order Rate Constants

Disappearance of:	k_{app} (min^{-1})
Whole histones ($A_{230\text{nm}}$)	0.00568
Free amino groups	0.00840
F1	0.04080
F2C	0.01850
F2B	0.00774
F2A1	0.00648
F2A2	0.00590
F3	0.00582

Fixation conditions: 4.0 mM glutaraldehyde, 5.0 mM MgCl_2 buffer, 0°C , 0–120 min.

glutaraldehyde concentration. Further, the observation that the y -intercept of Fig. 2 approaches zero, suggests that the fixation reaction is irreversible in the time scale of the reaction.

The kinetics of disappearance of the different histone classes in solutions of different glutaraldehyde concentration was also studied. Where measurable, each class of histones showed apparent first-order kinetics, the rate constants increasing with glutaraldehyde concentration. In the range from 0 to 5 mM glutaraldehyde, the apparent second-order rate constants followed the progression: $k(\text{F1}) > k(\text{F2C}) > k(\text{F2B}) > k(\text{F2A2}), k(\text{F2A1}), k(\text{F3})$. The data were insufficient, however, to give more precise definitions of second-order rate constants.

The Acid-Soluble Products of Glutaraldehyde Fixation

As mentioned earlier, several weak-staining bands appear during glutaraldehyde fixation when total histones are examined by the acid histone gels. In addition, staining at gel origins suggested the formation of polymeric material. Acid-soluble extracts were, therefore, dialyzed against SDS-containing buffers and examined by electrophoresis in neutral SDS gels (Fig. 3). It is clear that there is a rapid appearance of polymeric bands. As many as 6–8 polymer bands could be seen. The samples shown in Fig. 3 were, in fact, preparations from glutaraldehyde-fixed nuclei that had been solubilized with 2.0 M NaCl -5.0 M urea (as described in Materials and Methods). This procedure appears to solubilize nonhistone proteins with the histones

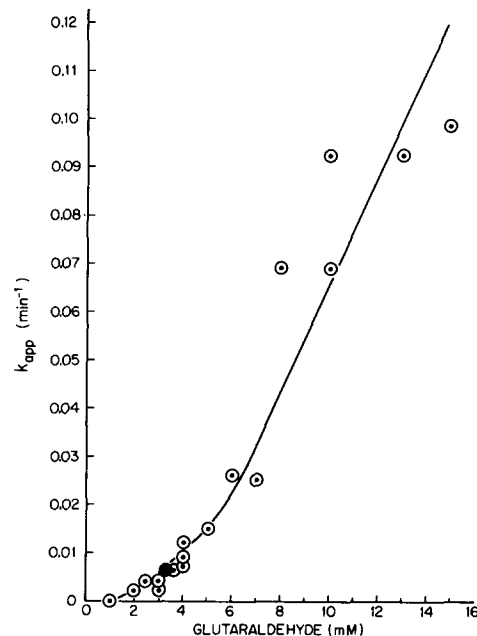


FIGURE 2 Dependence of apparent first-order rate constant (k_{app}) upon glutaraldehyde concentration. All data obtained in 5 mM MgCl_2 buffer except for the solid point, which was measured in 0.2 M KCl buffer.

(O'Malley et al., 1972). These are seen as the faintly staining bands of high molecular weight in the control samples (i.e., time = 0 min). Acid-soluble extracts of glutaraldehyde-fixed nuclei reveal the same polymeric bands, and extracts of control nuclei appeared to contain much less nonhistone protein than obtained by solubilization with NaCl -urea.

Measurements of approximate molecular weights for these presumptive histone polymers were performed with alkaline SDS gels, using the unreacted histone bands as internal molecular weight standards (Panyim and Chalkley, 1971). Assuming the molecular weights F2A1, 11,000; F2A2, 12,500; F2B, 14,000; and F2C, 25,000, we obtained the approximate molecular weights GLU-A, 39,000; GLU-B, 50,000; GLU-C, 62,000; and GLU-D, 72,000. Thus, each band appeared to increase 10,000–12,000 over each preceding, faster, band.

In order to identify the histones which were being cross-linked by glutaraldehyde, attempts were made to isolate the polymers by gel filtration in 6 M guanidine HCl (Fish et al., 1969). The results of fractionation of 100 mg acid-

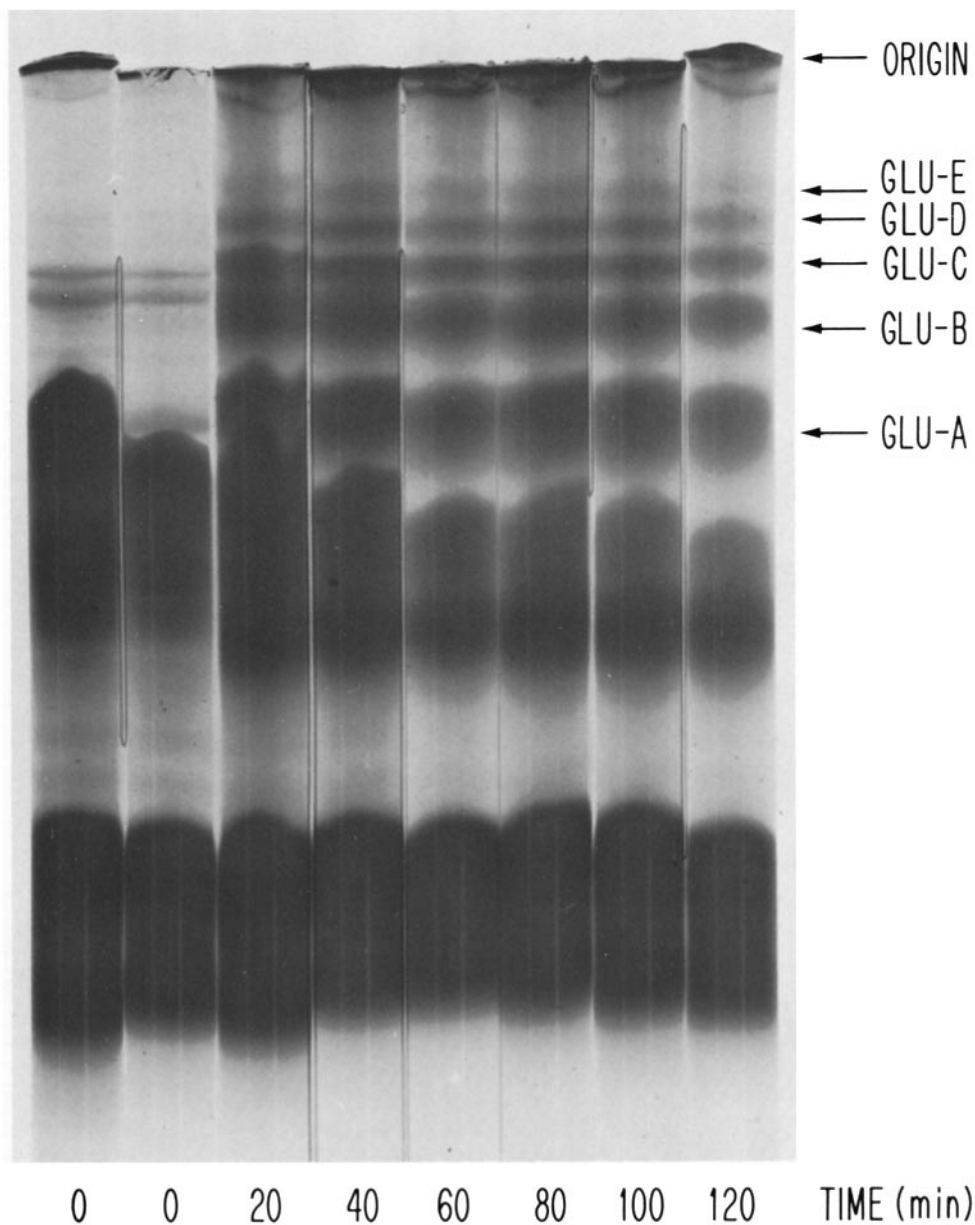


FIGURE 3 Neutral SDS-gel electrophoresis of urea-NaCl extracts from glutaraldehyde-fixed chicken erythrocyte nuclei. Conditions of fixation: 4 mM glutaraldehyde, 5 mM $MgCl_2$ buffer. Two control gels (unfixed nuclei, time = 0 min) are shown; the leftmost gel contains twice the volume of extract of all the other gels. The broad, deeply stained bands migrating ahead of GLU-A contain the remaining unreacted and monomer histone fractions.

extracted, glutaraldehyde-fixed histones (reduced and alkylated to disrupt F3 dimer) are shown in Fig. 4 *a*. In this manner, 300 mg histone were separated and the indicated fractions were concentrated and refiltered through the Sepharose

column. Fraction I was recycled and the resulting peak was denoted fraction 1. Fraction II was divided into two fractions, 2 and 3, and fraction III was obtained as a single peak (denoted fraction 4) as a consequence of rechromatography.

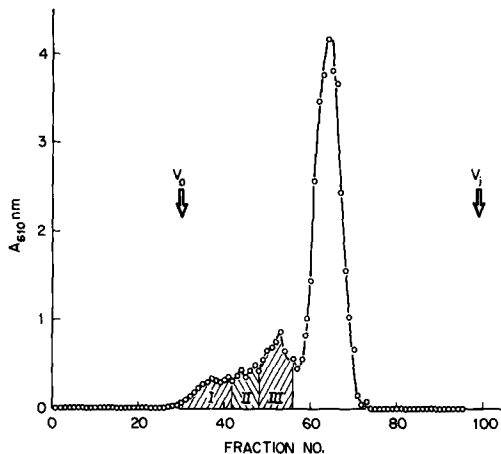


FIGURE 4 *a* Gel filtration of acid-soluble extract from glutaraldehyde-fixed chicken erythrocyte nuclei on Sepharose 6B in 6 M guanidine HCl. Column size: 90 x 1.5 cm. Fraction volume: 1.2 ml. V_0 and V_T : column void volume and total solvent volume, determined by elution positions of blue dextran and ϵ -DNP-lysine, respectively. $A_{610\text{nm}}$: absorbance by protein of xylene brilliant cyanin G.

The four resulting fractions were compared in neutral SDS-gel electrophoresis (Fig. 4 *b*). It is clear that fraction 1 is a mixture of high molecular weight polymers; 2 is a mixture of GLU-B, GLU-C, and others; 3 is a mixture of GLU-A, GLU-B, and GLU-C (approximately 60, 30, and 10%, respectively); and 4 migrates in the region of F2C and F1. None of these fractions contained detectable amounts of the rapidly migrating histone bands (i.e., F2A1, F2A2, F2B, and F3).

Table II contains a comparison of the amino acid compositions of fractions 1, 2, 3, and 4 with published values for the major classes of histones. Since prior workers (Wang and Tu, 1969) have shown that glutaraldehyde fixation of proteins results in a loss of lysine residues, the data were corrected for this loss. Parallel experiments indicated that 30 min fixation with 4.0 mM glutaraldehyde in 5 mM MgCl_2 buffer resulted in a loss of $\sim 25\%$ of the free amino groups, as assayed with 2,4,6-trinitrobenzene sulfonic acid. This percentage loss was assumed for all the isolated fractions. The first striking observation from these amino acid analyses is that the fractions 1, 2, 3, and 4 are remarkably similar to one another, supporting the idea of a series of polymers of the same subunit. It is also clear that

compositions of these fractions are not identical to any of those of the purified histone classes. However, the high lysine content (after correction for loss) and the lysine/arginine ratios most closely resemble F2C and possibly F2B, and least resemble F2A1, F2A2, and F3. In fact, if one assumes that fractions 1, 2, 3, and 4 contain $>80\%$ F2C, the difference could be accounted for by contamination with acidic nonhistone proteins (rich in aspartic and glutamic acids, glycine, valine, and leucine). Such a contamination by nonhistones is not unreasonable considering their high molecular weights. The amino acid composition data, therefore, suggest that the acid-soluble polymers isolated after fixation under defined conditions (30 min, 4.0 mM glutaraldehyde, 5 mM MgCl_2 buffer) are largely composed of F2C histone.

An additional factor favoring the view that F2C can be cross-linked into a series of polymers derives from studies on glutaraldehyde fixation of purified F2C histones. 2.2 mg of purified chicken F2C histone, in distilled H_2O , was lyophilized and redissolved in 100 μl of 1 M NaCl—conditions designed to promote histone folding and aggregation (Bradbury et al., 1967). Aliquots were made to 4.0 mM glutaraldehyde, 0°C . Samples were taken at 15, 30, 60, and 120 min; diluted 50-fold with 0.1% SDS, 0.1% β -mercaptoethanol, 0.01 M phosphate buffer (pH 7.0); and dialyzed against the 1% SDS buffer and 0.1% SDS buffers, as described in Materials and Methods. When examined by neutral SDS-gel electrophoresis, F2C polymer bands appeared by 15 min of fixation. Comparative electrophoresis with acid-extracted, unfractionated chick histones from glutaraldehyde-fixed nuclei revealed a similar pattern of polymer bands, with some slight differences in mobility. Comparison of these samples by alkaline SDS-gel electrophoresis, however, revealed greater differences in mobility. Thus F2C alone, in high concentration and at aggregating conditions, is readily susceptible to polymerization in the presence of low concentrations of glutaraldehyde, but the products formed are not identical to the histone polymers from fixed whole nuclei. Similar studies are in progress with other purified histones.

One attempt was made to isolate the GLU-X band observed with histone gel electrophoresis (see Fig. 1) by eluting the band from a portion of a slab electrophoresis. Enough material was ob-

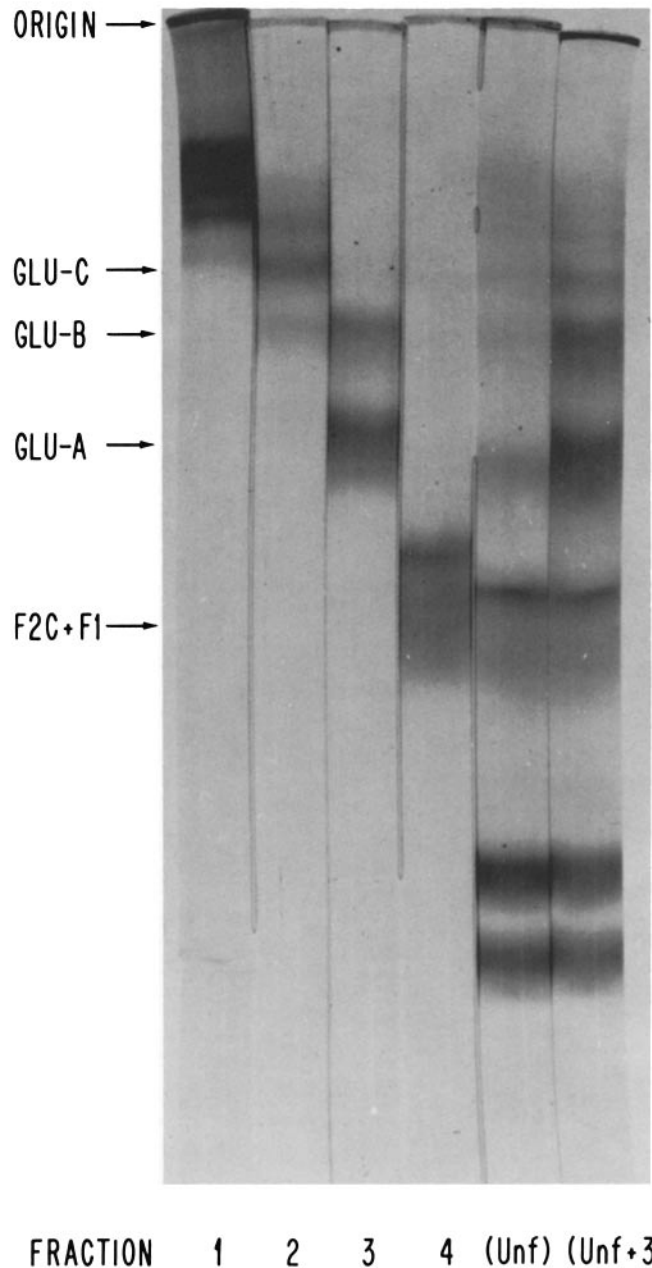


FIGURE 4 *b* Neutral SDS-gel electrophoresis of fractions from Sepharose filtration of glutaraldehyde-fixed histones. Fractions 1, 2, 3, and 4 derived from rechromatography (see text). (Unf): starting unfractionated acid-soluble extract of glutaraldehyde-fixed nuclei. (Unf + 3): Gel loaded with the same amounts of fraction 3 and unfractionated extract mixed just before electrophoresis.

tained for one amino acid analysis, which showed a strong resemblance to the fractions described in Table II. The relationship of GLU-X with GLU-A, GLU-B, etc., is, as yet, unclear.

Influence of Solvent Composition

When isolated interphase nuclei are washed in solvents free of divalent cations and at low

TABLE II
Amino Acid Compositions of Fractions from Glutaraldehyde-Treated Histones

Amino acid	Moles (%)										
	Column fraction					Histone fraction*					
	3‡	1§	2§	3§	4§	F2C	F1	F2B	F2A2	F2A1	F3
Lysine	16.8	22.2	23.6	21.1	20.9	21.0	26.8	14.1	10.2	11.4	10.0
Histidine	1.2	1.3	1.9	1.1	1.7	1.8	0	2.3	3.1	2.2	1.7
Arginine	8.9	7.9	8.8	8.4	8.9	10.6	1.8	6.9	9.4	12.8	13.0
Aspartic acid	4.0	3.6	4.2	3.8	3.9	2.3	2.5	5.0	6.6	5.2	4.2
Threonine	4.7	4.0	4.6	4.5	4.8	4.2	5.6	6.4	3.9	6.3	6.8
Serine	8.2	8.6	8.8	7.8	7.4	11.9	5.6	10.4	3.4	2.2	3.6
Glutamic acid	7.4	6.6	6.9	7.0	7.1	5.3	3.7	8.7	9.8	6.9	11.5
Proline	5.0	6.6	5.6	4.8	4.7	6.7	9.2	4.9	4.1	1.5	4.6
Glycine	7.4	7.0	7.9	7.0	7.0	5.4	7.2	5.9	10.8	14.9	5.4
Alanine	15.9	14.9	15.3	15.1	14.0	15.1	24.3	10.8	12.9	7.7	13.3
Half cystine	0	0	0	0	0	0	0	0	0	0	1.0
Valine	5.5	5.0	5.1	5.2	5.1	4.6	5.4	7.5	6.3	8.2	4.4
Methionine	0.5	0	0	0.5	0.4	0.2	0	1.5	0	1.0	1.1
Isoleucine	3.9	3.3	3.2	3.7	3.8	3.4	1.5	5.1	3.9	5.7	5.3
Leucine	7.0	6.0	6.5	6.7	7.1	4.7	4.5	4.9	12.4	8.2	9.1
Tyrosine	2.0	1.7	1.9	1.9	1.9	1.7	0.9	4.0	2.2	3.8	2.2
Phenylalanine	1.5	1.3	1.4	1.4	1.3	0.7	0.9	1.6	0.9	2.1	3.1
Lysine/Arginine	1.9	2.8	2.7	2.5	2.3	2.0	14.9	2.0	1.1	0.9	0.8

All data for serine and threonine are uncorrected for hydrolytic loss.

* Mole percent data of different histone fractions taken from Butler et al. (1968), Tables 4 and 5.

‡ Mole percent data uncorrected for lysine loss due to reaction with glutaraldehyde.

§ Mole percent data corrected assuming a loss of 25% of the lysyl residues by reaction with glutaraldehyde; see text.

ionic strength, they undergo characteristic swelling and decondensation of chromatin clumps (Ris and Mirsky, 1949; Anderson and Wilbur, 1952; Philpot and Stanier, 1956; Brasch et al., 1971; Olins and Olins, 1972; Leake et al., 1972). This decondensation reaction appears to occur in at least two steps, which can be partially resolved by careful control of solvent conditions (Olins and Olins, 1972). Washing nuclei in 0.02 M KCl-containing buffers (with no added divalent cations) produces a dispersal of chromatin clumps with little concomitant disruption of the periodic nucleohistone folding. Solvents at much lower ionic strength (i.e., 0.5 mM sodium phosphate buffer or distilled H₂O) probably promote an unfolding of the periodic structure.

It was of interest to determine what influence alterations in solvent composition (and nuclear

morphology) have upon the kinetics and products of glutaraldehyde fixation. Fig. 5 shows the results obtained from two sets of experiments where the apparent first-order rate constants for the disappearance of acid-extracted histone (i.e., A_{230nm}) are plotted vs. solvent composition. A rapid decrease in k_{app} was observed when nuclei were swollen in 0.02 M KCl buffers, or at lower ionic strengths. Clearly, the rate of formation of acid-insoluble products is inhibited by conditions that promote disruption of chromatin condensations.

An examination of the different histone classes, after glutaraldehyde fixation in solvents of varying ionic composition, is presented in Fig. 6. Solvents that promote nuclear swelling and decondensation of chromatin were seen to inhibit the disappearance (i.e., acid insolubility) of the dif-

ferent histones (data for F1 and F2A1 are shown in Fig. 6), and to inhibit the formation of at least one product of fixation, GLU-X. Preliminary estimates of changes in k_{app} for the different histones, derived from a different series of experiments, indicated that for F2C histone, k_{app} decreased approximately fivefold (from 5 mM $MgCl_2$ buffer to 0.001 M KCl buffer); whereas k_{app} (F2A1) decreased 10%, or less, over the same solvent range.

Nuclei fixed in various solvents for 30 min at 0°C were examined for the amounts of acid-soluble protein, free amino groups, and polymeric proteins on neutral SDS-gel electrophoresis (Table III). Decreased ionic strength results in a clear inhibition of the glutaraldehyde reaction, as measured by the percentages of acid-soluble histones and of free amino groups recovered. The formation of histone polymers is less severely affected by nuclear swelling than is the overall

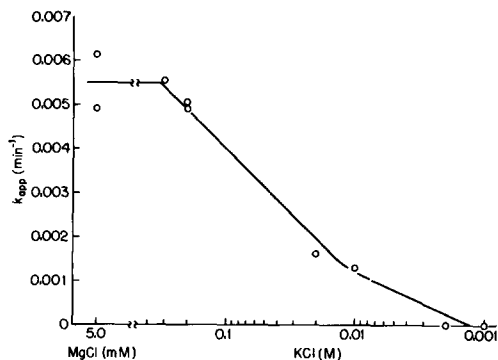


FIGURE 5 Dependence of apparent first-order rate constant (k_{app}) upon solvent ionic conditions. All data obtained at 4 mM glutaraldehyde.

fixation reaction. It would appear that only a few critically positioned amino groups need be cross-linked with glutaraldehyde in order to generate the histone polymers. Alternatively, cross-linking is accomplished via protein side chains, other than lysyl groups. At present, our prejudice is for the first explanation, although additional information is obviously required. Thus, disruption of chromatin condensations reduces, but does not obliterate, polymer formation. This data argues for a close proximity and a high relative reactivity of F2C molecules, even in nuclei with largely dispersed chromatin.

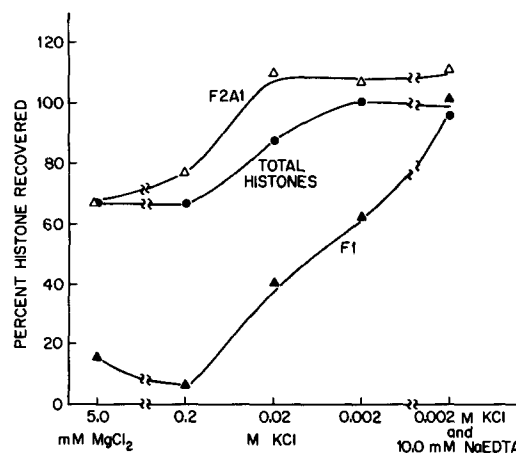


FIGURE 6 Yield of acid-soluble histones recovered from erythrocyte nuclei fixed with glutaraldehyde in solvents of varying ionic composition. Total histones (A_{230nm}), F1, and F2A1 (measured from densitometric scans) expressed relative to value for control unfixed nuclei (100%).

TABLE III
Solvent Effects Upon Glutaraldehyde Fixation

Solvent	Acid soluble*	Amino groups*	Polymers ‡
	%	%	%
5 mM $MgCl_2$, 0.02 M cacodylate (pH 7.5)	76.1	69.5	100
0.2 M KCl, 0.02 M cacodylate (pH 7.5)	74.0	79.0	93.9
0.02 M KCl, 0.001 M cacodylate (pH 7.5)	93.3	95.2	54.8
0.002 M KCl, 0.001 M cacodylate (pH 7.5)	86.3	102.0	81.9
0.001 M cacodylate (pH 7.5)	99.1	105.4	75.6

* Percentages measured relative to extracts from control nuclei, incubated in the same solvents without addition of glutaraldehyde.

‡ Measured from densitometric scans of neutral SDS gels. Percentage expressed relative to intensity of bands from nuclei fixed in 5 mM $MgCl_2$ buffer.

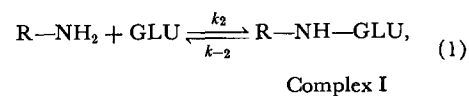
DISCUSSION

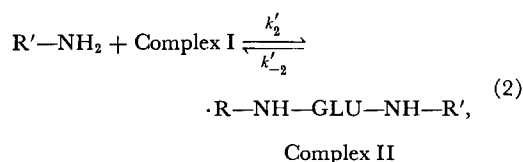
Fixation of nuclear histones by glutaraldehyde has been limited to the specified range of conditions: 0–20 mM glutaraldehyde, 0°C; nuclear DNA concentration, approximately 2 mg/ml; time, 0–2 h; and solvents of varying ionic strength at pH 7.5. Within this range of conditions, the disappearance of acid-soluble proteins, of free amino groups, and of individual histone fractions, appears to follow pseudo first-order kinetics. Although the decrease in acid-soluble histones (examined at A_{230nm}) behaves as a single kinetic class, it is clearly measuring the composite rates of disappearance of the different histone classes. Similarly, the disappearance of free amino groups might represent an average of classes with different rate constants that cannot be resolved by present methods. The sites of reaction of glutaraldehyde with protein molecules are not completely understood. Evidence has been presented that the order of reactivity of side-chain sites is lysine > cysteine > histidine and tyrosine; however, the adducts with histidine and tyrosine hydrolyze at acid pH (Habeeb and Hiramoto, 1968; Habeeb, 1969). Several observations in this study support the suggestion of significant reaction with lysyl residues: the rapid disappearance of free amino groups, the apparent loss of lysyl residues as determined by amino acid analysis, and the general correlation of first-order rate constants for the disappearance of individual histone fractions with the mole percent lysine of that histone class (i.e., F1 [26.8%] > F2C [24.1%] > F2B [14.1%] > F2A2 [10.2%] > F2A1 [11.4%] and F3 [10.0%]). This correlation also remains if the lysyl contents are expressed as residues per molecule of histone. Although it is not clear whether all amino groups constitute one kinetic class, it is highly probable that given sufficient time a large majority of lysyl residues can react with glutaraldehyde. Therefore, even lysine groups that are assumed in ionic linkages with DNA phosphates are capable of reaction.

Several additional points should be mentioned before postulation of a kinetic scheme. (a) Although there have been suggestions that glutaraldehyde exists as an aldol condensation product in aqueous solution (Richards and Knowles, 1968), recent nuclear magnetic resonance evidence argues for its existence and reaction as monomers in dilute aqueous solution (Korn et al., 1972). (b) The mechanism of reaction of glutaraldehyde with lysyl residues is not clear. The loss of lysyl resi-

dues, as determined by amino acid analysis, argues against simple Schiff base formation, which would be expected to cleave in strong acid (Jencks, 1964). (c) It is likely that not all reactions between glutaraldehyde and lysyl groups lead to a cross-linked structure. This would be in agreement with observations, made here, that the rate of disappearance of free amino groups is slightly faster than the rate of disappearance of acid-soluble histones. Unfortunately, it is difficult to judge the fraction of lysyl residues involved in cross-linkage, since we have little quantitative information about the relationship between cross-linking and acid insolubility. (d) The present experimental data could be complicated by the possibility of a reduced efficiency of acid extraction of monomer histones with increased time of fixation; i.e., a “trapping” of histones within the glutaraldehyde-fixed nuclei. A control experiment was therefore devised to test for this possibility. Nuclei were fixed for 0, 30, 60, and 120 min (4 mM glutaraldehyde, 5 mM $MgCl_2$ buffer) and extracted with acid in the usual manner. The acid-insoluble nuclear pellets were neutralized with several drops of 1 M sodium phosphate, pH 7.0, dispersed in 1% SDS, 1% β -mercaptoethanol, 0.01 M sodium phosphate, pH 7.0, stirred overnight, dialyzed against 0.1% SDS buffer, brought to constant volume, and centrifuged 18 h at 50,000 rpm. The supernatant SDS-soluble, acid-insoluble proteins were quantitated, employing the method of Bramhall et al. (1969), and compared with the acid-soluble extracts by neutral SDS-gel electrophoresis. Although the total amounts of acid-soluble proteins decreases markedly with time of fixation, the total amounts of SDS-soluble, acid-insoluble proteins remained almost constant for the 2 h period. Therefore most of the protein that became acid insoluble was also refractory to extraction by SDS. Comparison of the SDS-soluble proteins by SDS-gel electrophoresis did reveal some increase (i.e., trapping) of monomer F2C and F1 by 30 min fixation, but not enough to account for their rapid decrease in acid solubility.

Given these assumptions and uncertainties, the following kinetic model of consecutive second-order reactions may be presented.





where GLU represents bifunctional glutaraldehyde, and R and R' represent lysine residues on the same or different histone molecules. On the basis of data presented in Fig. 2, it was suggested that the reverse reaction, involving the hydrolysis of the cross-linked bond, was essentially irreversible in the time scale of the present experiments, suggesting that $k_2 \gg k_{-2}$ and $k'_2 \gg k'_{-2}$. The pseudo first-order property of the overall reaction scheme and of the disappearance of free amino groups could arise if the glutaraldehyde concentrations are in sufficient excess to remain essentially constant throughout the course of reaction.

The products of the reaction of glutaraldehyde with histones have been only partially characterized in the present study. Our data suggest that there are at least three classes of products: (1) acid-soluble intramolecular adducts, with or without intramolecular cross-linkages; (2) acid-soluble intermolecular complexes; and (3) acid-insoluble intermolecular complexes. It is not entirely clear why histones become acid insoluble during the course of glutaraldehyde fixation. There are several possible mechanisms that could account for this observation: (1) a decreased efficiency of acid extraction due to trapping of monomer histones (discussed earlier); (2) the formation of cages of cross-linked proteins around the DNA; and (3) the formation of DNA-protein cross-linkages. Brutlag et al. (1969) have examined the properties of formaldehyde-treated soluble nucleohistone. Employing [^{14}C]formaldehyde fixation followed by extensive digestion with pronase, these authors concluded that $\sim\frac{2}{3}$ of the formaldehyde linkages were involved in protein-protein bridges, with some of the remaining linkages between protein and nucleic acid.

In the present studies, the acid-soluble intermolecular products appear to consist primarily of cross-linked F2C histones. Amino acid analyses of the polymer fractions (Table II) constitutes the principal evidence supporting this view. However, apparent molecular weights, derived from alkaline SDS-gel electrophoresis, are seemingly inconsistent with the present suggestion of F2C polymers; molecular weights go up in steps of 10,000–12,000, rather than 25,000, the approximate

molecular weight of F2C histone. Reynolds and Tanford (1970) have presented evidence that protein-SDS complexes behave as rigid rodlike molecules, and that electrophoresis in SDS gels fractionates these complexes on the basis of their exclusion volume, which varies with peptide chain length and molecular weight. Covalently cross-linked side-by-side dimers (or polymers) would exhibit smaller exclusion volumes than corresponding end-to-end polymers, showing a faster mobility on SDS gels, and yielding lower molecule weights than predicted from the parent monomers. Such a mechanism could account for the low molecular weights determined for presumptive F2C polymers from glutaraldehyde-fixed erythrocyte nuclei. Additionally, electrophoretic mobility of these cross-linked histones might be affected by alterations in charge-to-mass ratios produced by modification of the lysyl residues (Tung and Knight, 1972; Panyim and Chalkley, 1971).

A class of acid-soluble intramolecular adducts can be identified with fraction 4 (see Fig. 4 *b* and Table II). On neutral SDS gels and on acid-urea gels this fraction migrated similarly to F2C and F1 histones, yet by amino acid analysis revealed a loss of lysyl residues comparable to the other cross-linked polymer fractions.

It is somewhat surprising that the amino acid composition data do not reveal the presence of F1 histone in any of the polymer fractions. The relatively low proline and alanine contents would not be consistent with a major contribution by F1 histone. We suggest, therefore, that F1 histone adducts become acid insoluble more rapidly than those of F2C. Isolation and characterization of polymer fractions after shorter fixation times might reveal the presence of F1 histone. A search for F1 polymer formation in glutaraldehyde-fixed chicken erythrocyte nuclei is made somewhat more difficult by the relatively low amounts of F1 relative to F2C. Panyim et al. (1971) have determined the relative proportions of the different histone classes in chicken erythrocyte nuclei to be: F1, 6.4%; F2C, 23.2%; F2B, 23.4%; F2A2, 14.1%; F2A1, 13.3%; F3, 18.7%. Experiments directed toward identification of F1 cross-linked polymers from other types of eucaryotic nuclei are currently in progress. Rat liver nuclei, which contain F1 histone instead of F2C, readily yield glutaraldehyde cross-linked polymers on neutral SDS-gel electrophoresis, after conditions of fixation comparable to those used with isolated chick erythrocyte nuclei. We have employed the tech-

nique of comigration electrophoresis to compare the acid-soluble extracts of glutaraldehyde-fixed chick erythrocyte and rat liver nuclei. These methods have demonstrated almost identical mobilities for corresponding polymer bands in both neutral and alkaline SDS gels. Obviously these presumptive F1 polymer bands must be further fractionated and characterized to make the parallel more convincing. None of the conclusions of this present study should be taken to imply that only F2C (and possibly F1) might exist in close proximity within the isolated erythrocyte nucleus. Any of the other histone classes might be capable of forming polymers, but would escape our attention because they have not been solubilized. Indeed, Clark and Felsenfeld (1972) have presented evidence favoring the view that arginine-rich histones might be clustered upon guanine and cytosine-rich regions of DNA.

It is possible that the rapid reaction of glutaraldehyde with F1 and F2C histones, and the formation of polymers of F2C, does not reflect their *in situ* proximity on the nucleohistone, but rather a reaction with histone molecules freely diffusing within the nucleus. Clark and Felsenfeld (1972) have demonstrated the ready exchangeability of some histones on chromatin at $\geq 5 \times 10^{-5}$ M CaCl_2 . We have reasons, however, for believing that F2C polymer formation is not occurring among nonbound histones. Isolated F2C histones are capable of being cross-linked by glutaraldehyde. But the polymers formed revealed clear differences in mobility, on alkaline SDS-gel electrophoresis, to the presumptive F2C polymers generated *in situ*. Swelling of isolated nuclei in solvents of low ionic strength, conditions expected to disfavor histone dissociation, does not eliminate F2C cross-linking by glutaraldehyde. Migration and subsequent clustering of histones during the isolation and washing procedures, before fixation by glutaraldehyde, represents an additional possible complication. We have extensively washed isolated chicken erythrocyte nuclei and chromatin with 5 mM MgCl_2 and with 0.2 M KCl. The washes were concentrated and examined by neutral SDS-gel electrophoresis. Although considerable nonhistone protein was extracted (especially by 0.2 M KCl), no dissociated histones could be detected. The possibility of histone migration along the DNA, without the formation of nonbound histones, remains untested.

Chromatin may be considered, schematically, to possess several levels of molecular organization: 1°, the arrangement of histones and nonhistones along the DNA; 2°, periodic foldings of the chromatin fiber (Pardon et al., 1967; Pardon and Wilkins, 1972; Bram and Ris, 1971); 3°, unique configurations of the chromatin fiber (Crick, 1971); and 4°, condensations of the periodically folded nucleohistone structure. Swelling of nuclei in the absence of divalent cations and at low ionic strength indicates that disruption of 4° structure can occur without complete destruction of the 2° periodic organization (Olins and Olins, 1972). The present observation that F2C polymer formation is inhibited ~25% by nuclear swelling at low ionic strength (Table III) suggests that some F2C molecules may be brought in close proximity by chromatin condensation. Littau et al. (1956) has presented ultrastructural evidence that the very lysine-rich histones are responsible for chromatin condensation. We would note one other observation consistent with this view. Treatment of isolated chicken erythrocyte nuclei with glutaraldehyde (4 mM) for 3–5 min in 5 mM MgCl_2 buffer completely prevents subsequent swelling and chromatin decondensation induced by washing in 0.02 M KCl or in distilled H_2O (Olins and Olins, unpublished observation). The persistence of significant F2C polymer formation at very low ionic strength could be a consequence of several alternative mechanisms: (a) clusters of adjacent F2C molecules along the DNA (1° arrangement); or (b) residual foldings of the nucleohistone fiber. Consistent with this latter interpretation, we have obtained electron micrographs of nuclei swollen in water which reveal a nonuniform structure, i.e., spherical particles ~70 Å diameter connected by thin strands ~20 Å wide.¹ Further, these spherical particles show varying amounts of close packing along a chromatin fiber. We wish to suggest the possibility that F2C histone is on the outside of these chromatin particles and brought into proximity by close packing of the particles into a periodically folded chromatin structure.

The authors gratefully acknowledge the criticisms and advice of Drs. Ada L. Olins and Mayo Uziel during the course of these studies. Dr. F. C. Hartman deserves our particular appreciation for performing the amino

¹ Olins, A. L., and D. E. Olins. 1973. *Science (Wash. D.C.)*. Submitted for publication.

acid analyses. Further, we wish to thank Drs. F. C. Hartman, O. L. Miller, F. H. Gaertner, and Ada L. Olins for their criticisms of the manuscript.

This investigation has been supported in part by the United States Atomic Energy Commission, under contract with the Union Carbide Corporation, and in part by a Public Health Service Research Grant to Dr. D. E. Olins (no. 1 R01 GM19334-01) from the National Institute of General Medical Sciences. Dr. D. E. Olins is a recipient of Public Health Service Research Career Development Award (no. 5 K04 GM40441-03) from the National Institute of General Medical Sciences.

Received for publication 12 February 1973, and in revised form 20 June 1973.

REFERENCES

- ANDERSON, N. G., and K. M. WILBUR. 1952. *J. Gen. Physiol.* **35**:781.
- BONNER, J., G. R. CHALKLEY, M. DAHMUS, D. FAMBROUGH, F. FUJIMURA, R.-C. C. HUANG, J. HUBERMAN, R. JENSEN, K. MARUSHIGE, H. OHLENBUSCH, B. OLIVERA, and J. WIDHOLM. 1967. *Methods Enzymol.* **12**(pt. B):3.
- BRADBURY, E. M., C. CRANE-ROBINSON, H. GOLDMAN, H. W. E. RATTLE, and R. M. STEPHENS. 1967. *J. Mol. Biol.* **29**:507.
- BRAM, S., and H. RIS. 1971. *J. Mol. Biol.* **55**:325.
- BRAMHALL, S., N. NOACK, M. WU, and J. R. LOEWENBERG. 1969. *Anal. Biochem.* **31**:146.
- BRASCH, K., V. L. SELIGY, and G. SETTERFIELD. 1971. *Exp. Cell Res.* **65**:61.
- BRUTLAG, D., C. SCHLEHUKER, and J. BONNER. 1969. *Biochemistry.* **8**:3214.
- BUTLER, J. A., E. W. JOHNS, and D. M. P. PHILLIPS. 1968. *Prog. Biophys. Mol. Biol.* **18**:211.
- CLARK, R. J., and G. FELSENFELD. 1972. *Biophys. Soc. Annu. Meet. Abstr.* **12**:246a.
- CRICK, F. C. 1971. *Nature (Lond.)*. **234**:25.
- FISH, W. W., K. G. MANN, and C. TANFORD. 1969. *J. Biol. Chem.* **244**:4989.
- HABEEB, A. F. S. A. 1966. *Anal. Biochem.* **14**:328.
- HABEEB, A. F. S. A. 1969. *J. Immunol.* **102**:457.
- HABEEB, A. F. S. A., and R. HIRAMOTO. 1968. *Arch. Biochem. Biophys.* **126**:16.
- JENCKS, W. P. 1964. *Prog. Phys. Org. Chem.* **2**:63.
- KAHAN, L., and E. KALTSCHMIDT. 1972. *Biochemistry.* **11**:2691.
- KORN, A. H., S. H. FEAIRHELLER, and E. M. FILACHIONE. 1972. *J. Mol. Biol.* **65**:525.
- LEAKE, R. E., M. E. TRENCH, and J. M. BARRY. 1972. *Exp. Cell Res.* **71**:17.
- LITTAU, V. C., C. J. BURDICK, V. G. ALLFREY, and A. E. MIRSKY. 1956. *Proc. Natl. Acad. Sci. U. S. A.* **54**:1204.
- MITCHELL, J. 1953. In *Organic Analysis*. J. Mitchell, Jr., I. M. Kolthoff, L. S. Proskauer, and A. Weissberger, editors. Interscience Publishers Inc., New York. **1**:282.
- OLINS, D. E., and A. L. OLINS. 1971. *J. Mol. Biol.* **57**:437.
- OLINS, D. E., and A. L. OLINS. 1972. *J. Cell Biol.* **53**:715.
- O'MALLEY, B. W., T. C. SPELSBERG, W. T. SCHRADER, F. CHYTLIL, and A. W. STEGGLES. 1972. *Nature (Lond.)*. **235**:141.
- PANYIM, S., D. BILEK, and R. CHALKLEY. 1971. *J. Biol. Chem.* **246**:4206.
- PANYIM, S., and R. CHALKLEY. 1969 a. *Arch. Biochem. Biophys.* **130**:337.
- PANYIM, S., and R. CHALKLEY. 1969 b. *Biochemistry.* **8**:3972.
- PANYIM, S., and R. CHALKLEY. 1971. *J. Biol. Chem.* **246**:7557.
- PARDON, J., and M. H. F. WILKINS. 1972. *J. Mol. Biol.* **68**:115.
- PARDON, J., M. H. F. WILKINS, and B. M. RICHARDS. 1967. *Nature (Lond.)*. **215**:508.
- PHILPOT, J. ST. L., and J. E. STANIER. 1956. *Biochem. J.* **63**:214.
- REYNOLDS, J. A., and C. TANFORD. 1970. *Proc. Natl. Acad. Sci. U. S. A.* **66**:1002.
- RICHARDS, F. M., and J. R. KNOWLES. 1968. *J. Mol. Biol.* **37**:231.
- RIS, H., and A. E. MIRSKY. 1949. *J. Gen. Physiol.* **32**:489.
- SPACKMAN, D. H., W. H. STEIN, and S. MOORE. 1958. *Anal. Chem.* **30**:1190.
- SUBRAMANIAN, A. R. 1972. *Biochemistry.* **11**:2710.
- TUNG, J.-S., and C. A. KNIGHT. 1972. *Anal. Biochem.* **48**:153.
- VON HIPPEL, P. H., and K.-Y. WONG. 1971. *J. Mol. Biol.* **61**:587.
- WANG, J. H.-C., and J.-I. TU. 1969. *Biochemistry.* **8**:4403.
- WEBER, K., and M. J. OSBORN. 1969. *J. Biol. Chem.* **244**:4406.
- WOLD, F. 1967. *Methods Enzymol.* **11**:617.
- ZENTGRAF, H., B. DEUMLING, and W. W. FRANKE. 1969. *Exp. Cell Res.* **56**:333.