AN IRREVERSIBLE DISSOCIATION OF THE HISTONE H3-H4 TETRAMER

by

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THIS IS AS RECEIVED FROM CUSTOMER.

Introduction

In a typical eucaryotic cell, about 100 cm of DNA is folded into a volume of less than 10^{-10} cm^3 at the time of DNA replication (1). Histones may play a role in this remarkably efficient packaging of DNA. In addition to this, histones may be involved in non-specific repression of transcription (2).

Johns (3) has proposed a model of the chromatin structure, in which part of each histone molecule attaches itself firmly to the DNA, the remainder being available for interactions with other nuclear macromolecules. Weintraub (4) reports that it is the histone N-terminal that binds to some regions of DNA, while the histone C-terminal is free for other interactions. In this way the histones control the physical state of the chromatin and the ability of chromosomal DNA to serve as a template for RNA synthesis.

Recent studies show that at the lowest level of organization, the DNA of chromatin is folded together with histones, to form repeating morphological units (called bodies or nucloesomes) consisting of an octameric core of the four small histones surrounded by a segment of the DNA fiber. H1[1] is suggested to be on the outside of this globular subunit. These nucleosomes

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[1]The new histone nomenclature used here was accepted by participants in the CIBA foundation symposium on the structure a and function of chromatin, April 1974. The new nomenclature is as follows for each histone, where the previous names are given given in parentheses: H1(F1,I,KAP), H5(F2C,V,KAS),H2A(F2A2,IIb1, AUK), H2B(F2B,IIb2,KSA), H3(F3,III,AUK), and H4(F2A1,IV,GRK).

are composed of two each of the four histones, H2A, H2B, H3 and H4, complexed to 180-200 base pairs of DNA (5, 6, 7).

There are two schools of thought on the nucleosomal structure of chromatin. Noll et al. (8) for example, suggest that all the DNA of the chromatin subunits strongly interacts with the histone core to form a nucleoprotein "bead", whereas others (9, 10) support a "bead and bridge" model whereby only a portion of the DNA is in the nucleoprotein conformation, while the remaining DNA functions as a spacer. Griffith (11) has shown that at low ionic strengths (I=0.015), a bead and bridge model might exist, while at higher ionic strengths (I=0.15), the beads become more closely spaced.

Thomas and Kornberg (7) demonstrated that the octamer can exist free in solution at pH 9.0 and ionic strength of 2.0, by cross-linking studies performed on histones after DNA removal by gel filtration. This observation applied only to chromatin samples at high concentrations (2.0 mg/ml). If the chromatin concentration was lowered to 0.1 mg/ml, the octamer began to dissociate into dimers of H2A and H2B, and hexamers containing more H3 and H4 than H2A and H2B. The above investigators also suggested that the octamer contained roughly equal amounts of all the four small molecular weight histones and their arrangement in the octamer, free in solution was the same as in chromatin.

In attempting to understand the organization of histones in chromatin, and the function and replication of the eucaryotic chromosome, it would be desirable to be able to reconstitute

the nucleosome from free histone pairs in solution. However, it may be difficult to do so in the absence of DNA, as is observed in reconstitution experiments of ribosomes in the absence of RNA. Weintraub and Van Lente (12,1) have shown that in 2 M NaCl, histones H2A, H2B, H3 and H4 adopt their native conformation in the absence of DNA. However attempts to reconstitute the octamer from histone pairs in the absence of DNA failed, possibly because the histones were damaged during isolation, despite the mild methods used (7).

Studies by Isenberg and his colleagues (13) on DNA-free histones have demonstrated specific pairwise interactions between different histones, producing subcomplexes that may be related in structure to the nucleosome. These interactions are accompanied by an increase in α-helical structure. Individual histones also self-associate (often with the formation of β-sheet structures). Specific interactions between different histones tend to suppress the self association of individual histones. D'Anna and Isenberg (14), by the use of circular dichroism and fluorescence anisotropy have monitored changes in the secondary and tertiary structures of mixtures of histones and have concluded that H3-H4, H2A-H2B, and H2B-H4 are all strong complexes, albeit with differing association constants. Weak complexes are found within H2A-H4 and H3-H2B. There is still much experimental uncertainity in determining the strength of the H3-H2A interaction. There is a marked change in the secondary structure when any of the three strong complexes form, implying that there is mutual adjustment as the partners

bind to form a compact structure. The slightly lysine rich pair H2A-H2B seems to form either dimers (15),or multiples of dimers (16), while the arginine rich pair, H3-H4 can form a tetramer of molecular weight 54,000 daltons in solution, consisting of two molecules of each histone (16, 17, 18).

There is as yet no compelling evidence that the complexes of histones observed in solution accurately reflect the arrangement of histones in chromatin, but there have been a few suggestive observations. In salt dissociation studies on chromatin, Bartley and Chalkley (19) demonstrated that H2A and H2B dissociated coincidentally, and so too did H3 and H4 when the salt concentration was increased. This suggests that these histones exist as subcomplexes in chromatin as they do in solution. From the above information, it can be seen that the cross-linking studies of DNA-bound histones and the solution studies complement each other, in that the former elucidates the chromatin structure, while the latter identifies specific histone interactions that may contribute to that structure.

A considerable amount of work has been done on the characteristics of the H3-H4 tetramer. The complex has a sedimentation coefficient of 3 S (16), with a corresponding frictional ratio of 1.99 (17). An unusual characteristic of the tetramer is that it elutes at an apparent molecular weight of approximately 100,000 daltons from a Sephadex G-100 column (17), although its true molecular weight is 54,000 daltons. From the above facts, it appears that the

tetramer is not a compact structure.

The unusual hydrodynamic behavior of the complex is consistent with the model of Moss et al.(20). In this model, the relatively hydrophobic carboxyl terminal regions of the four polypeptide chains are folded into a central compact core; the remaining residues are randomly coiled and fully flexible in solution. This is in accordance with the amino acid sequences for H3 and H4, where the charged groups are clustered in the amino terminal regions, while the hydrophobic groups are predominantly in the carboxyl terminal region (21, 22).

In a preliminary report, Roark et al.(23) demonstrated by equilibrium centrifugation that the H3-H4 tetramer undergoes a concentration dependent dissociation to its dimer ($2(H3\text{-}H4) \rightleftharpoons (H3\text{-}H4)_2$). However, due to the limitations of the sedimentation equilibrium technique, the lowest concentration that could be used was 0.2 mg/ml. At this concentration, the tetramer was found to be little dissociated, and so the measured association constant was somewhat uncertain. It seemed useful to find an alternative method applicable at lower concentrations, so that the complex could be examined under conditions where its dissociation was more nearly complete.

The objective of my work was to obtain a more reliable value for the equilibrium constant for the dissociation reaction. For this purpose, we employed broad-zone gel permeation chromatography. By this method, concentrations as low as 0.05 mg/ml could be used, this limit being determined by the sensitivity of the spectrophotometer which was used

for protein estimation.

The elution profiles from the broad-zone experiments strongly suggested that the reaction was not a simple dimer-tetramer equilibrium. From the narrow-zone experiments that followed, it appeared that the reaction involves the dissociation of H3 and H4 followed by the irreversible aggregation of H3.

Experimental

Preparation of histones

There are two general methods for displacing histones from DNA under non-denaturing conditions, one reported by Van der Westhuyzen et al. (17), and the other by Bidney and Reeck (27). The procedure adopted in this work combines features of both methods.

The procedure of Van der Westhuyzen involves the isolation of chromatin from calf thymus nuclei. The histones are displaced from deoxynucleoprotein by protamine, the precipitate of deoxynucleoprotamine is removed by centrifugation, and the supernatant is concentrated by ultrafiltration. Excess protamine is separated from histones by gel filtration on a Sephadex G-50 column (14 X 70 cm) equilibrated with 0.05 M sodium acetate and 0.05 M sodium bisulfite buffer (pH 5.0). The resultant histones are then further fractionated on a Sephadex G-100 column (2.5 X 95 cm) equilibrated and developed with the same buffer used for the Sephadex G-50 column. The elution profile displays two predominant peaks. Peak A contains the very lysine-rich histones H1 together with the arginine-rich histones H3 and H4, while peak B contains the slightly lysine-rich histones H2A and H2B. Peak A is further fractionated with solid ammonium sulfate to 70% (w/v) saturation, which precipitates histones H3 and H4 and leaves histone H1 in solution.

Bidney's procedure involves the isolation of chromatin

from chick erythrocyte nuclei. The histones are displaced and separated from DNA by a two-phase salt dissociation technique. To perform this transaction, the chromatin is made up to 4% (w/v) dextran, 15% (w/v) polyethylene glycol (PEG), 5 M NaCl and 0.01 M Tris Cl buffer (pH 7.5). The mixture is vigorously shaken for thirty minutes, and complete phase separation is achieved by centrifugation. The histones and PEG constitute the top phase, while the bottom phase contains DNA and dextran. The top phase is carefully removed, diluted tenfold with 0.01 M Tris Cl buffer (pH 7.5) and applied to a BioRex-70 ion exchange column, equilibrated with 0.01 M Tris Cl buffer (pH 7.5), in order to remove the polymer and to concentrate the proteins. The total histones are then fractionated by the method of Van der Westhuyzen on a Sephadex G-100 column equilibrated with 0.05 M sodium acetate buffer (pH 5.0).

It should be noted particularly that Bidney's procedure was designed for chick erythrocyte chromatin, which differs from calf thymus chromatin in that it contains practically no non-histone chromatin proteins and has an additional histone, H5, which is similar to histone H1. Moreover, the chick erythrocyte chromatin lacks the protease that is common in most tissues (28). In the procedure of Van der Westhuyzen, bisulfite was added to the chromatographic solvents in order to suppress the protease activity that accompanies calf thymus chromatin; bisulfite was presumed to be unnecessary in the purification of chick erythrocyte histones.

For our study, the isolation of total histones from chick

erythrocyte chromatin was achieved by the two-phase method of Bidney and Reeck with the modifications explained below. Further fractionation of total histones was developed, as in Van der Westhuyzen's method, on a Sephadex G-100 column with no bisulfite in the solvent, followed by ammonium sulfate precipitation to obtain pure H3-H4 tetramer. The procedure to obtain the H3-H4 complex is given below.

Materials

Chick blood (with heparin) was obtained from Pel-Freeze biologics. Polyethylene glycol 600 (PEG) was a product of Union Carbide. Dextran 500 and Sigmacote SL-1 were purchased from Sigma Chemical Company. Bio-Rex-70 was obtained from BioRad. Sephadex G-100, G-150 and G-25 were obtained from Pharmacia. Special enzyme grade, ultrapure ammonium sulfate was purchased from Schwarz/Mann. N,N'-methylene bisacrylamide (Bis) and technical grade acrylamide was obtained from Eastman Kodak Company and Ames Company respectively. Glass columns were purchased from Glenco.

Sodium acetate buffer (pH 5.0) and Tris Cl buffer (pH 7.5) were prepared by dilution from 1 M stock solutions. All buffer pH values were measured at 25°C. All the steps were carried out at 4°C.

Preparation of nuclei

400 ml of chick blood was suspended in 4 liters of 0.15

M NaCl and centrifuged at 200 g for 10 min in a Lourdes 30-R Clinifuge swinging bucket rotor (#4300). The top layer of white cells was removed with an aspirator. Nuclei were collected by homogenizing the erythrocytes three times in two volumes of ice cold Buffer A (0.25 M sucrose--3 mM CaCl--0.01 M Tris Cl) made up in 1% (v/v) Triton X-100, using a Potter-Elvehjem homogenizer. Each homogenization was followed by centrifugation in a Beckman J-21 B centrifuge at 4000 g for 10 min (JA-14 rotor). The final pellet from this series was washed twice by homogenization in ice cold Buffer A. The purified nuclei recovered in the pellet from the final homogenization were stored in batches at -20°C. About 20 batches of approximately 2 ml each were obtained from 400 ml chick blood.

Preparation of chromatin

All centrifugations for this preparation were carried out in a Beckman J-21B centrifuge using a JA-14 rotor.

Chromatin was prepared by homogenizing 2 ml of thawed nuclei in 200 ml Tris Cl buffer in decreasing concentrations of 0.05 M, 0.01 M, 0.005 M and 0.001 M. Each homogenization was followed by centrifugation at 4,000 g for 10 min. The gel obtained from this series of washes was made up to 220 ml in 0.001 M Tris Cl, sheared in a Waring blendor at 90 V for 120 sec, and centrifuged at 16,000 g for 15 min. The resulting translucent supernatant was the chromatin and its DNA content was determined by absorbance measurements at

260 nm in 1% sodium dodecyl sulfate (SDS) assuming $A^{0.1\%}_{260}$ =21. The protein content can be obtained from this value by assuming a 1:1 mass ratio between the DNA content and the protein content in chick chromatin. From 2 ml of nuclei, about 180-230 mg of protein were recovered.

Two phase separation of histones and DNA

To 200 ml of sheared chromatin, with a DNA concentration of 0.8 mg/ml, were added 8.0 g of dextran, 20 g of PEG, 58.4 g of NaCl and 2 ml of 1 M Tris Cl buffer (pH 7.5). The mixture was then shaken vigorously for 30 min in the cold until the polymers were dissolved. Phase separation was achieved by centrifuging the composite in a Lourdes swinging bucket rotor (#4200) at 700 g for 10 min, so as to prevent interphase mixing while the rotor was decelerating. The top layer containing histones and PEG was carefully removed in one continuous stream by suction, using a long piece of narrow diameter tubing and a decent pressure head (about 75-100 cm). The bottom phase containing DNA and dextran was discarded, together with about 5% of the top phase.

Polymer removal and histone concentration

150 ml of the top phase was diluted tenfold with 0.01 M Tris Cl to lower the salt concentration and was applied to a BioRex-70 column (2.5 X 5.0 cm). The BioRex was previously washed with three volumes of 1 M NaOH and equilibrated with

0.01 M Tris Cl, until the pH of the effluent was about 7.5. The diluted histone sample was applied to the column at approximately 1 l/hr, and the column washed with 0.01 M Tris Cl. Histones were eluted with 3 M NaCl, 0.01 M Tris Cl and were collected in 2 ml fractions. Protein content was detected on a Beckman Spectrophotometer, Model 25, at 260 and 280 nm. Fractions with an A_{280} reading greater than 1 (3 mg/ml), and with an A_{280}/A_{260} ratio greater than or equal to 1.7 were considered to be pure histones, uncontaminated by DNA, and were pooled to be used in further fractionation on a preparative Sephadex G-100 column. Protein yields from BioRex ranged from 100-170 mg histones per 2 ml nuclei.

Fractionation of histones by gel chromatography

400 µl of 1 M sodium acetate buffer (pH 5.0) was added to 8 ml of histone sample from above, in 3 M NaCl, 0.01 M Tris Cl, and applied to a Sephadex G-100 column (2.6 X 90 cm) equilibrated with 0.05 M acetate buffer. The column was developed at 12 ml/hr with the equilibration buffer collecting 6 ml fractions. The protein content was determined by absorbance at 280 nm. The resulting elution profile displays a minor peak followed by two predominant peaks. The minor peak contained DNA. Histones H1, H5, H3 and H4 were found in the first predominant peak, and histones H2A and H2B in the second predominant peak. The fractions in the first peak were pooled to be fractionated by ammonium sulfate (below). Approximately 50-75 mg of protein were recovered in this step, from the first peak.

Fractionation of histones by salt precipitation

34.6 g of ultrapure solid ammonium sulfate was slowly added with stirring to 72 ml of pooled material from the Sephadex G-100 effluent, under carefully monitored conditions (pH 5.0, 4°C). The pH was adjusted when required with 1 M NaOH. The suspension was allowed to settle for 30 min and the precipitated H3 and H4 were pelleted by centrifuging at 44,000 g for 10 min in a JA-20 rotor. The pellet was then dissolved in 0.05 M acetate buffer to the appropriate concentration and volume to be used in broad and narrow zone chromatography. About 35-55 mg of protein were recovered, which is pure H3 and H4.

Alternative isolation procedure

In an attempt to speed up the isolation procedure, we tried to replace BioRex-70 with hydroxyapatite (prepared by the method of Siegelman (30)), which in addition to removing PEG and concentrating the histones, also separated H1 and H5 from the rest of the histones in a one-step operation. However, this procedure failed due to the erratic reproducibility of the binding capacity of hydroxyapatite from one experiment to another. We also assayed Ultrogel AcA 44 (LKB Produkter), as a substitute for Sephadex G-100, since this medium is reported to give high resolution, fast flow rates and short separation times. However, in our experience, histones were not well resolved on Ultrogel, and the protein recoveries were

low, and so we returned to Sephadex G-100.

Notice that throughout the procedure for histone isolation, the use of dialysis sacs was avoided. This was because, during the time of histone isolation, our laboratory was experiencing contamination of dialysis sacs by a fungus, which appeared to secrete a protease that clipped off the tails of histones H3 and H4 being dialysed, causing the histones to leak out of the bag within 5 hours of dialysis.

Gel electrophoresis

Histones were identified and their purity was assessed by discontinuous polyacrylamide gel electrophoresis in the presence of SDS by the method of Laemmli (29). The gel tubes (i.d. 5 mm) were coated with 2% (v/v) Sigmacote for 15 min and dried in an oven at 90°C for 30 min. The separating gels were 7.5 cm long, of 15% (w/w) acrylamide, 0.2% (w/w) bis-acrylamide at a pH of 8.9, while the spacer gels were 1 cm long and contained 3% (w/w) acrylamide and 0.04% (w/w) bis-acrylamide at a pH of 7.5. For very low protein concentrations and large samples, 10 cm long separating gels were made up with 4 cm long spacer gels. Gels were run at 1.5 mA/tube until the bromophenol blue tracking dye had reached the bottom. The gels were stained for 15 min in 0.5% (w/v) amido black in 7% (v/v) glacial acetic acid and 40% (v/v) ethanol and destained overnight in 40% (v/v) ethanol, and 7% (v/v) glacial acetic acid, with the aid of Dowex-1 anion exchange resin.

Fig. 1. Flow chart of procedure followed to obtain pure histone H3-H4, as in Preparation.

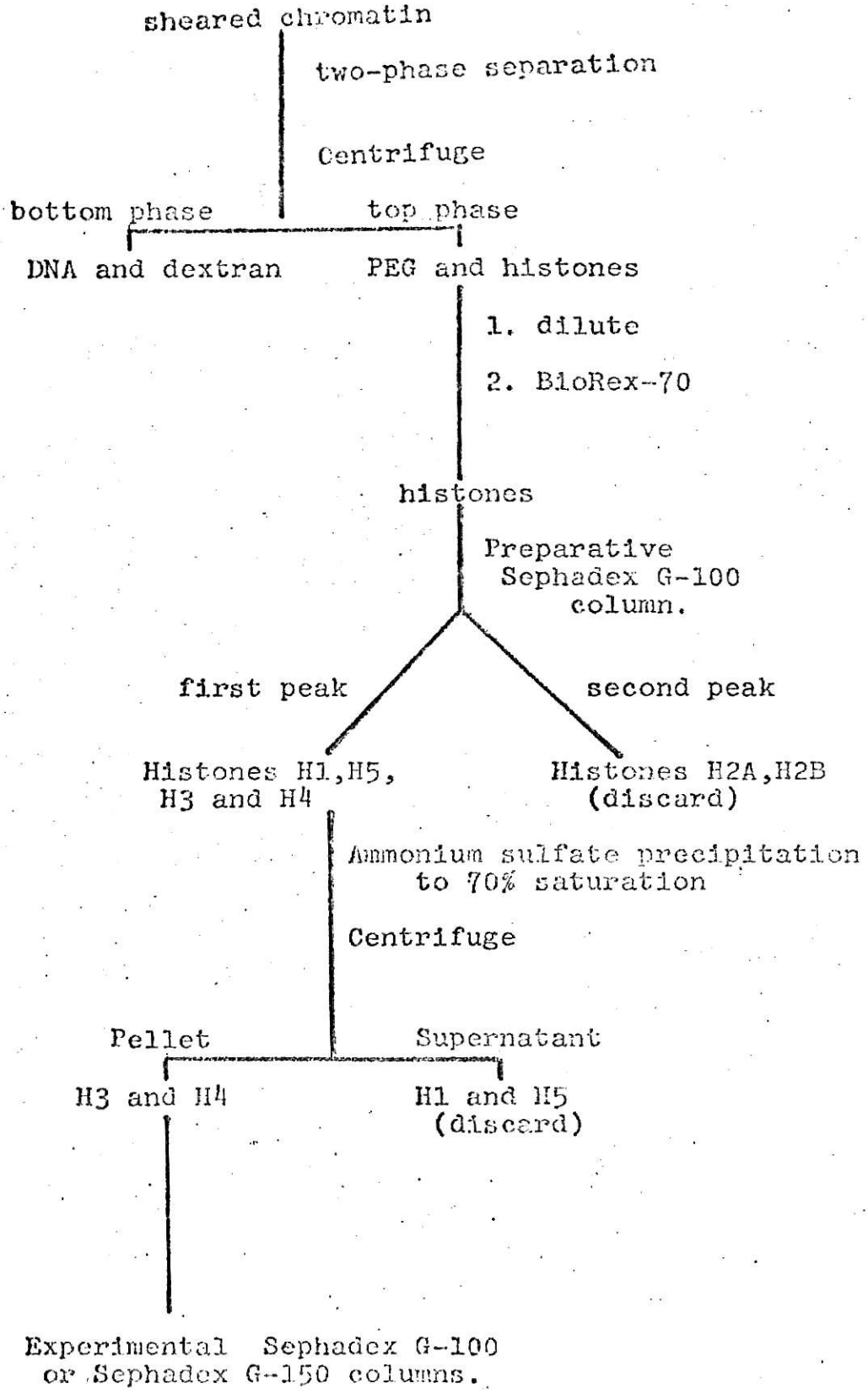

sheared chromatin
two-phase separation
Centrifuge
bottom phase
top phase
DNA and dextran
PEG and histones
1. dilute
2. BioRex-70
histones
Preparative Sephadex G-100 column.
first peak
second peak
Histones H1,H5, H3 and H4
Histones H2A,H2B (discard)
Ammonium sulfate precipitation to 70% saturation
Centrifuge
Pellet
Supernatant
H3 and H4
H1 and H5 (discard)
Experimental Sephadex G-100 or Sephadex G-150 columns.

Figure 1a

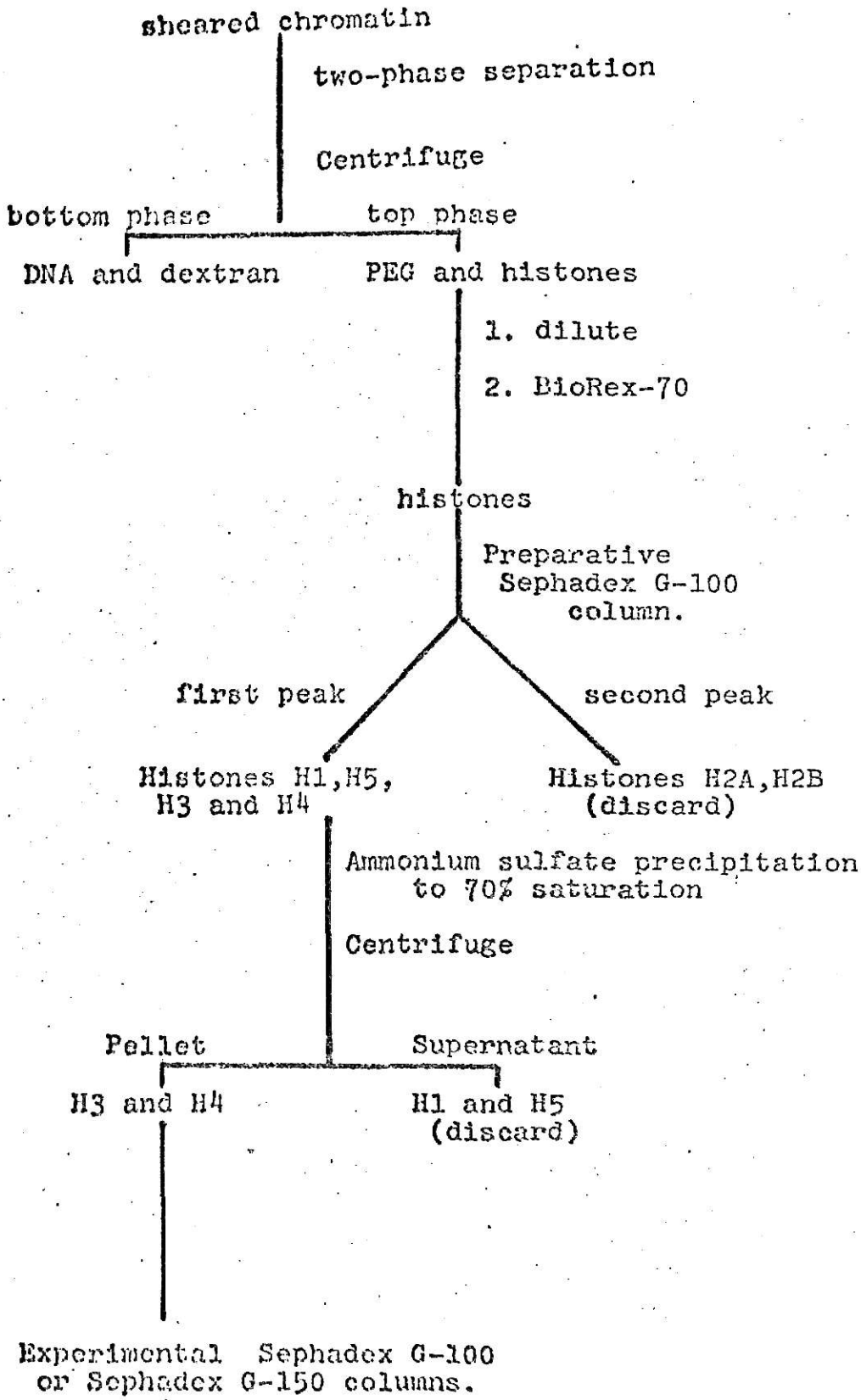

sheared chromatin
two-phase separation
Centrifuge
bottom phase
top phase
DNA and dextran
PEG and histones
1. dilute
2. BioRex-70
histones
Preparative Sephadex G-100 column.
first peak
second peak
Histones H1,H5, H3 and H4
Histones H2A,H2B (discard)
Ammonium sulfate precipitation to 70% saturation
Centrifuge
Pellet
Supernatant
H3 and H4
H1 and H5 (discard)
Experimental Sephadex G-100 or Sephadex G-150 columns.

Figure 1b

Chromatography

Columns (0.988 X 90 and 2.6 X 90 cm) were packed with preswollen beads of Sephadex G-100 or Sephadex G-150 as appropriate, and equilibrated with 0.05 M sodium acetate buffer (pH 5.0). For the larger Sephadex G-100 column (2.6 X 90 cm), a flow rate of 12 ml/hr was maintained by keeping a constant pressure head of about 60 cm. A constant flow rate of 2 ml/hr was maintained for the smaller Sephadex G-100 and for the Sephadex G-150 columns (0.988 X 90 cm), by means of an LKB Varioperpex peristaltic pump.

Homogeneity of the beds was checked by running a colored substance (dextran blue) through the column and observing the column visually. Columns were calibrated with the following standards of known molecular weight: bovine serum albumin, dextran blue, chymotrypsinogen and glycylglycine. The elution volumes of dextran blue and glycylglycine were used for the void volume (V_o) and the total column volume (V_T) respectively, which were used to calculate the partition coefficient (σ). The partition coefficient is a measure of the fraction of the internal volume of beads accessible to the solute and is calculated by the equation, $\sigma = \frac{V_e - V_o}{V_T - V_o}$, where V_e is the elution volume of the solute under investigation. The value of σ ranges from zero (for a particle that is totally excluded from the gel) to one (for a particle that is totally included in the gel). The spreading of peaks in the elution profile depends on the amount of time spent by the molecules in the

column. In addition, for equal residence times on the column, the spreading is more rapid for solutes that are partly included in the gel beads than for solutes that are either totally included or entirely excluded. For large molecules that are entirely excluded from the internal volume of the gel beads and spend little time in the column, the spreading is minimal. For small molecules, the spreading is greater, since these are included into the gel beads and therefore spend a lot of time in the column. For the intermediate sized molecules which penetrate only part of the internal volume and hence spend a fair amount of time in the column, the resultant peaks are broader than those of the other two extreme-sized molecules (Fig. 2).

The sharpest possible elution profiles were obtained by: positioning the peristaltic pump upstream of the column, which minimized mixing of the sample which occured when the pump was placed downstream; using short lengths of very small diameter tubing between the outlet of the column and the fraction collector, to minimize dilution and prevent remixing of the sample; operating the column at slow flow rates (2 ml/hr); and by collecting small fraction volumes (0.5 ml/tube), using the time feature of the fraction collector rather than the drop counter, due to small changes in the solvent density and surface tension.

Initially Sephadex G-100 columns (0.988 X 90 cm) were used for the broad and narrow zone experiments on samples of the H3-H4 complex, and it was observed that the sample eluted very close to the void volume. Under such a situation

Fig. 2. Schematic elution profile to illustrate the three types of solute behavior:

(a) total exclusion

(b) a penetrant molecule

(c) a small, totally non-excluded molecule.

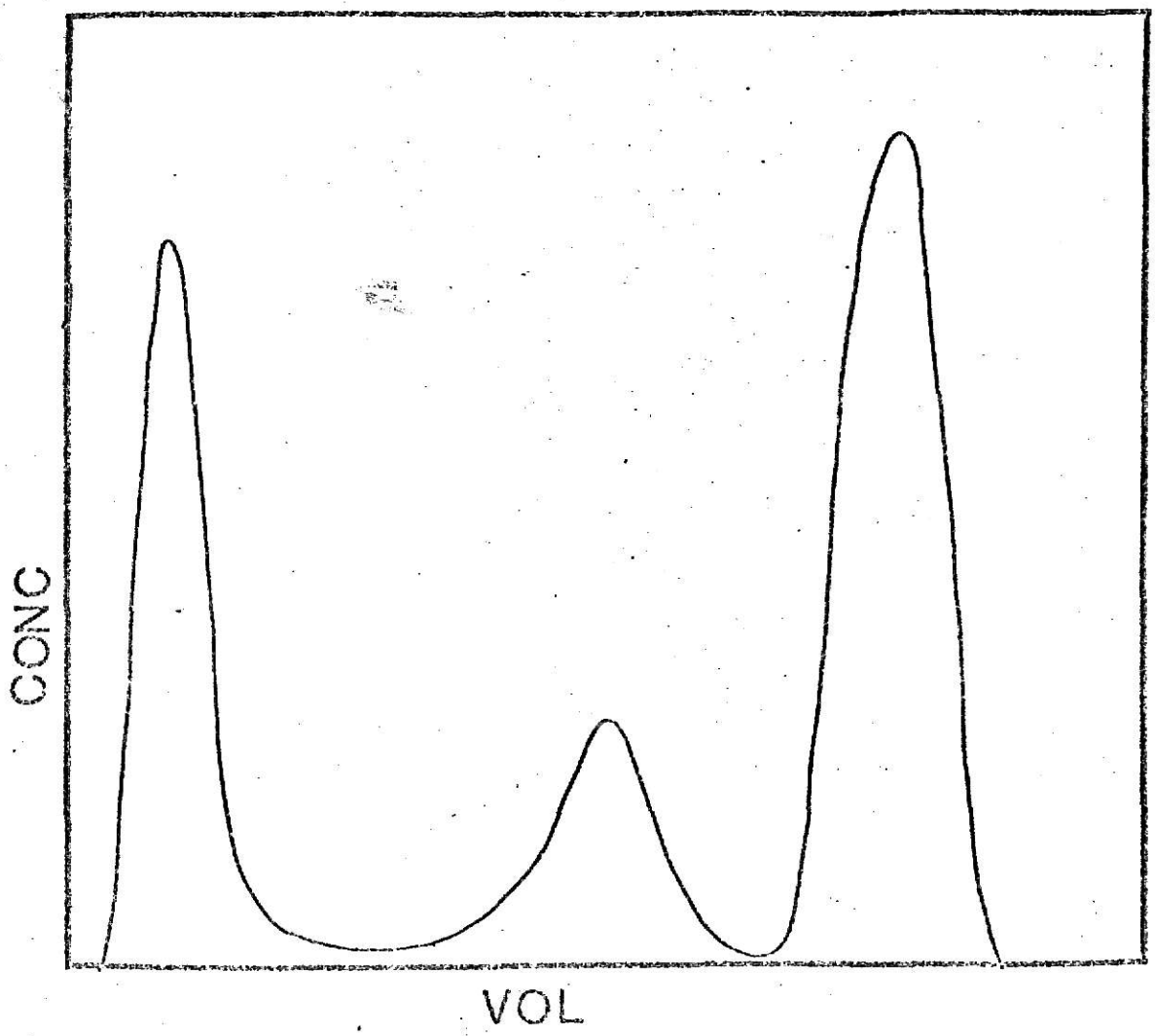


Figure 2

the elution volume is not sensitive to small changes in the state of aggregation of the solute. Therefore we replaced Sephadex G-100 with Sephadex G-150, which due to the larger internal volume available to the solute molecules, resulted in improved resolution, since samples eluted further away from the void volume.

The precision of measurement of the elution volume becomes very important for experiments designed to measure the variation of the apparent molecular weight of the H3-H4 complex as a function of protein concentration. A given small relative error in the determination of the elution volume would have a devastating effect on the accuracy of the measured association constant for the complex. We used three methods to measure the precise fraction volumes. Method I involved pooling the contents of the first twenty tubes into a volumetric cylinder and then dividing the total volume by twenty to obtain the individual fraction volumes. This did not prove accurate enough as it was impossible to transfer the entire contents of the tubes into the volumetric cylinder. In Method II, preweighed tubes were used and fraction volumes were obtained by reweighing the tubes, followed by a little computation. By this method, it was noticed that the flow rate varied negligibly (± 0.07 ml/hr). There was an occasional tube whose volume did not quite match the average fraction volume. This was due to the fact that the time interval between tube changes was not an integral multiple of the time interval between drops. some tubes had one drop more or less than the others. This proc-

edure proved to be too cumbersome, since an experiment involved about 200 tubes. To compensate for this and knowing that the flow rate remained constant within narrow limits, fractions of approximately 3 ml effluent were collected in three preweighed tubes at the begining and end of an experiment (Method III). By reweighing these tubes and with the aid of a simple calculation, the average flow rate and fraction volume were obtained. This method gave accurate results and was the method that we used in most of our experiments to calculate the fraction volumes.

To check for any column irregularities that may occur during an experiment, an internal calibration technique was devised. This involves the application of a small volume (0.7 ml) of sample containing a large and a small molecular weight standard immediately prior to application of the broad zone to the column. The volume of the broad zone was selected so that the high molecular weight solute eluted ahead of the leading boundary, while the low molecular weight solute eluted in the plateau region. A change in the elution volumes of the two standards between successive experiments indicated gel shrinkage or packing of the column matrix during the experiment. For the broad zone experiments on the histone H3-H4 tetramer, the use of a high molecular weight solute (blue dextran) was avoided, because it eluted so close to the void volume along with the H3-H4 tetramer, and so obscured the profile of the leading boundary. Glycylglycine was a good standard in that it eluted on the plateau region, provided the plateau was large enough. Final elution volumes were calculated by taking into

consideration the volume of sample required for the narrow zone of glycylglycine, while centroid elution volumes were calculated from the time the sample for the broad zone was applied to the column.

Ultracentrifugation

To obtain the molecular weight of the H3-H4 tetramer, a high speed sedimentation equilibrium or meniscus depletion experiment was conducted according to the method of Yphantis (31), on a Beckman Spinco Model E ultracentrifuge, at a speed of 28,000 r.p.m., using interference optics. Photographs of interference fringe patterns were made at 12-hr intervals until the pattern no longer changed. "Blank" experiments (both cell sectors containing water) were subtracted from each photograph prior to molecular weight computations. The baseline-corrected fringe displacements were converted to lists of ln C versus r^2 by a computer program that also measured the local slopes of the data and reported molecular m k weight as a function of solute concentration (M.M.St. Clair, unpublished). The partial specific volume for the H3-H4 complex was estimated from the amino acid composition by Haschemeyer (32) and corrected for the Donnan non-ideality effect by Roark (33), was 0.733 ml/g.

Histone concentrations were determined spectrophotometrically, assuming $A_{280}^{1\ mg/ml} = 0.35$ and $A_{220}^{1\ mg/ml} = 8.75$.

Broad-zone chromatography

Gel permeation chromatography is potentially more effective in characterizing the H3-H4 tetramer than ultracentrifugation, since the solute is detectable at lower concentrations in chromatography than in ultracentrifugation. For the concentration-dependent self associating species, frontal analysis (or broad-zone chromatography) is preferred over narrow zone chromatography for the following reason: in order to define the association equilibrium, one seeks the variation of the partition coefficient as a function of solute concentration. In narrow zone experiments, the solute is progressively diluted during the passage of the sample through the column, resulting ultimately in a ten- or twenty-fold dilution. In a broad zone experiment, provided that there is no irreversible binding of the solute to the column matrix, there is virtually no sample dilution. For a concentration-dependent system, this is of prime importance, since dilution results in dissociation of the solute and therefore in slowing down the migration of the solute through the column. As a result of this, the migration velocity varies continuously during the experiment.

A broad zone experiment is conducted in the following way: a sample is introduced to a column in a volume (V_s), which is large in relation to the total bed volume (V_T), and is followed by a large volume of solvent. The concentration of the solute in the effluent is measured as a function of the volume (V_e) that has flowed through the column. From the schematic profile,

Fig. 3. it is seen that a plateau region, of constant solute concentration equal to the initial concentration, is bounded by leading and trailing boundaries. For such a non-associating system, the boundaries are mirror images of one another (enantiographic). As a result of axial dispersion, these boundaries are not sharp. However, an equivalent sharp boundary (or centroid volume V_c) can be defined on the basis of conservation of mass (24, 20).

Consider an arbitrary position, V_p, within the plateau region of the elution profile, Fig. 3. The centroid volume is chosen in such a way that the mass of solute, $C_p(V_p-V_c)$, represented by the area under the idealized diagram (dotted lines) equals the true mass represented by the area under the experimentally determined curve (solid lines). The centroids of the leading and trailing boundaries move at the same rate and their velovity is equal to the migration velovity of the solute in the plateau region. The centroid elution volume is thus unambiguously related to the partition coefficient of the solute at the plateau concentration. Notice, from Fig. 3, that the volume between the centroids is equal to the sample volume.

Typically, in a self-associating system, boundary sharpening is present on the leading boundary, followed by a corresponding boundary spreading on the trailing edge. This effect arises from the tendency of the larger molecules to move faster through the column than the smaller ones. In the plateau region, the concentration of solute is high and there is a high proportion of aggregation. Since the solute has relatively little access to the internal volume of the beads, it moves rapidly. However,

Fig. 3. Typical broad zone diagram for an non-associating solute. Broken lines represent the "idealized diagram. Solid lines represent the experimentally determined elution curve.

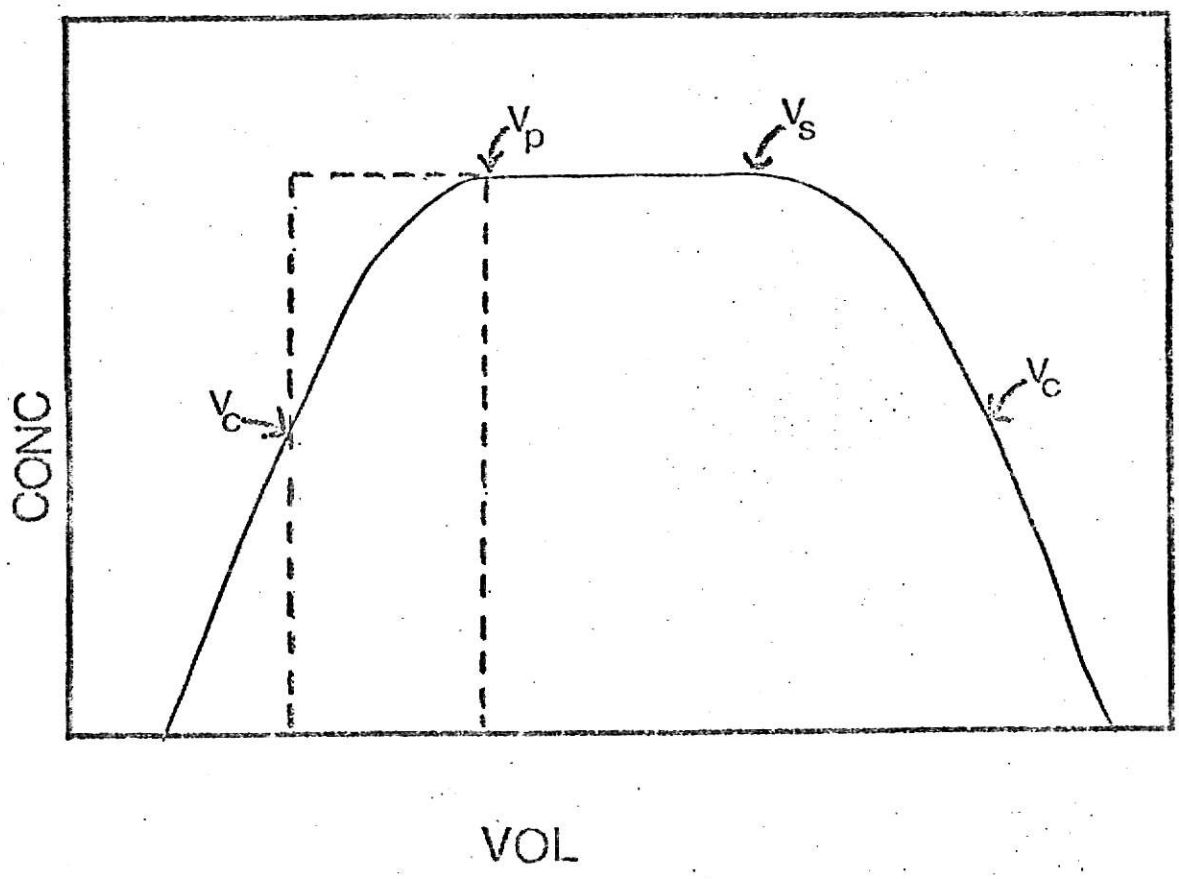


Figure 3

at the outer edges of the boundaries, where the solute concentration is low, there is a relatively high proportion of monomer, which has access to the internal volume of the beads and therefore moves slowly. The overall result is a continuous operation with boundary sharpening on the leading edge followed by a boundary spreading on the trailing edge; that is for a self-associating species, the boundaries will be non-enantiographic (25). Notice in Fig. 4, that as the plateau concentration is changed, the centroid elution volumes also change. This change in elution volume is the same for both boundaries of the profile.

Gradient profiles, analogous to the schlieren patterns given by sedimentation velocity experiments, can be obtained from broad zone chromatographic data, by taking the first derivative (26).

There are several disadvantages of this method. Molecular weights can only be determined indirectly. The fundamental quantity derived from the gel experiment is the partition coefficient which is a complex function of molecular volume and shape. Large sample volumes are required (about 50 ml). The experiments take a long time (about three days); and the calculations that follow are slightly more complex than for a narrow zone experiment.

However, the chief advantage of this method is that it relates the partition coefficient unambiguously to the solute concentration In contrast, the narrow zone technique yields a complicated average of the migration velocities over a wide range of concentrations experienced by the solute as it is diluted during its passage through the column.

Fig. 4. Predicted behavior for a self associating system, at two different solute concentrations.

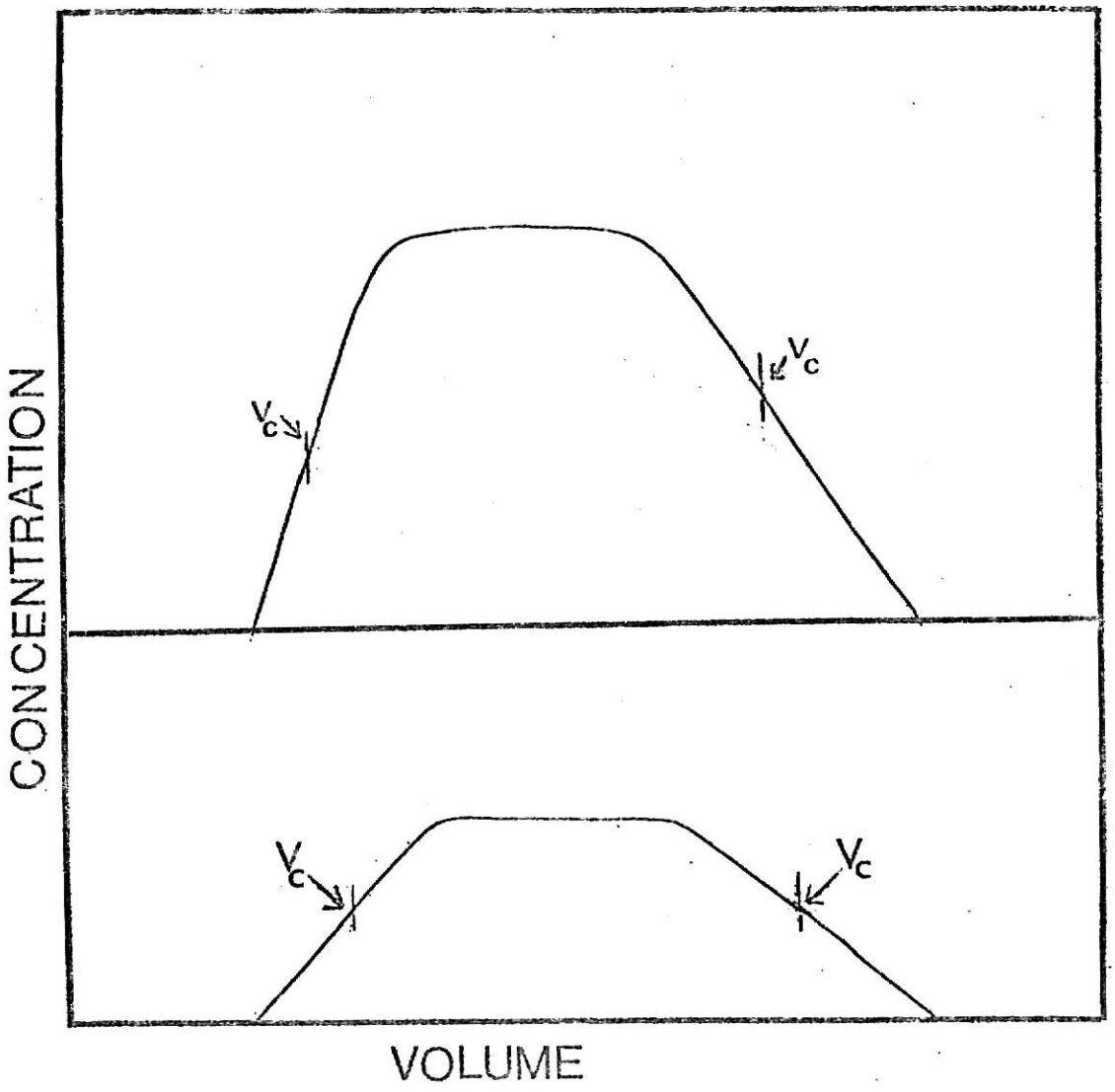


Figure 4

Results and discussion

In order to optimize the mechanical arrangements for broad zone chromatography, a number of experiments were done using a standard protein (BSA). Commercially obtained BSA contains a fair proportion of dimer (about 15%) that is irreversibly formed from monomer during isolation. Fig. 5 displays a typical elution profile for a broad zone experiment. The leading and trailing boundaries each displayed the presence of two non-interacting components, monomer and dimer. In addition to this, the enantiography of the two boundaries for each component, typical of that for a non-interacting solute, can be observed. Owing to the small volume of sample applied, the plateau region was not very prominent. However, the plateau concentration was equal to that of the sample applied, indicating that there was no irreversible binding of the solute to the column matrix. Notice particularly that the volume of sample applied was close to the volume between the centroid volumes for each component. Theoretically these volumes should have been identical. The discrepancy observed here was due to the method used in this early experiment for determining fraction volumes. Fraction volumes and consequently elution volumes were obtained by Method I (see experimental) which, as was seen later, was not accurate enough for experiments such as these.

The next step was to apply this technique to the study of the self-associating H3-H4 tetramer, at a concentration

Fig. 5. Broad zone profile on Sephadex G-100 column of BSA. 91 ml of 0.05 M sodium acetate (pH 5.0) containing 9.1 mg BSA were applied to the column equilibrated with 0.05 M sodium acetate buffer (pH 5.0). Bed dimensions were 2.5 X 85 cm. Flow rate 12 ml/hr. Fraction volume 4 ml.

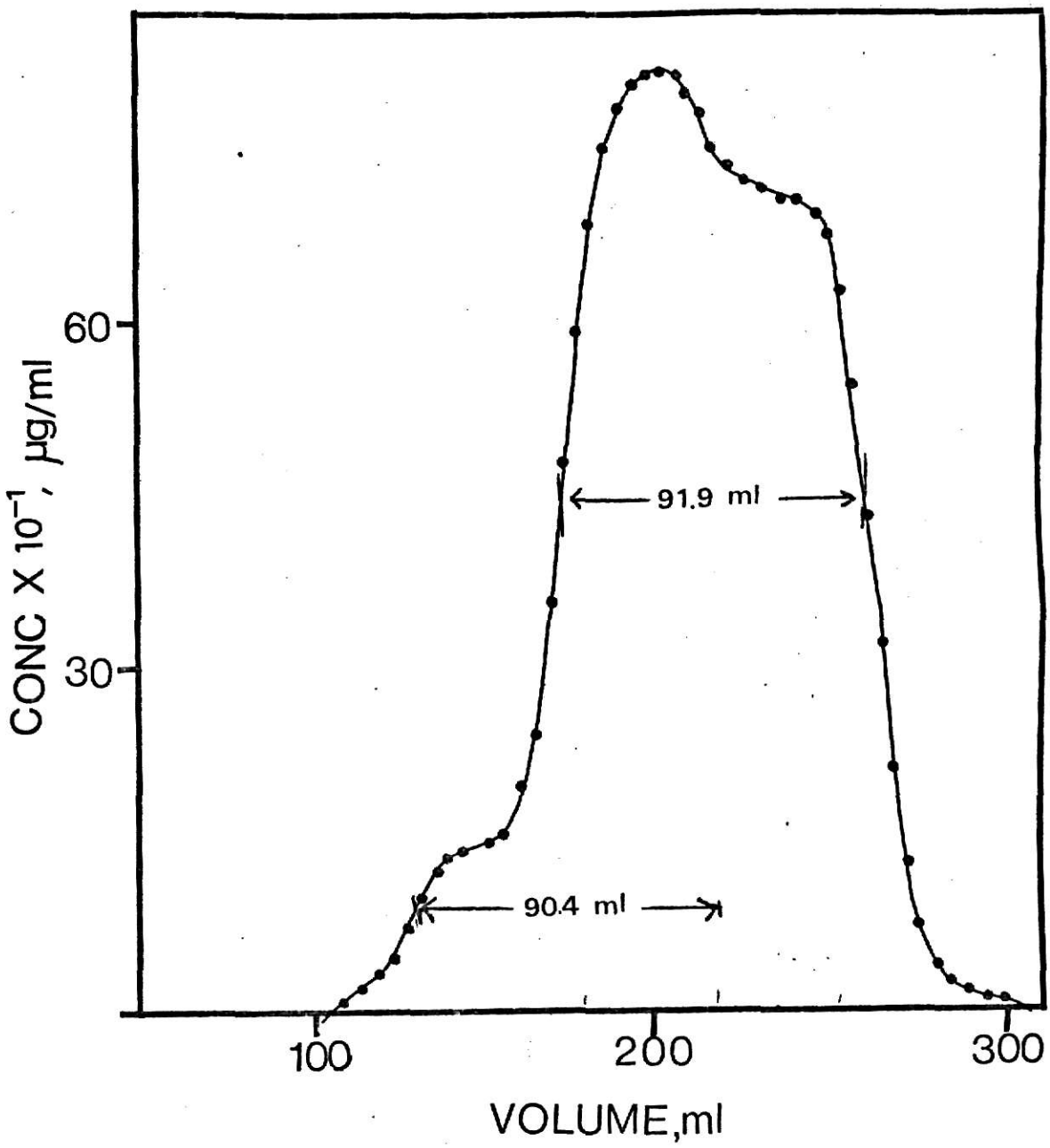


Figure 5

in the range examined by Roark et al. (0.4 mg/ml). For such a self-associating species, we expected the elution profile to exhibit asymmetry resulting from the concentration dependence of the migration rate i.e. a sharp boundary on the leading edge and a corresponding boundary spreading at the trailing edge. However, it came as quite a surprise to observe that the elution profile (Fig. 6) did not exhibit the predicted characteristics. Both the boundaries possessed two plateaus, indicating the presence of at least two components, a large amount of a high molecular weight fraction followed by a relatively small amount of a smaller molecular weight fraction. The peak superimposed on the plateau was due to glycylglycine, which was used as an internal standard to check for any changes in column packing subsequent to calibration. The elution volume of glycylglycine was unchanged in this experiment, and so the column behaved normally. Close investigation of the boundary shapes showed that both the leading and trailing boundaries were mirror images for each component i.e. slope ab = slope and slope bc = slope . Such an elution profile resembled that of a non-interacting mixture. These results implied that the system under investigation was not a reequilibrating species, and that perhaps, at low concentrations, the equilibrium between the H3-H4 tetramer and its dimer became a more complex situation, which involved an irreversible dissociation of the tetramer into its parent subunits.

Narrow zone experiments were conducted on samples of pure H3-H4 tetramer, with the intention of resolving the components detected in the broad zone experiment and identifying them by

Fig. 6. Broad zone profile on Sephadex G-100 column of histones $(H3\text{-}H4)_2$ from chick erythrocytes. 48.4 ml of 0.05 M acetate buffer (pH 5.0) containing 7.15 mg tetramer were applied to the column equilibrated with 0.05 M acetate buffer. Narrow zone consists of 0.7 ml of glycyl-glycine, for internal calibration (see text), in 0.05 M sodium acetate buffer (pH 5.0). Bed dimensions 0.988 X 100 cm. Flow rate 1.73 ml./hr. Fraction volume 0.52 ml.

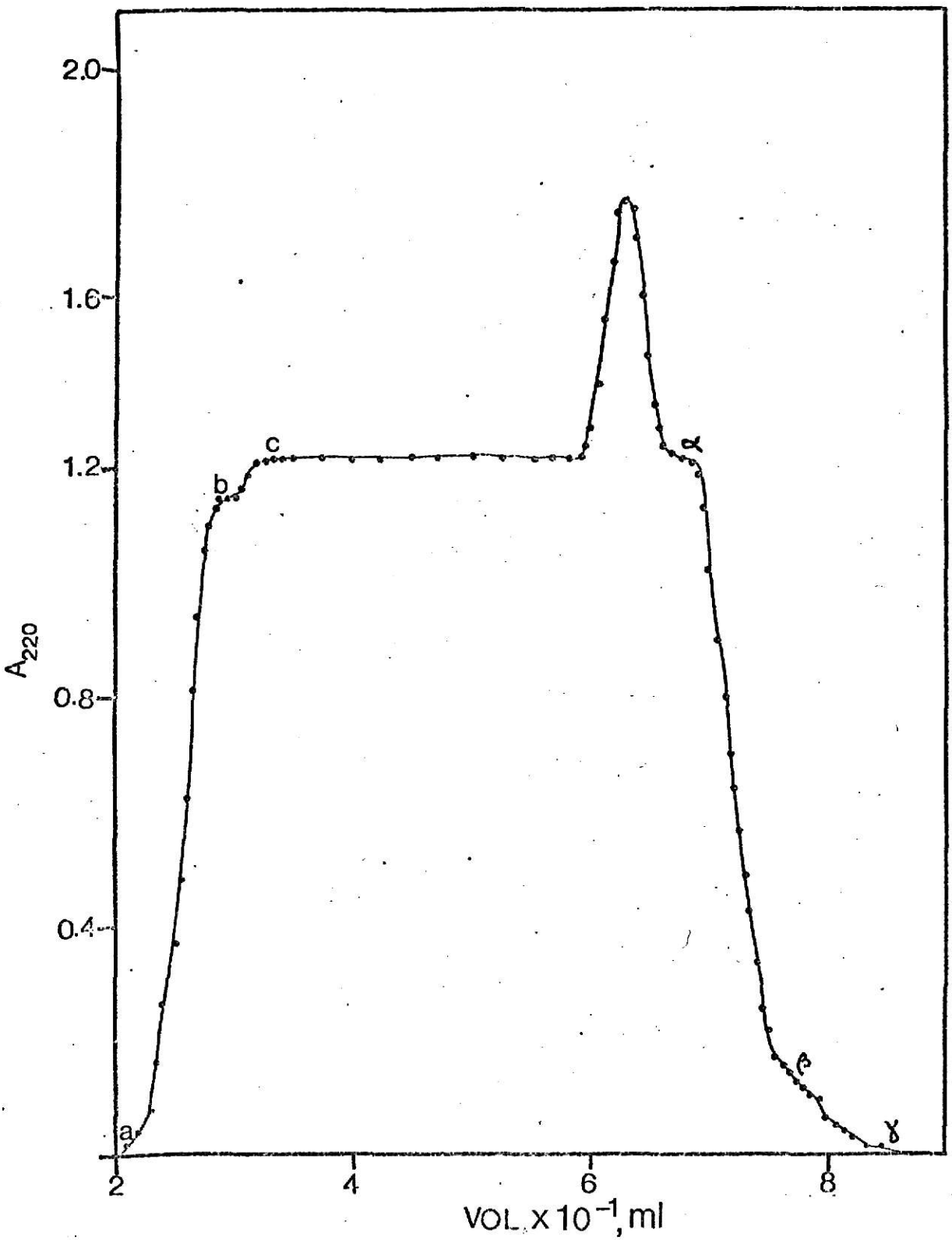


Figure 6

SDS gel electrophoresis. The elution profile, Fig. 7, displayed two peaks, a predominant peak (Peak I), followed by a comparatively smaller peak (Peak II). The elution volume of Peak I was similar to that of the H3-H4 tetramer, while that of Peak II was for a compound with an apparent molecular weight of 30,000 daltons. The shapes of the edges of Peak I and the progressive development of Peak II suggested that the tetramer was dissociating irreversibly. Since this information was acquired from a number of experiments on the H3-H4 tetramer, obtained from separate preparations, the possibility that this effect could have been an artifact of a particular sample was ruled out. The components of these peaks were identified by subjecting representative samples from various points of the profile to SDS gel electrophoresis (Fig. 8). Peak II contained only H4. Moreover, the leading peak was found to be heterogenous. The trailing half of Peak I appeared to be unchanged tetramer, but the leading half of the zone contained a considerable excess of H3. This verified our surmise that the tetramer was dissociating irreversibly into its parent subunits, under the conditions we were using (I=0.05, pH=5.0).

It was surprising that H3, with a molecular weight of 15,300 daltons would elute ahead of the H3-H4 tetramer of molecular weight 54,000 daltons. It seemed probable, that after dissociation, the H3 underwent a self-association step to form aggregates, in which the arrangement of individual H3 molecules differed from that present in the tetrameric H3-H4 complex. H4 could elute either as a monomer or dimer. The reaction for such a complex could proceed as follows:

$$(H3\text{-}H4)_2 \longrightarrow (H3)_n + (H4)_m$$

Fig. 7. Gel filtration on Sephadex G-150 of histones $(H3\text{-}H4)_2$ tetramer. 0.7 ml 0.05 M sodium acetate (pH 5.0) containing 1.28 mg histone $(H3\text{-}H4)_2$ were applied to the column. equilibrated with 0.05 M sodium acetate buffer (pH 5.0). Bed dimensions 0.959 X 100 cm. Flow rate 1.93 ml/hr. Fraction volume 0.573 ml.

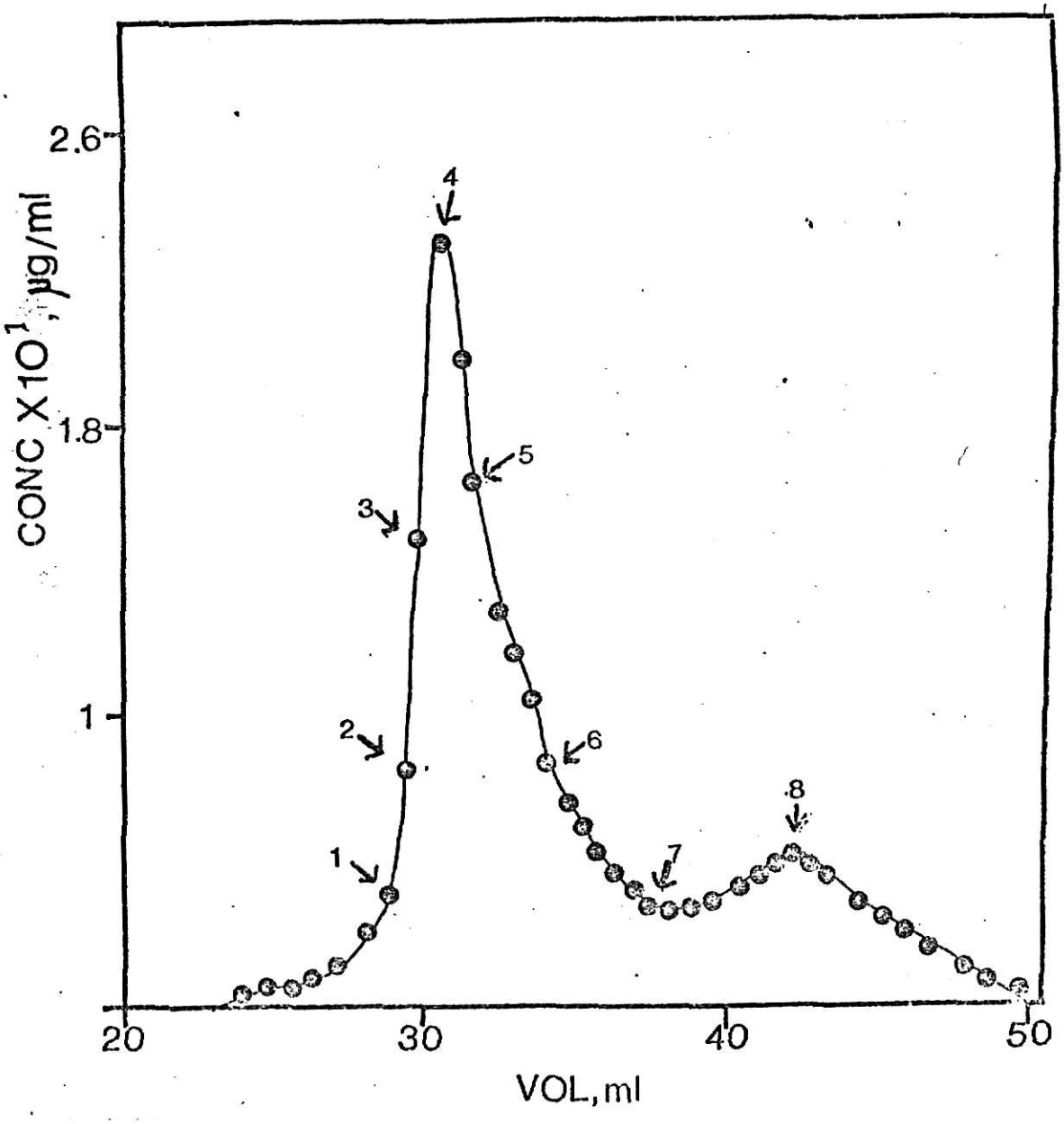


Figure 7

Fig. 8. SDS polyacrylamide gel electrophoresis of samples from chromatogram in Fig. 7. The number below each gel indicates the position the sample was taken from the chromatogram. Migration was from the top to the bottom.

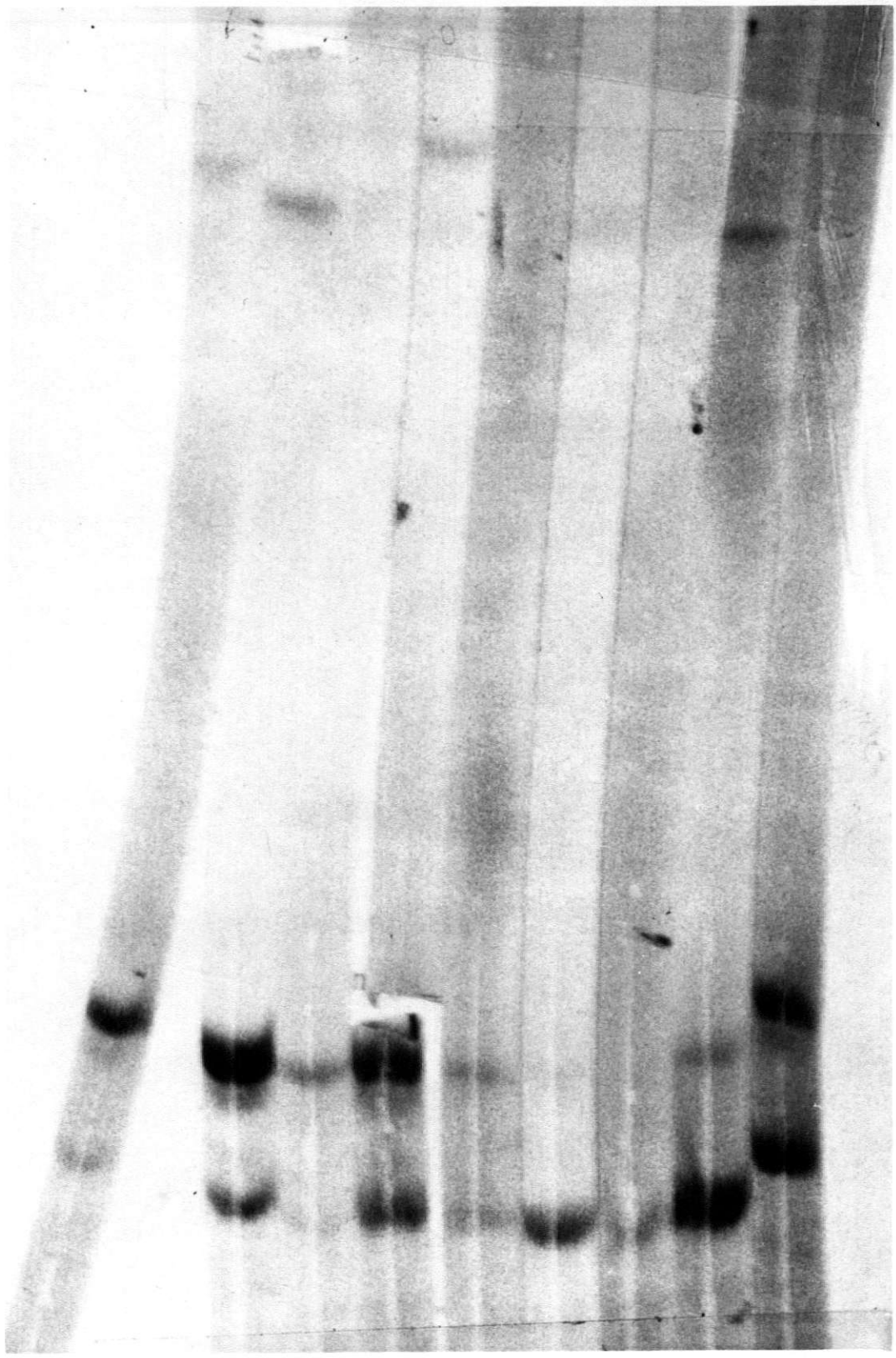


Figure 8

where n is large and m is either 1 or 2.

With this in mind, the observed broad zone profile (Fig. 6) could be theoretically explained, in accordance with the reaction given above. Fig. 9 illustrates a schematic profile of the behavior of the components of the reaction. The H3 aggregate would elute near the void volume, followed closely by the H3-H4 tetramer and eventually by the H4 dimer or monomer, in order of decreasing molecular weights. In theory, both the leading and trailing boundaries would be expected to possess three plateaus. However the intact H3-H4 tetramer elutes close to the void volume and so might not be well resolved from the H3 aggregate, even if the latter were quite large. The overall picture for the irreversible dissociation of the H3-H4 tetramer is illustrated in Fig. 9.

The ability of H3 and H4 to aggregate into high molecular weight structures has previously been reported by other investigators. The aggregation is dependent on protein concentration, ionic strength and temperature. Diggle and Peacock (34) showed that the self-association of H3 or H4 molecules involved a fast structural change with the formation of α-helical structures and aggregation, followed by a slow structural change with the formation of β-structure. The first step in the assembly process was observed, under an electron microscope, by the appearance of bent rods. Sperling and Bustin (35) have found bent rods for H3 at an ionic strength of 0.01, while for H4 bent rods began to appear at an ionic strength of 0.15. Under the conditions used in our experiments, one would expect aggregation of H3 but not H4.

Fig. 9. Schematic representation for the broad zone chromatogram. Broken lines represent the H3 aggregate. Solid lines represent the H3-H4 tetramer. Dotted lines represent H4 molecules.

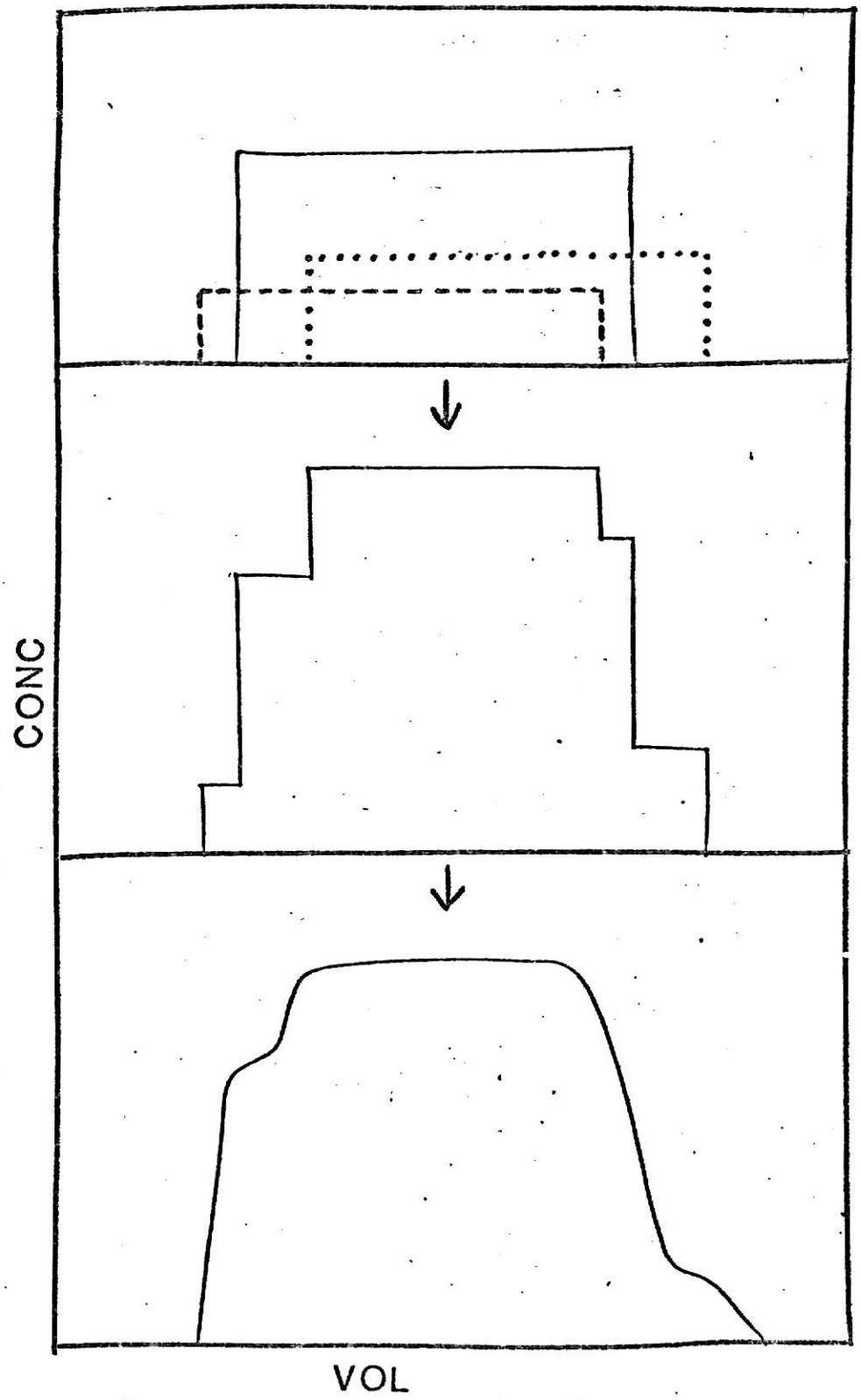


Figure 9

To try to determine the cause for this irreversible dissociation, and to find conditions under which it might be prevented, we conducted a series of narrow zone experiments with varying solute concentrations (Figs. 10 and 12). Both these elution profiles exhibited two peaks as was observed in Fig. 7. As the initial solute concentration was decreased, Peak I was shifted further away from the void volume and Peak II became more prominent. Gels from various points of these profiles (Figs. 11 and 13) indicated the presence of aggregated H3 on the leading edge, followed by a region containing equal amounts of H3 and H4, indicating the presence of intact H3-H4 tetramer. Peak II contained only H4. The spreading of the bands in the gels in Fig. 13 was due to overloading of sample. These experiments indicated, as might be expected, that the dissociation of the tetramer proceeded more rapidly at low concentrations.

It seemed possible that aging of the samples might lead to progressive oxidation of histone H3 that could account for the gradual loss of the H3-H4 tetramer. Oxidation of H3 molecules would result in the formation of disulfide bonds between cysteine residues. Since the H3 molecule of chick erythrocytes contains only one cysteine residue, H3 dimers would form irreversibly. Aggregates of H3 would be formed by self-association of the dimers, and due to their large molecular weight would elute close to the void volume. The remaining H4 from the H3-H4 tetramer would then elute separately as monomers or dimers. It seemed worthwhile, therefore, to examine more systematically the effect of age on the elution profiles given

Fig. 10. Elution profile from a Sephadex G-150 column. The sample was 2.5 mg of H3-H4 tetramer dissolved in 0.7 ml 0.05 M sodium acetate buffer (pH 5.0). Bed dimensions were 0.959 X 100 cm. Flow rate 1.810 ml/hr. Fraction volume 0.543 ml. Sample was 5 days old.

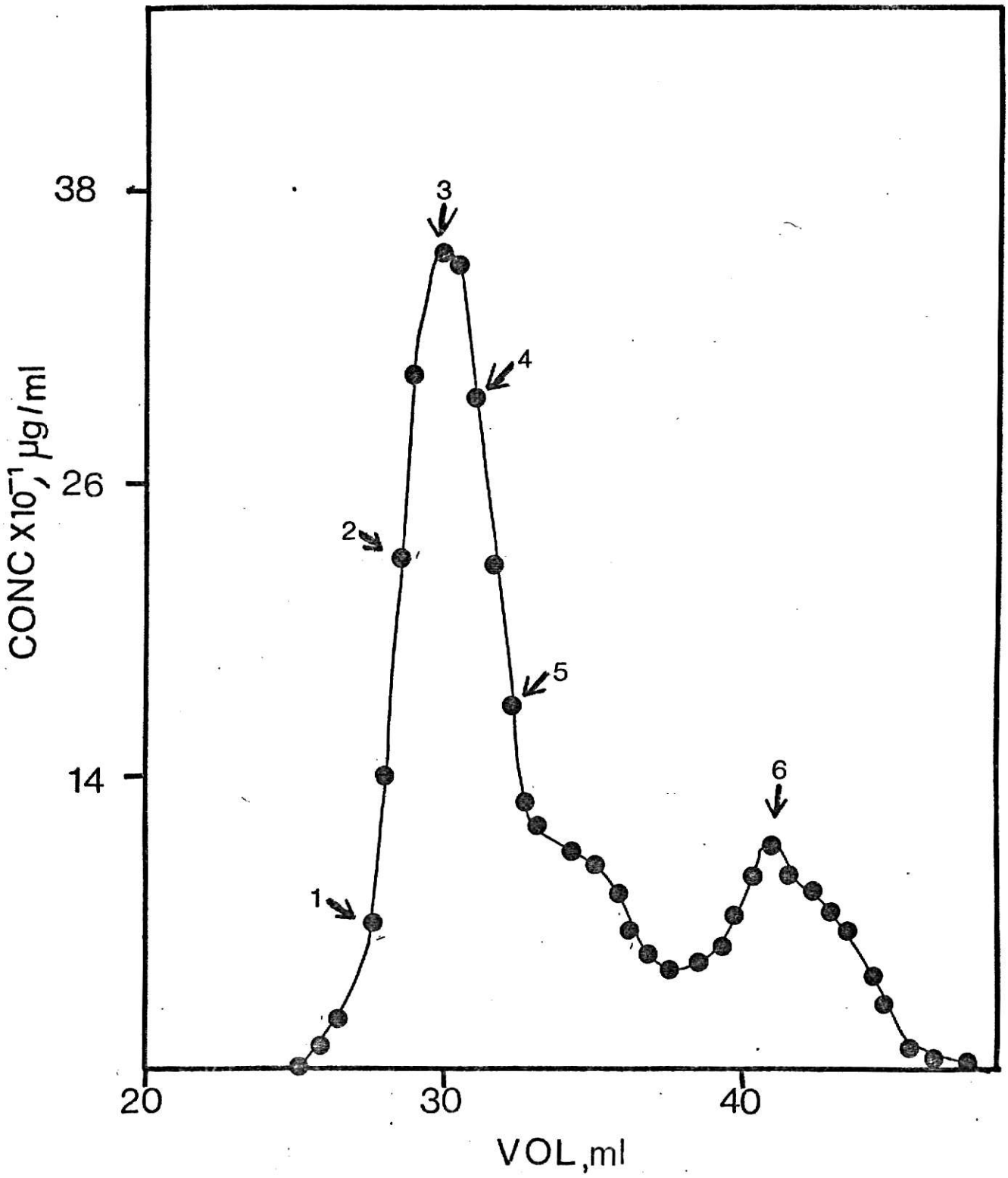


Figure 10

Fig. 11. SDS polyacrylamide gel electrophoresis of samples from chromatogram in Fig. 10. Migration was from the top to the bottom. The number below each gel indicates the position the sample was taken from.

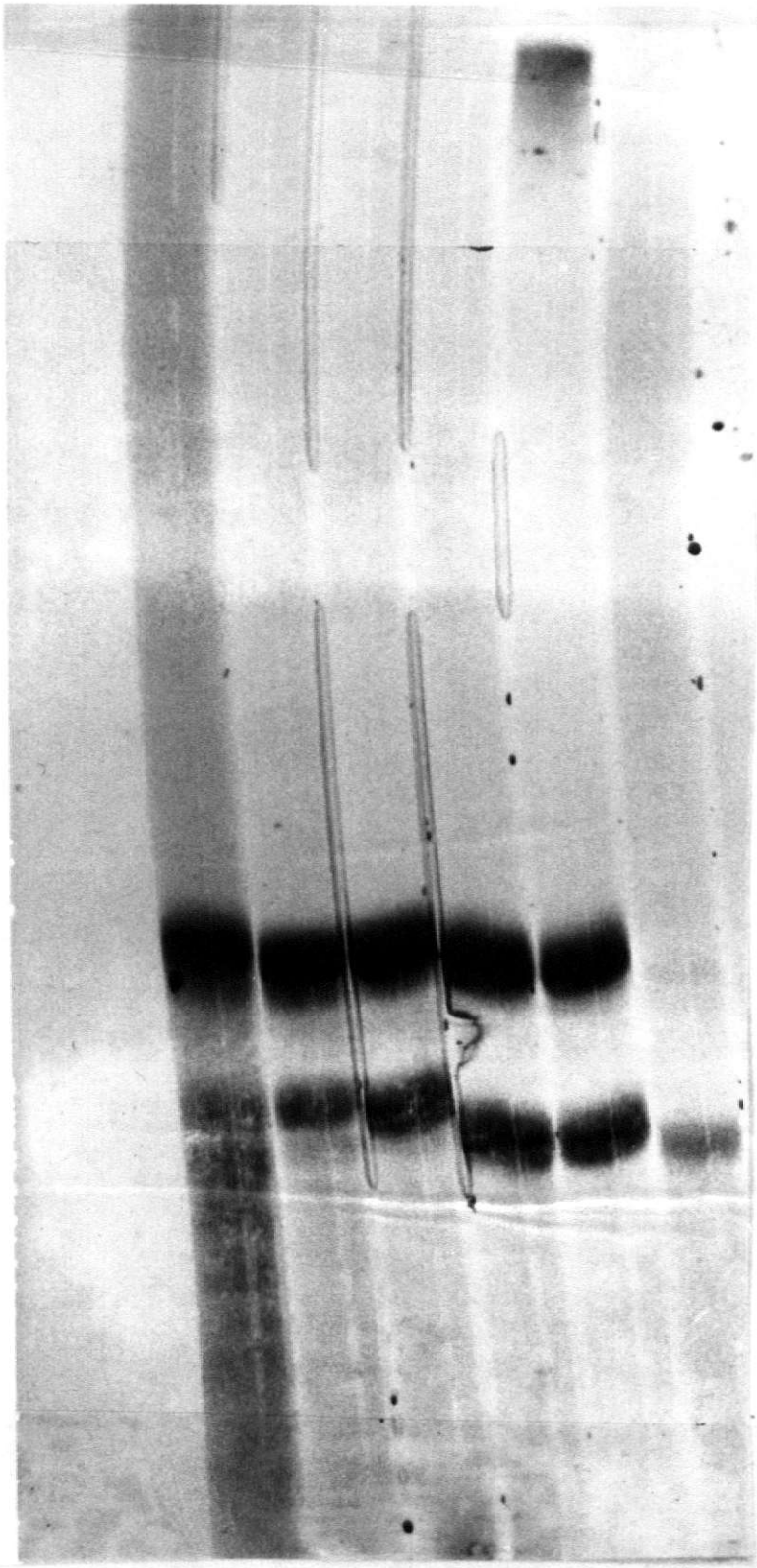


Fig. 11

Fig. 12. Chromatography of histone H3-H4 tetramer on a Sephadex G-150 column. The sample was 0.50 mg dissolved in 0.7 ml 0.05 M sodium acetate buffer (pH 5.0). Bed dimensions 0.959 X 100 cm. Flow rate 1.996 ml/hr. Fraction volume 0.599 ml. Age of sample before application was 5 days.

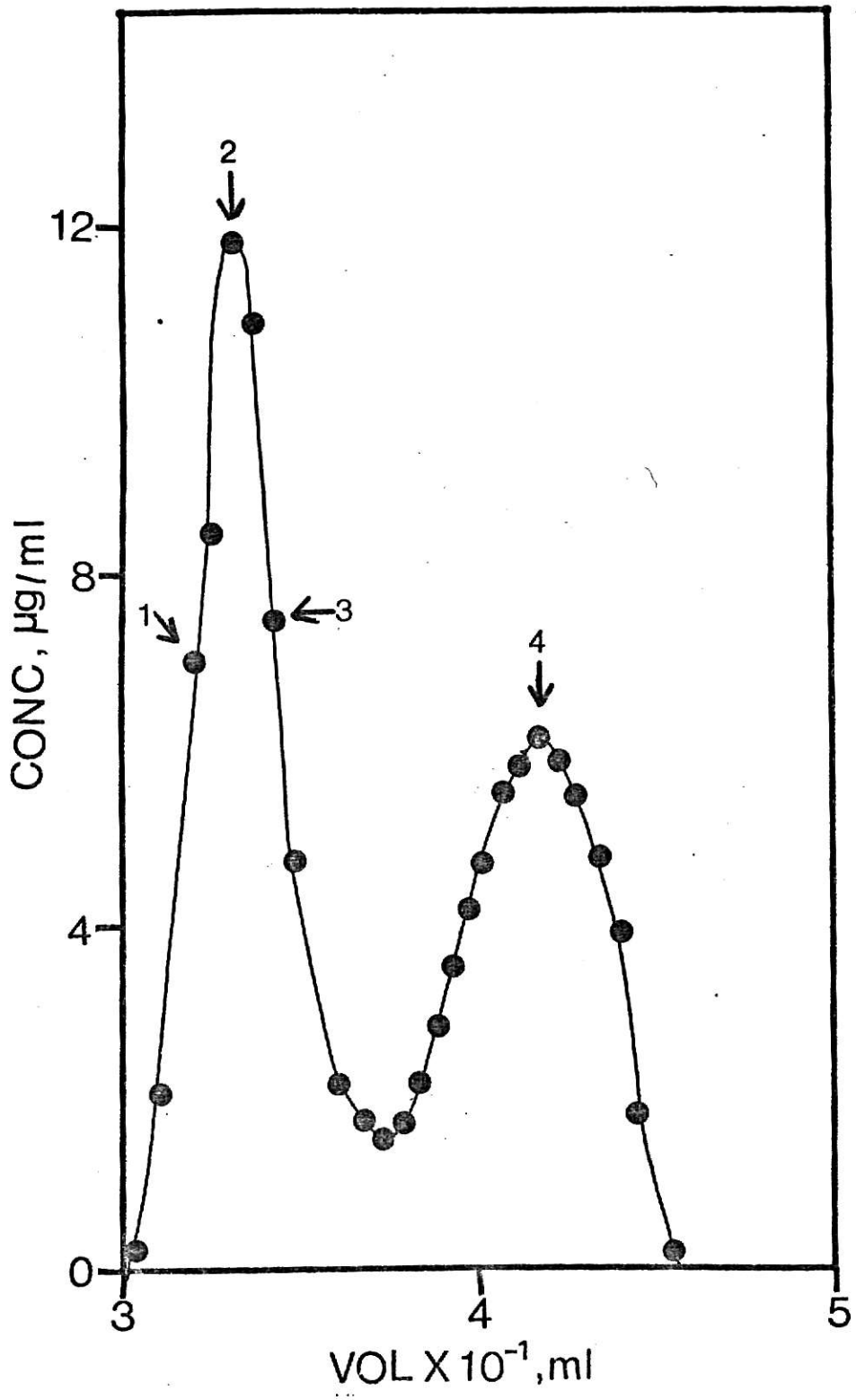
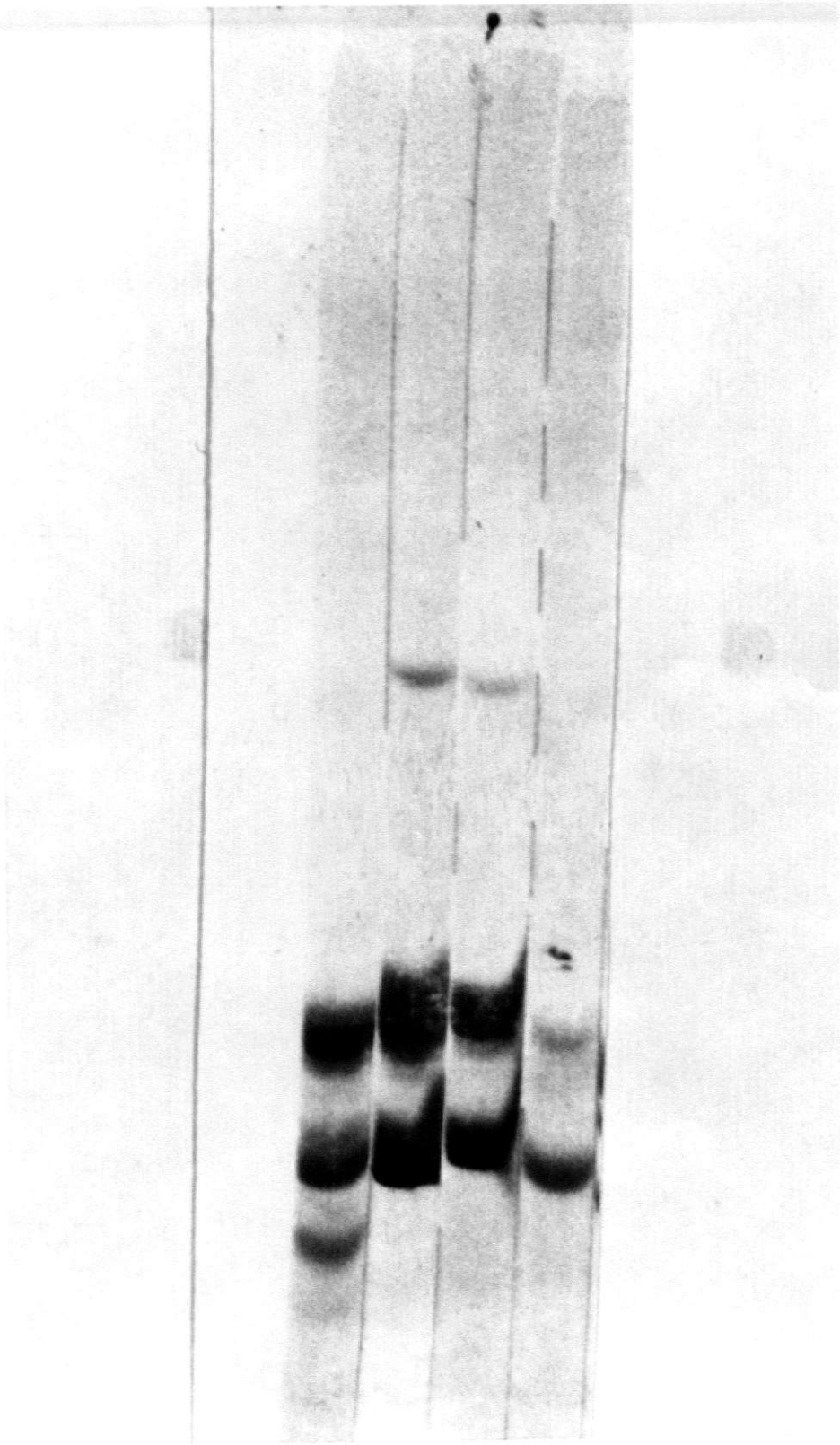


Figure 12

Fig. 13. SDS polyacrylamide electrophoresis of samples from chromatogram in Fig. 12. Migration was from top to bottom. The number below each gel indicates the position, on the chromatogram, that the sample was taken from.



1 2 3 4

Fig. 13

by the H3-H4 tetramer. The elution profiles for samples at varying ages are shown in Figs. 14,16 and 18 as specified by the legends. Age was determined by the number of days the sample was stored at 4°C, day one being the day the sample of total histones was applied to the preparative Sephadex G-100 column. Figs. 15,17 and 19 show the gels obtained for each of the profiles. When a sample was run immediately after isolation, Fig. 14, a small peak consisting almost entirely of H3 was resolved from the remaining H3-H4 tetramer. The second peak, whose elution volume was within the elution range for H3-H4 tetramer, contained H3 and H4 in equal amounts, as seen in Fig. 15. The trailing edge possessed a slight hump which was seen in the gels to be predominantly H4. After 4 days, Fig. 17, the amount of aggregated H3 had increased sufficiently, that it was no longer resolved from the residual tetramer, and the amount of H4 released by the disaggregation of the tetramer had increased. This was what we would have expected for such a sample in which the extent of oxidation was high.

The sample whose elution profile is shown in Fig. 18, was both older and more dilute than the ones shown in Figs. 14 and 16. Here the H3 peak was very much larger, and the elution volume of the remaining tetramer had shifted substantially, giving a lower apparent molecular weight. The gels in Fig. 19 showed that this peak still contained H3 and H4 in approximately equal amounts. However, from the position of the peak in the profile, we suspected that the stoichiometry of the H3-H4 complex here, was different from that of the H3-H4

Fig. 14. Chromatography of the H3-H4 tetramer on a Sephadex G-150 column. The sample was 1.03 mg of H3-H4 tetramer dissolved in 0.7 ml 0.05 M sodium acetate buffer (pH 5.0). Bed dimensions 0.959 X 100 cm. Flow rate 2.053 ml/hr. Fraction volume 0.616 ml. Age of the sample before application was 1 day.

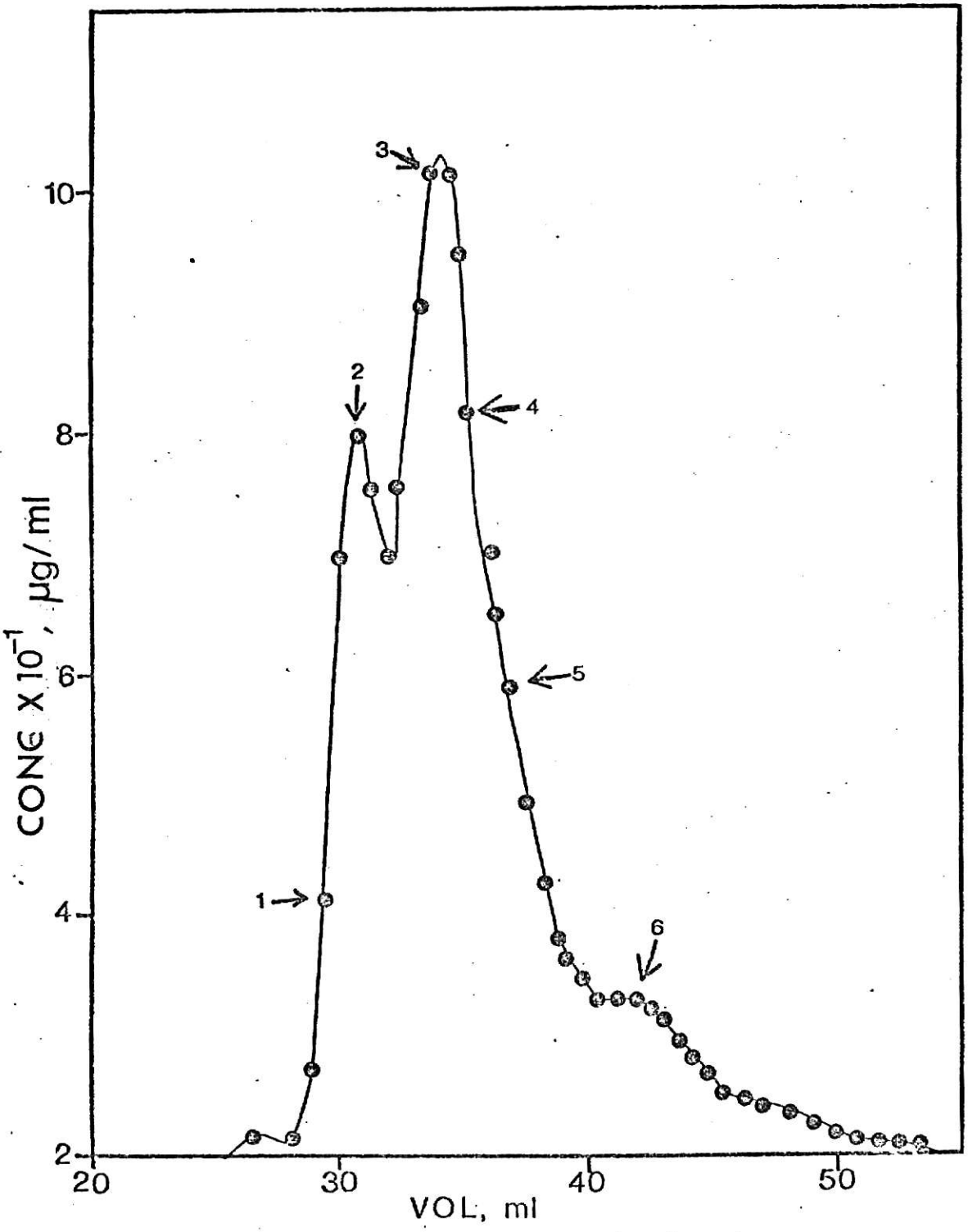
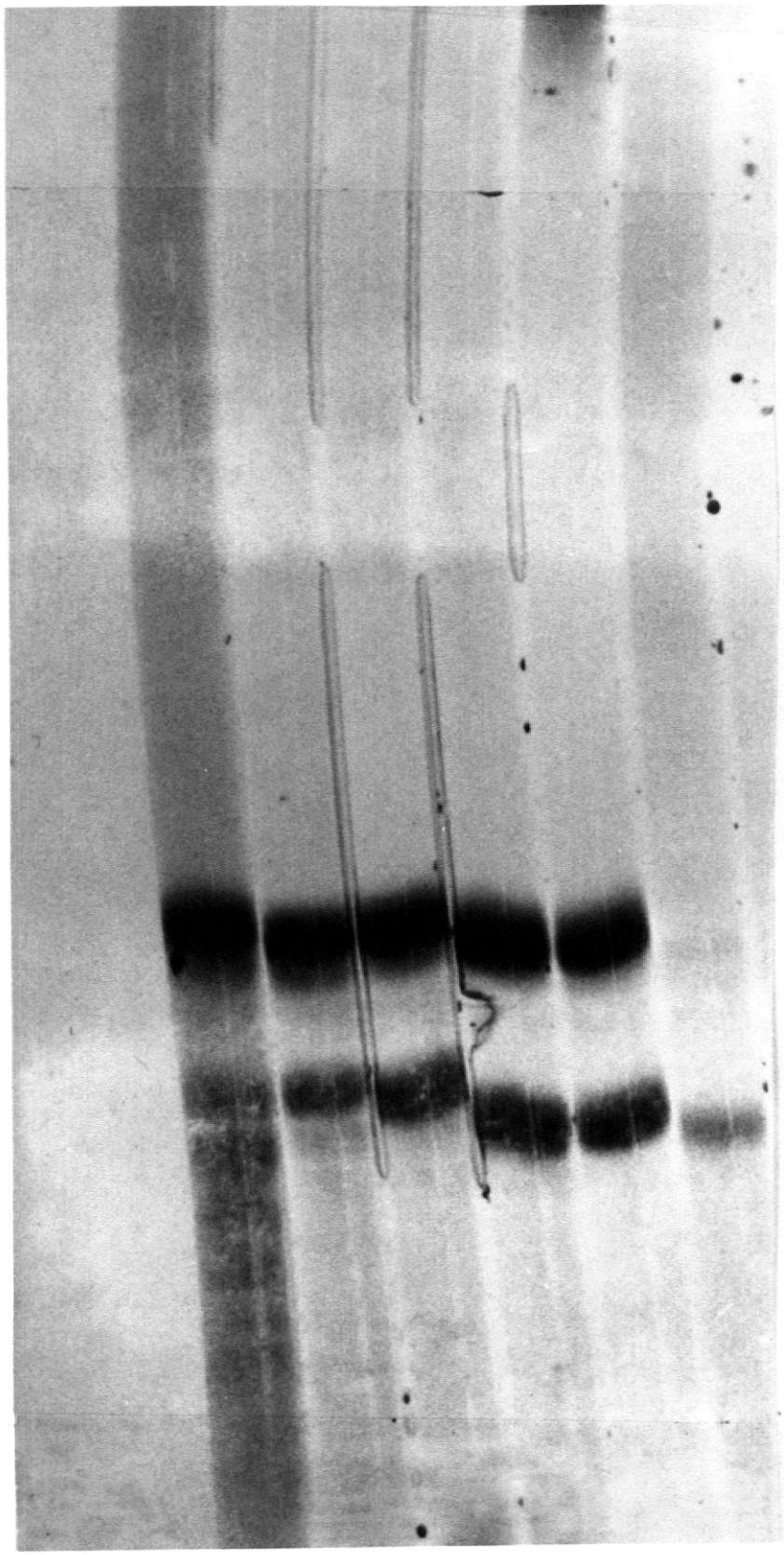


Figure 14

Fig. 15. SDS polyacrylamide gel electrophoresis of samples from the chromatogram shown in Fig. 14. The migration was from top to bottom. The number below each gel represents the position the sample was taken from the chromatogram.



1 2 3 4 5 6

Fig. 15

Fig. 16. Gel filtration on Sephadex G-150 of histones H3-H4 tetramer. 0.7 ml 0.05 M sodium acetate buffer (pH 5.0) containing 1.02 mg H3-H4 tetramer was applied to the column. Bed dimensions 0.959 X 100 cm. Flow rate 2.05 ml/hr. Fraction volume 0.615 ml. Age of the sample before application was 4 days.

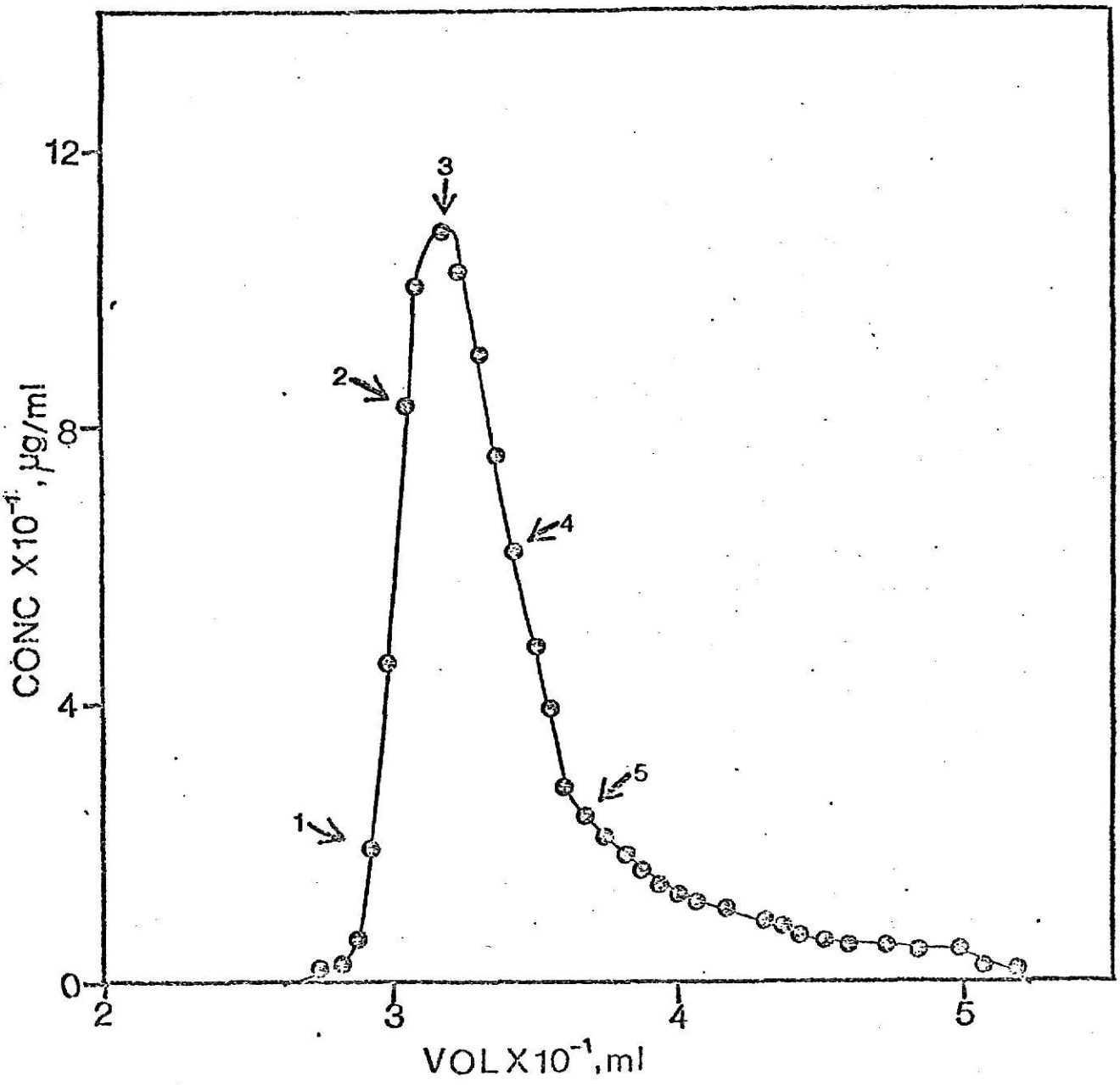


Figure 16

Fig. 17. SDS polyacrylamide gel electrophoresis of samples from the chromatogram shown in Fig. 16. The migration was from top to bottom. The number below each gel represents the position the sample was taken from the chromatogram.

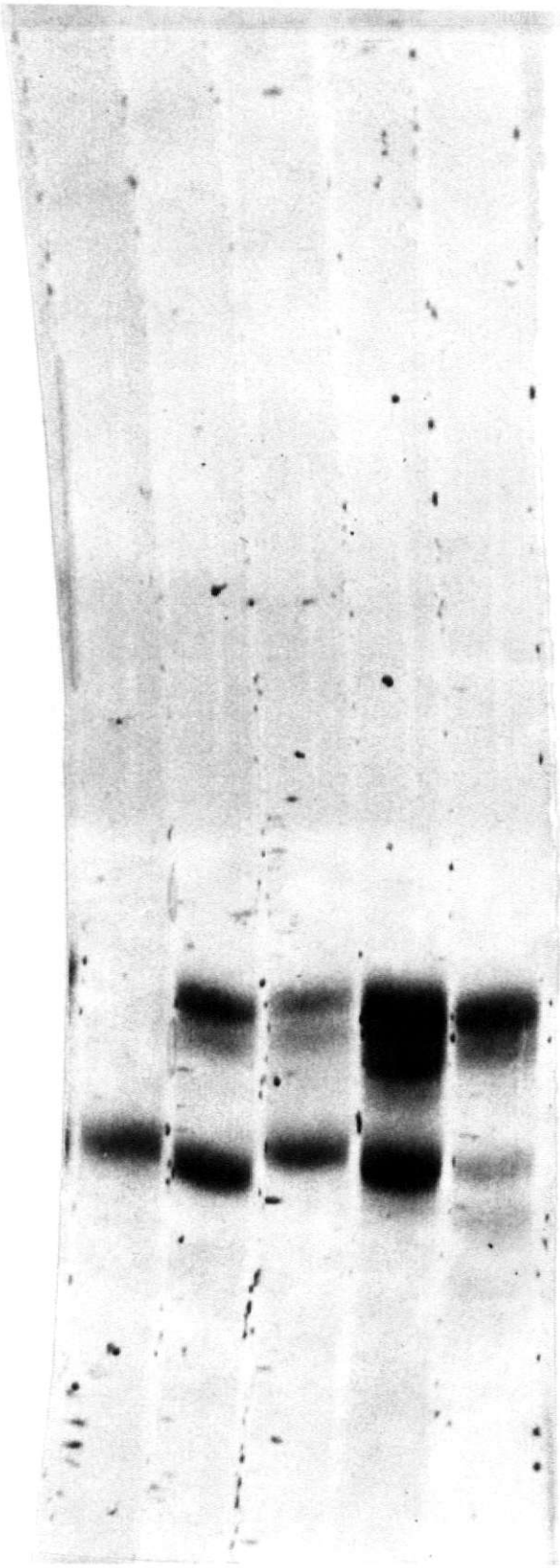


Fig. 17

Fig. 18. Gel filtration on Sephadex G-150 of the histone H3-H4 tetramer. 0.7 ml, 0.05 M sodium acetate buffer (pH 5.0) containing 0.532 mg histone were applied to the column. Bed dimensions 0.959 X 100 cm. Flow rate, 1.956 ml/hr. Fraction volume, 0.587 ml. Age of the sample before application was 7 days.

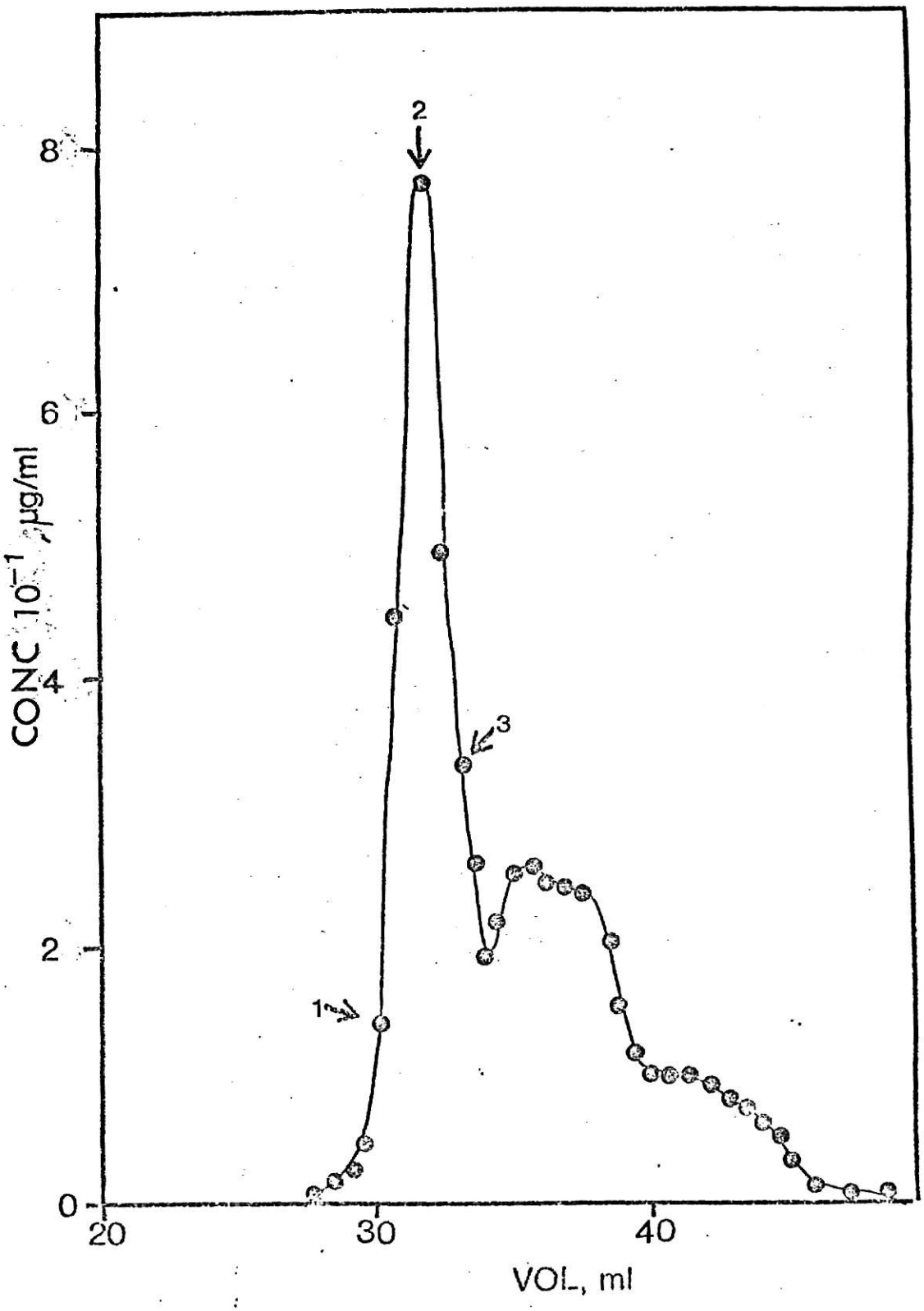


Figure 18

Fig. 19. SDS polyacrylamide gel electrophoresis of samples from the chromatogram shown in Fig. 18. The migration was from top to bottom. The number below each gel represents the position the sample was taken from the chromatogram.

1 2 3

Fig. 19

tetramer. The shift in elution volume may perhaps reflect partial dissociation to H3-H4 dimers of the kind reported by Roark et al. It was not possible to obtain a gel pattern for the trailing shoulder of the peak due to the extremely low concentration. However, since H4 was found at the outer edge of the trailing boundary in Figs. 14-16, we presume that H4 is at the trailing shoulder of the peak in Fig. 18, and is present in much larger quantities than in Figs. 14-16.

The above observations suggested that the system in our hands was behaving in a much more complex way than the quite similar samples examined by Roark et al. We suspected that this effect could possibly be caused by some step in our isolation procedure, perhaps by precipitation of the tetramer with solid ammonium sulfate and subsequent dissolution at low salt concentration. Further, it seemed worthwhile to consider whether the presence of histones in addition to H3 and H4 might tend to suppress the progressive dissociation of the tetramer. A sample of histones, of the highest concentration from Peak I of the preparative Sephadex G-100 column, was directly applied to an experimental Sephadex G-150 column. Notice that this sample had not been exposed to ammonium sulfate and therefore contained histones H1 and H5 in addition to the H3-H4 tetramer. Roark (2) has shown by separate chromatography on H3-H4 fractions and H1 fractions, that both gave elution volumes identical to that of the original peak indicating the absence of interaction between H1 and the H3-H4 complex. Here, we assume that H5, being almost identical to H1, would similarly not interact with the

H3-H4 tetramer. The elution profiles from this experiment, Fig. 20, resembled that of the other elution profiles for samples containing only H3-H4 tetramer. Due to the raised baseline it was not possible to observe a peak for the H4, however the gels displayed in Fig. 21, showed the excess H3 early in the profile and the trailing excess of H4 that were seen with isolated tetramer of the same age, in addition to the H1 and H5 bands. Ammonium sulfate apparently did not affect the histone complex in any way, and the presence of H1 and H5 did not prevent the dissociation of the H3-H4 tetramer.

To determine whether the irreversible change in the tetramer occured only after the removal of histones H2A and H2B, a sample was examined that had not been subjected to preparative chromatography on Sephadex G-100. A sample of histones, obtained from BioRex-70 in 3 M NaCl was desalted over a Sephadex G-25 column (2.2 X 20 cm) equilibrated and developed with 0.05 M sodium acetate buffer (pH 5.0). The resultant peak was pooled and H1 and H5 were removed by precipitating histones H3, H4, H2A, and H2B with solid ammonium sulfate to 70% (w/v) saturation. The pellet was then dissolved in 0.05 M sodium acetate buffer (pH 5.0), and a small volume (0.7 ml) was applied to an experimental Sephadex G-150 column. The resultant elution profile, Fig. 22, displayed two predominant peaks, the first one representative of the H3-H4 tetramer, while the second of H2A-H2B dimer. The small peak seen on the leading edge of the first peak was possibly due to H3 aggregate. The gels obtained from samples of this

Fig. 20. Chromatogram from a Sephadex G-150 column. The sample was 1.12 mg of H3, H4, H1 and H5 dissolved in 0.7 ml 0.05 M sodium acetate buffer (pH 5.0). Bed dimensions 0.959 X 100 cm. Flow rate 1.930 ml/hr. Fraction volume 0.591 ml. The age of the sample before application was 2 days.

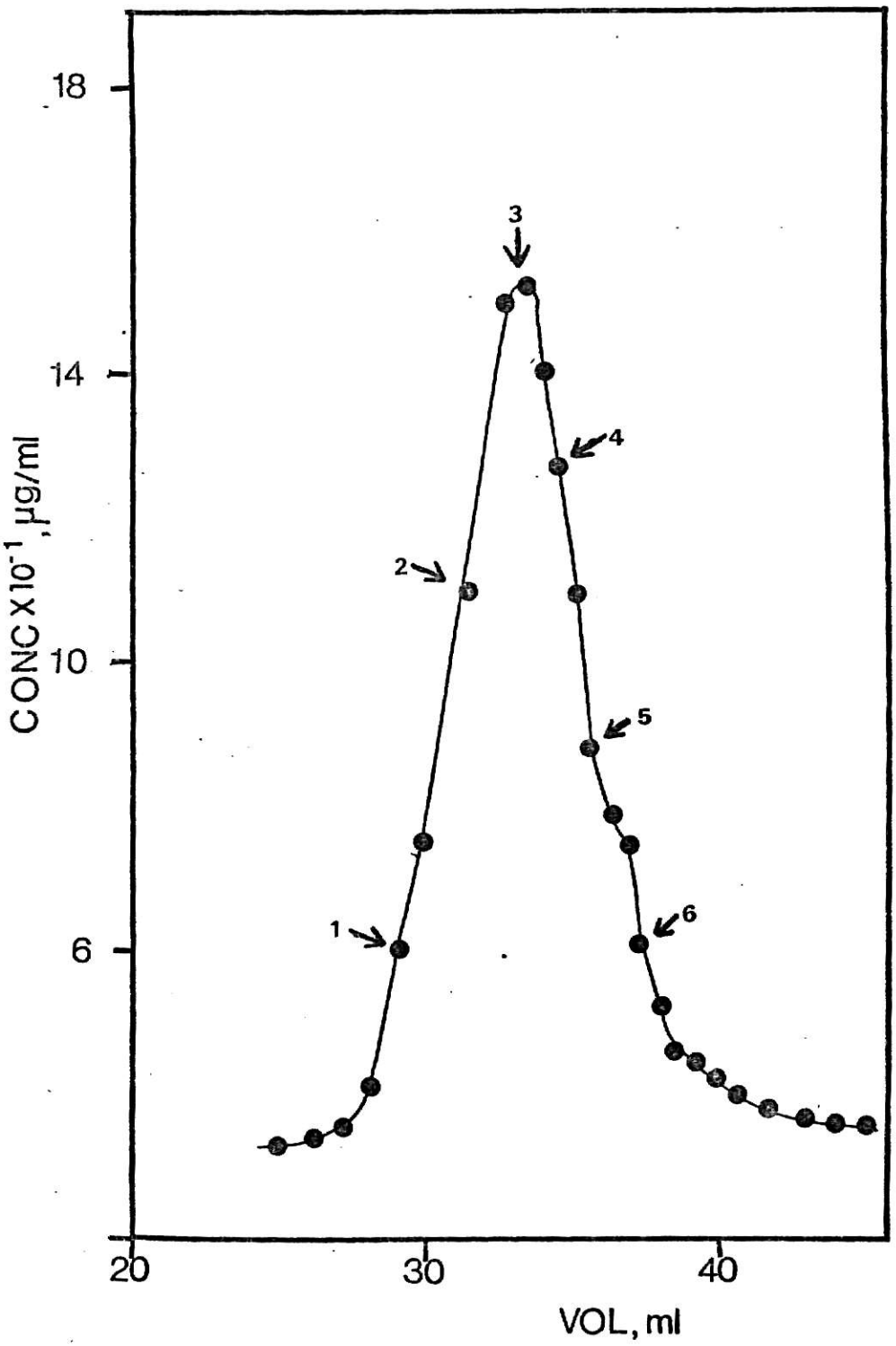


Figure 20

Fig. 21. SDS polyacrylamide gel electrophoresis of samples from the chromatogram shown in Fig. 20. The migration was from top to bottom. The number below each gel represents the position the sample was taken from the chromatogram.

Fig. 21

Fig. 22. Elution profile of histones H2A, H2B, H3 and H4 on a Sephadex G-150 column (0.959 X 100 cm) equilibrated in 0.05 M sodium acetate buffer (pH 5.0). The sample contained 9.1 mg of histones in 0.7 ml 0.05 M sodium acetate buffer (pH 5.0). Flow rate 1.976 ml/hr. Fraction volume 0.593 ml. The age of the sample before application was 2 days.

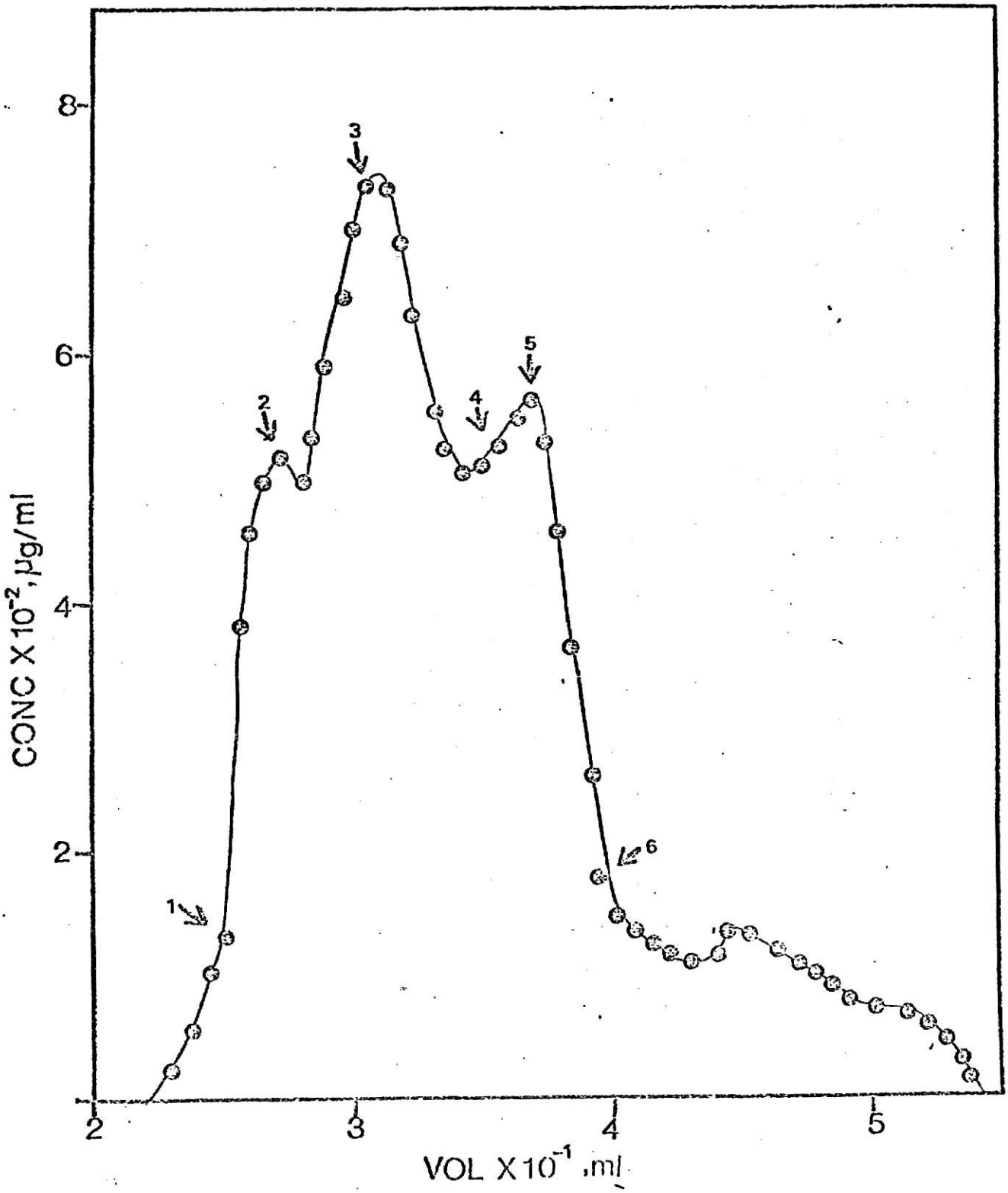


Figure 22

Fig. 23. SDS polyacrylamide gel electrophoresis of samples from the chromatogram shown in Fig. 22. The migration was from top to bottom. The number below each gel represents the position the sample was taken from the chromatogram.

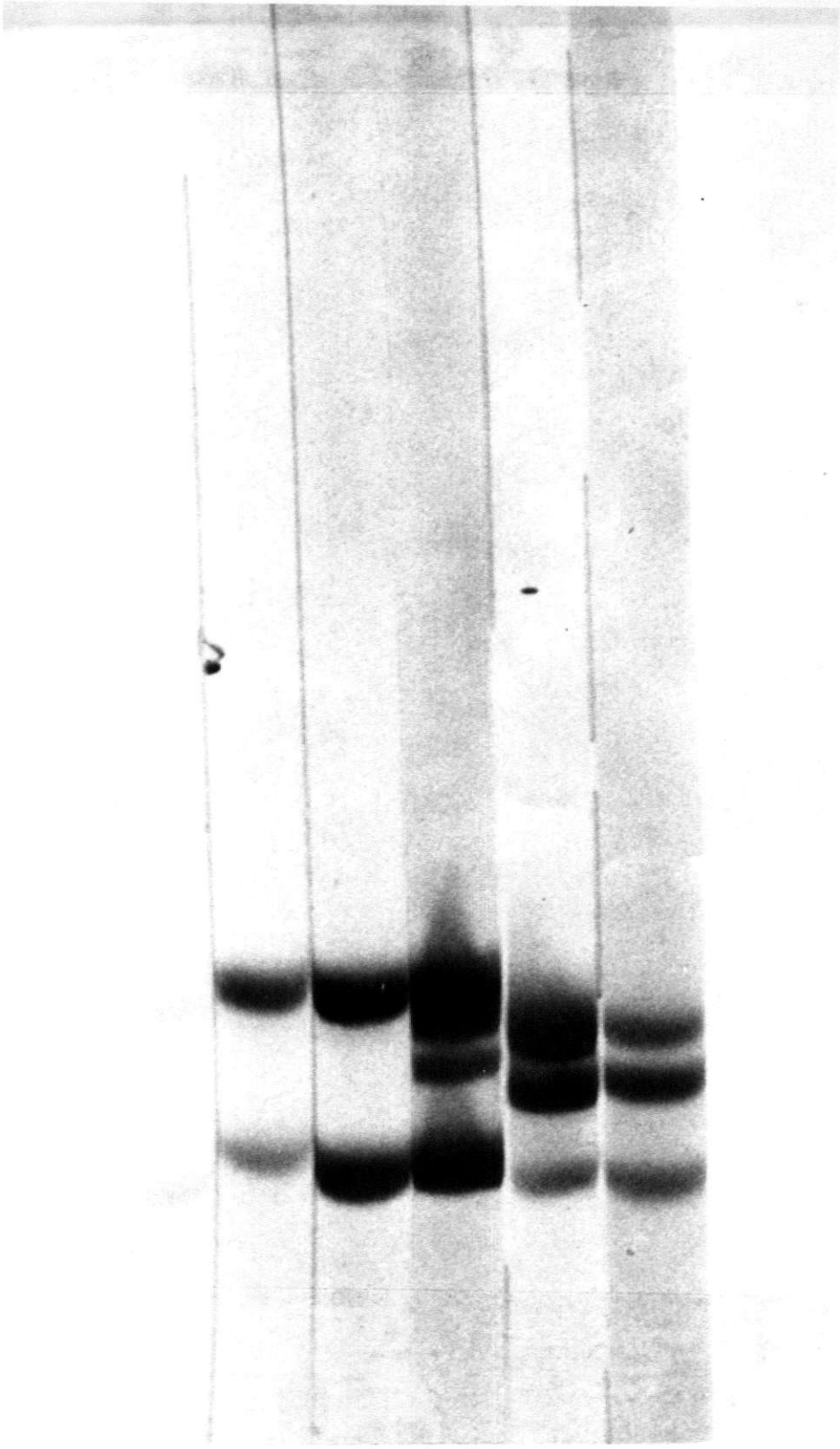


Fig. 23

profile, Fig. 23, in addition to indicating the fractionation of the H3-H4 tetramer and the H2A-H2B dimer, also showed an excess of H3 at the leading edge of the first peak, while H4, but not H3 was present in the second peak. These results indicated that the irreversible dissociation of the H3-H4 tetramer still occured in the presence of H2A and H2B.

The above experiments indicated that the tetramer dissociation equilibrium was shifted by the formation of disulfide bonds between the cysteine residues of the H3 molecule, followed by the aggregation of H3 and the consequent release of H4 from the H3-H4 tetramer. One would expect, therefore, that the effect could be prevented and perhaps reversed by agents capable of splitting disulfide bonds. Lewis (36) has shown that the H3 H4 complex can be formed from separated H3 and H4 only if the cysteine residues in the calf thymus H3 remain reduced. If the sulfhydryl group was allowed to oxidize, under conditions as in our experiments (pH = 5.0, I = 0.05), a mixture of complex with aggregated and monomeric forms of H3 and H4 would be formed. In order to test this model, a sample of pure H3-H4 tetramer was applied to a Sephadex G-150 column equilibrated with 0.05 M sodium acetate buffer (pH 5.0) and 2% β-mercaptoethanol (ME). The ME would reduce any disulfide bonds, if present. Due to the inability to determine the protein content in the effluent spectrophotometrically, caused by the presence of ME in the solvent, the protein content was determined by the Lowry method (37). The data (not shown) obtained by this method, consisted of only one peak at 33.68 ml. It was impossible to observe a peak, if

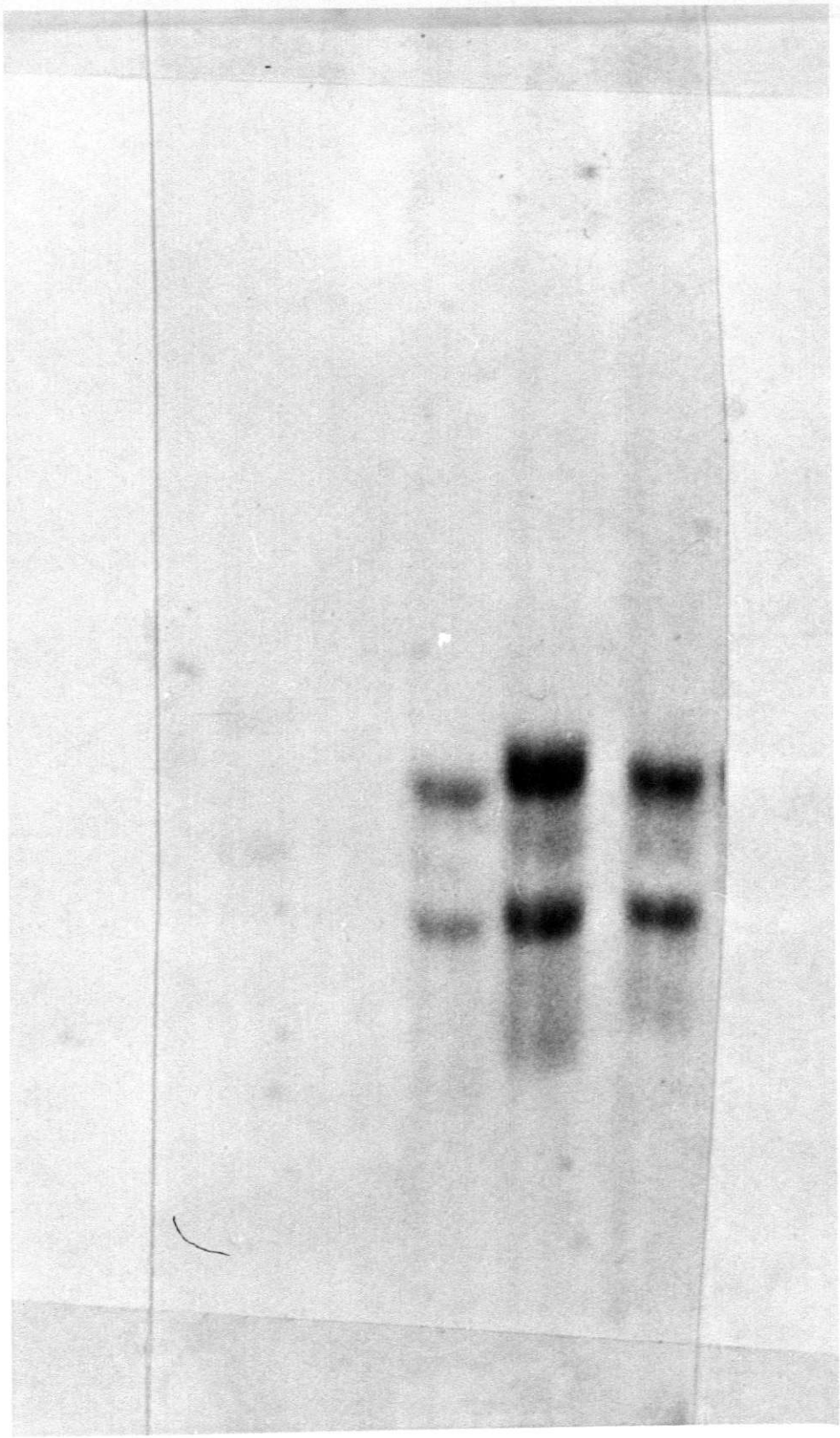
any, for H4 due to the raised baseline. However, gels shown in Fig. 24 indicated the presence of H3-H4 tetramer through the entire elution peak, with no indication of any excess H3 at the leading edge or any free H4. This result was consistent with the hypothesis that dissociation was observed due to the formation of disulfide bonds between the H3 molecules.

In the ultracentrifuge, non-interacting mixtures are not easily distinguished from self-associating solutes, and so it was of some interest to consider the possibility that Roark et al. had misinterpreted the behavior of samples that were in fact, undergoing the complex irreversible process described here. The results of a high speed equilibrium experiment, similar to the one reported by Roark et al. are shown in Fig. 25. It can be seen that within the concentration range of 0.2 mg/ml to 0.57 mg/ml, the variation of M_{app} with solute concentration was quite similar to what Roark described. However, at higher concentrations, molecular weights as high as 65,000 daltons were observed; these values presumably reflect the presence of the H3 aggregate seen in the narrow zone chromatograms. Roark et al. found no molecular weights higher than that of the tetramer, and so substantial amounts of H3 aggregate were probably absent from their system.

Considering that our columns were operated under the same conditions as the experiments conducted by Roark et al. (pH, 5.0, I, 0.05), it was puzzling that their data could not be reproduced in our laboratory. In the isolation procedure used by Roark et al., bisulfite was included in the chromatographic solvents with the stated purpose of suppressing

Fig. 24. SDS polyacrylamide gel electrophoresis of samples from the experiment of histones H3-H4 on a Sephadex G-150 column, in the presence of β-mercaptoethanol. The migration was from top to bottom.

gel 1. outer edge of the leading boundary
gel 2. tip of peak of the elution profile
gel 3. outer edge of the trailing boundary.



1 2 3

Fig. 24

Fig. 25. Plot of M_{app} versus protein concentration for histone H3-H4 tetramer in 0.05 M sodium acetate buffer (pH 5.0). Loading concentration was 0.4 mg/ml.

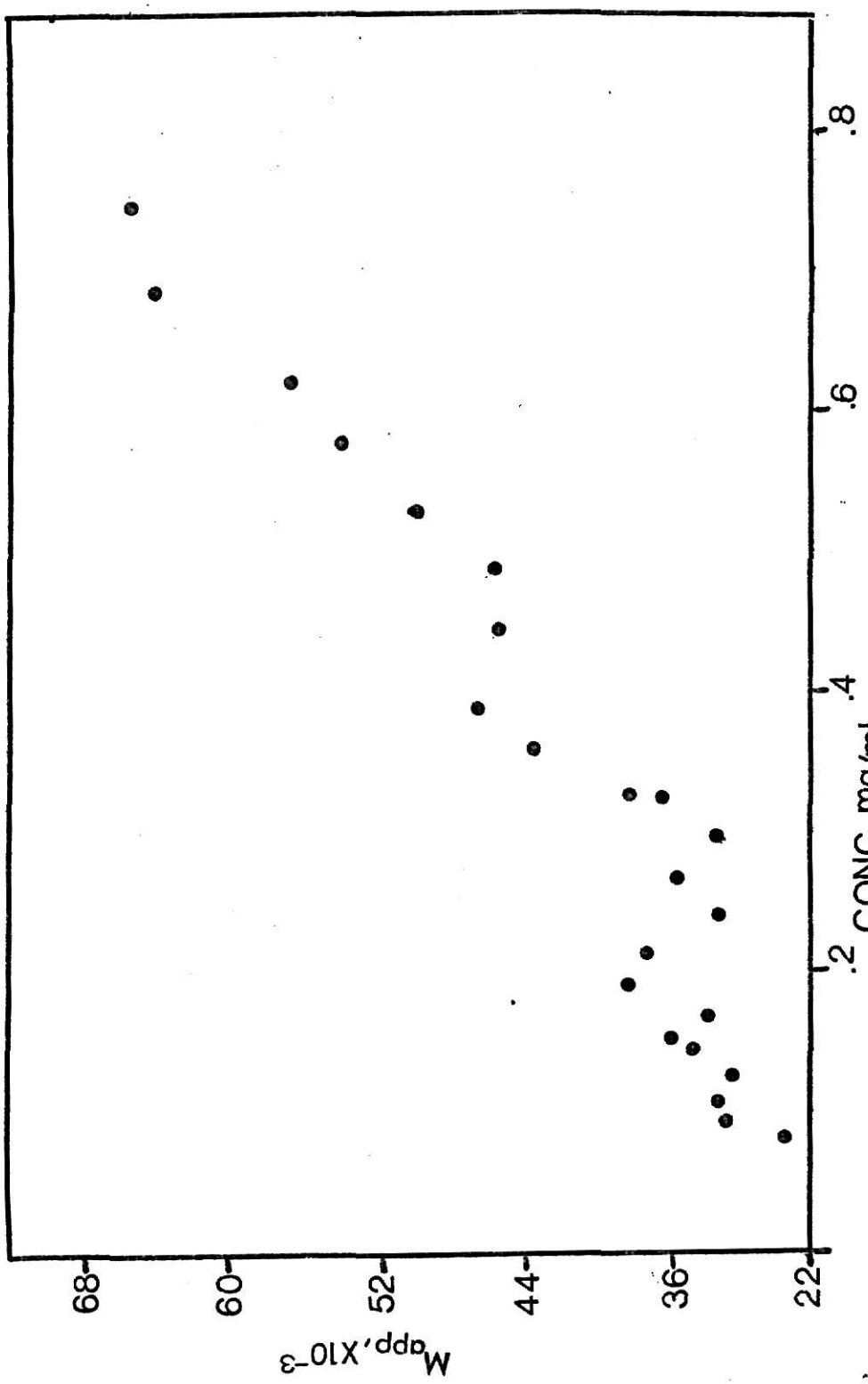


Figure 25

protease activity that is usually associated with calf thymus chromatin. In our experiments, the use of bisulfite was considered unnecessary due to the absence of protease in chick chromatin. However, Cole (38), has shown that sulfite ions can split disulfide bonds to form S-Sulfonates.

$$R\text{-}S\text{-}S\text{-}R + SO_3^{=} \rightleftharpoons R\text{-}S^{-} + R\text{-}SO_3^{-}$$

This suggests that the most significant effect of bisulfite might be the splitting of H3 dimers rather than the nonspecific inhibition of a protease. To test this possibility, a sample of pure H3-H4 tetramer was applied to an experimental Sephadex G-150 column, equilibrated and developed in 0.05 M sodium bisulfite and 0.05 M sodium acetate buffer (pH 5.0). The elution profile, Fig. 26, displayed only one peak whose elution volume was similar to that for the intact H3-H4 complex. Gels of samples taken from various points of the elution profile, Fig. 27, showed equal amounts of H3 and H4 everywhere in the zone, indicating that the H3-H4 tetramer does not dissociate in the presence of bisulfite.

From the above experiments, it appeared that the bisulfite was reversing the dimerization of H3 and so stabilizing the tetramer. If so, then other workers, including Roark et al., have been working with a chemically modified (S-sulfo) histone preparation. It must be asked whether the behavior of these modified subunits can safely be considered relevant to the assembly of the nucleosome <u>in vivo</u>.

Fig. 26. Chromatogram from Sephadex G-150. The sample was 1.9 mg of H3-H4 tetramer dissolved in 0.7 ml 0.05 M sodium acetate buffer (pH 5.0). and 0.05 M sodium bisulfite. Bed dimensions 0.959 X 100 cm. Flow rate 2.023 ml/hr. Fraction volume 0.607 ml. The age of the sample before application was 2 days.

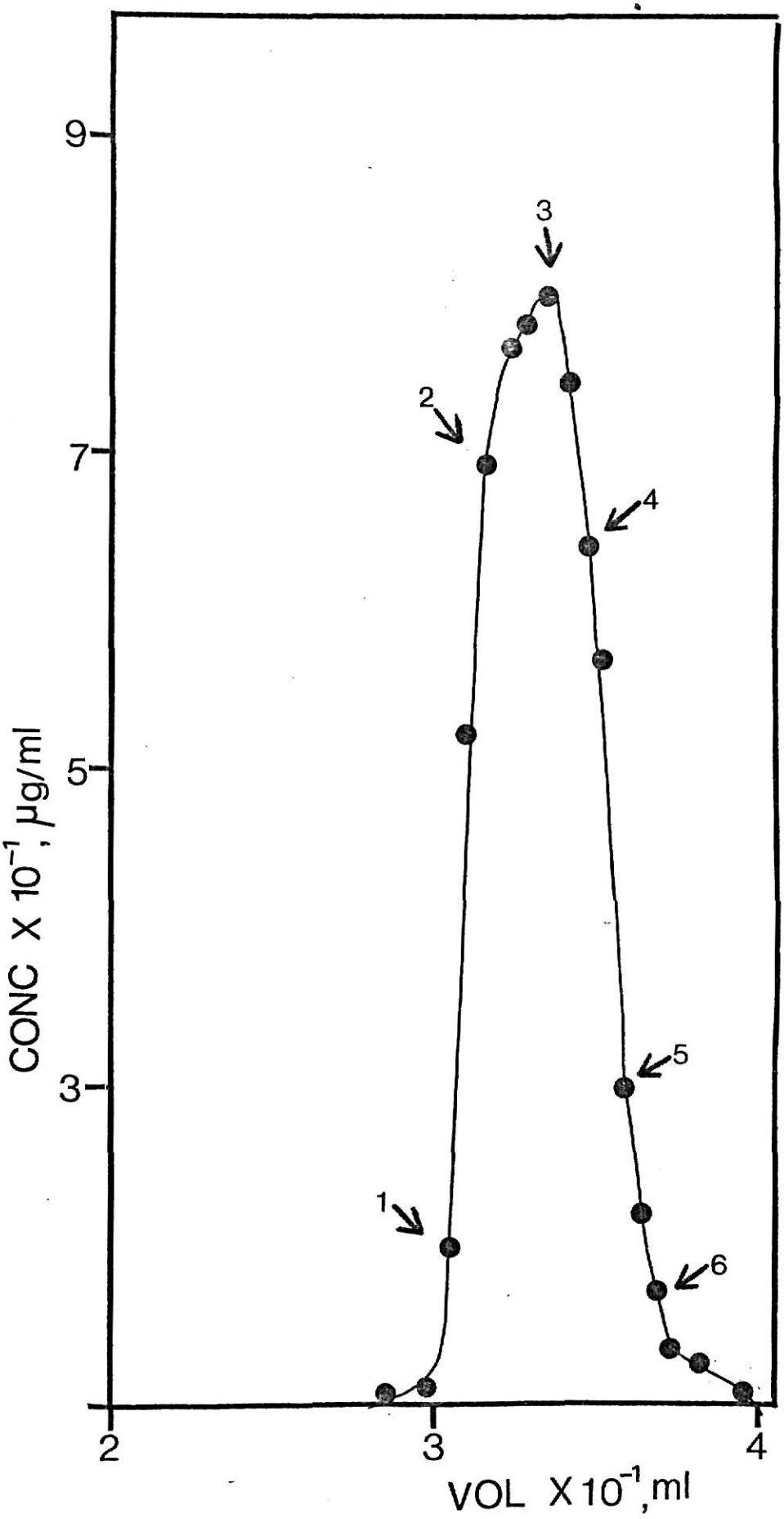
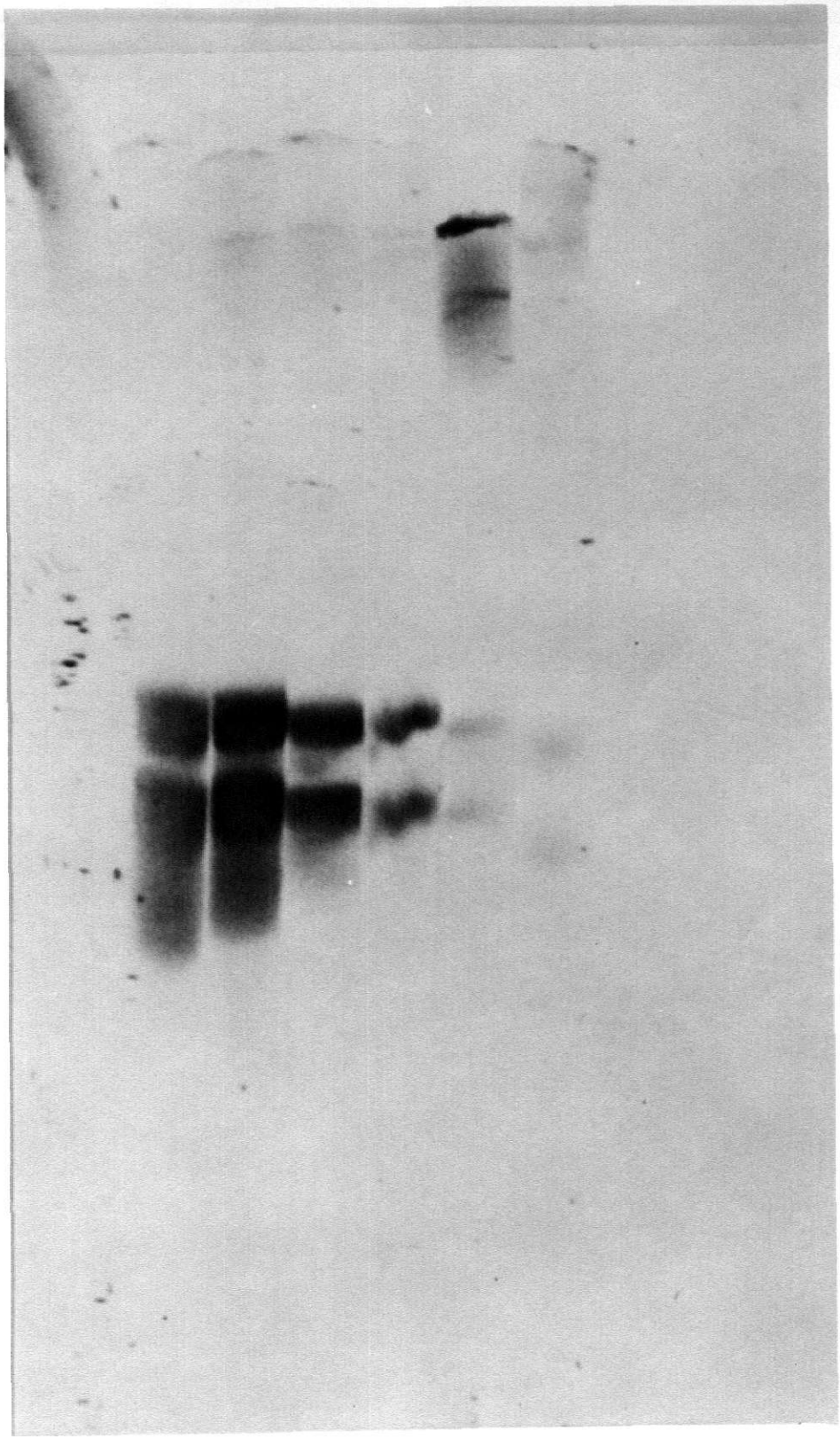


Figure 26

Fig. 27. SDS polyacrylamide gel electrophoresis of samples from the chromatogram shown in Fig. 26. Migration was from top to bottom. The number below each gel represents the position the sample was taken from the chromatogram.



1 2 3 4 5 6

Fig. 27

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AN IRREVERSIBLE DISSOCIATION OF THE HISTONE H3-H4 TETRAMER

by

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AN ABSTRACT OF A MASTER'S THESIS

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Roark et al. (23) interpreted sedimentation equilibrium data as indicating that the histone H3-H4 tetramer isolated from calf thymus undergoes a concentration dependent dissociation to its dimer ($2(H3\text{-}H4) \rightleftharpoons (H3\text{-}H4)_2$). At the lowest concentration accessible to Roark et al. the tetramer was found to be little dissociated and so the dissociation constant was somewhat uncertain. The objective of this study was to obtain a more reliable value for the dissociation constant for the equilibrium reaction by studying the complex at much lower concentrations than those used by Roark et al. where the dissociation would be more nearly complete. We employed broad zone gel permeation chromatography of the H3-H4 tetramer from chicken erythrocyte for this purpose.

The broad zone chromatograms showed that the H3-H4 tetramer appeared to behave as a non-interacting mixture, rather than a self-associating system. Further investigations of this behavior were done by narrow zone chromatographic experiments and the components resolved by chromatography were identified by SDS polyacrylamide gel electrophoresis. In all the experiments, the narrow zone chromatogram of the isolated H3-H4 tetramer displayed two peaks. A predominant one (Peak I), which had an elution volume within the range for the tetramer, was followed by a comparatively smaller peak (Peak II), with an elution volume corresponding to an apparent molecular weight of 30,000 daltons. Peak II contained only H4. The leading peak was found to be heterogeneous. The trailing

half of Peak I appeared to be unchanged tetramer, while the leading half of the zone contained a considerable excess of H3. These results suggested that the tetramer was dissociating irreversibly into its parent subunits, and that, after dissociation, the H3 molecule underwent self-association to form aggregates. H4 could elute either as monomers or dimers. A reaction for the complex was proposed as follows:

$$(H3\text{-}H4)_2 \longrightarrow (H3)_n + (H4)_m$$

where n is large and m is one or two.

The dissociation of the tetramer proceeded more rapidly at low initial solute concentrations than at high concentrations. The behavior of the tetramer was also found to be age dependent. The state of aggregation of the H3 molecules increased from a 2-day old sample to a 7-day old sample, and increased amounts of H4 were released by the disaggregation of the older sample. The isolation procedure, in particular the preparative Sephadex G-100 column and the precipitation of histones with solid ammonium sulfate, appeared to have no effect on the behavior of the tetramer. The tetramer still dissociated progressively and irreversibly in the presence of H1, H5, H2A and H2B.

The results of a high speed equilibrium experiment showed that at low concentrations, the variation of M_{app} with solute concentration was quite similar to that reported by Roark et al. However, at high concentrations, molecular weights as high as 65,000 daltons were observed

which probably reflects the presence of H3 aggregates. Large aggregates were not observed in the experiments of Roark et al.

It appeared that the tetramer dissociation equilibrium was shifted by the formation of disulfide bonds between cysteine residues of the H3 molecule, to form dimers, followed by their aggregation and the consequent release of the H4 molecule from the tetramer. This effect could be prevented in the presence of reducing agents eg. β-mercapto-ethanol or bisulfite.

The only difference between Roarks experimental conditions and ours was that the solvent used by Roark contained bisulfite to suppress the protease activity normally associated with calf thymus chromatin. Our results show that the more significant effect of bisulfite was to reverse the formation of disulfide bonds between the H3 chains, and so to prevent the irreversible dissociation of the tetramer. It appears that Roark et al. have been working with a chemically modified (S-sulfo) histone preparation. The question now asked is whether the behavior of this modified subunit can safely be considered relevant to the assembly of the nucleosome in vivo.