A Study of Calf Thymus Histone Fraction F2(b) by Gel Filtration

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The gel-filtration behaviour of calf thymus histone fraction F2(b) was studied at three different salt concentrations (0.01 M-, 0.10 M- and 1.00 M-sodium chloride) and two different pH ranges (pH 3–4 and pH 6.7–7.1). Other histone fractions [F1, F2(a) and F3] were also utilized to assist interpretation of the data. It was found that the Stokes radius of histone fraction F2(b) was not significantly changed when the salt concentration was increased, implying that the aggregation of the individual histone molecules (Edwards & Shooter, 1969) resulted in only relatively minor changes in the hydrodynamic volume. Aggregation would appear to be due to the salting out of hydrophobic regions giving rise, in the aggregate, to a compact core of hydrophobic groups from which protrude the remaining basic parts of the molecule. Repulsion between charged groups on the basic regions of individual histone molecules would give the aggregate approximately spherical symmetry, the diameter of the aggregate approximating to the length of a single histone molecule.

It has been shown (Edwards & Shooter, 1969) that fraction F2(b) from calf thymus histone undergoes progressive self association to form aggregates of increasing size as the salt concentration of the solution is raised. The nature of the aggregation process is of interest in view of the possible role of one or more histone fractions in maintaining the complex supercoiled structure of DNA in chromosomes as suggested by Zubay (1964) and by Pardon, Wilkins & Richards (1967).

Sedimentation-velocity experiments with histone fraction F2(b) (Edwards & Shooter, 1969) gave little information on the shape or hydrodynamic volume of single molecules of histone or of aggregates containing a few molecules, since under the conditions where aggregation was absent or small up to one-half of the apparent change in frictional coefficient with concentration was due to charge effects. Although measurements of the diffusion coefficient could, in principle, give information on the effective hydrodynamic radius of solute particles, calculation again suggested that charge effects would be even more marked than in sedimentation velocity.

This paper reports on the use of gel filtration, a technique that can be used to measure the hydrodynamic volumes of particles, to obtain further information on the nature of the aggregation process of histone fraction F2(b) induced by increasing the salt concentration. Experiments were also carried out with other fractions of calf thymus histone. The

information so obtained was used to help interpret the observed behaviour of fraction F2(b).

MATERIALS AND METHODS

Calf thymus histone fractions F1, F2(a), F2(b) and F3 were prepared and supplied by Dr E. W. Johns and Dr D. M. P. Phillips. Bovine γ -globulin, bovine serum albumin and bovine ribonuclease were obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.; horseradish peroxidase, bovine chymotrypsinogen and equine cytochrome *c* were from Seravac Laboratories, Maidenhead, Berks., U.K. Sephadex G-200 and Blue Dextran 2000 were obtained from Pharmacia, Uppsala, Sweden. Other chemicals were of reagent grade.

A column (95 cm $\times$ 2.5 cm) containing Sephadex G-200 was prepared and fitted with an upward-flow adaptor. Solvent was pumped through the gel bed at a rate of 3.7 ml cm⁻² h⁻¹. Experiments were performed at 4–6°C. The gel was equilibrated with each buffer for at least 18 h before use. Protein samples at a concentration of 1.5–2.0 mg ml⁻¹ were applied in a volume of 2.0 ml. Protein concentration in the eluate was measured by u.v. absorption. The void volume, V_0 , of the column was determined by using either Blue Dextran 2000 or a high-molecular-weight DNA prepared by the method of Berns & Thomas (1965). The internal volume, V_i , of the column was calculated from the dimensions of the gel bed and the weight of dry Sephadex used. Distribution coefficients, K_D , were calculated from elution volumes, V_e , by using the equation

$$K_D = \frac{V_e - V_0}{V_i}$$

Table 1. *Macromolecules used for standardizing Sephadex G-200 columns*

The Stokes radius of γ -globulin was taken from Ackers (1964). The Stokes radii of albumin, peroxidase, chymotrypsinogen, cytochrome *c* and ribonuclease were calculated from diffusion coefficients (Laurent & Killander, 1964). The source of molecular weights were: γ -globulin (Whitaker, 1963); albumin (Creeth & Knight, 1965); peroxidase (Whitaker, 1963); chymotrypsinogen (Schwert, 1951); cytochrome *c* (Margoliash & Lustgarten, 1962); ribonuclease (Van Holde & Baldwin, 1958).

Macromolecule	Stokes radius (Å)	$10^{-3} \times \text{Mol.wt.}$
Blue Dextran 2000		2000
DNA		10000
Bovine γ -globulin	52.2	156
Bovine serum albumin	36.1	66.5
Horseradish peroxidase	30.2	40.2
Bovine chymotrypsinogen	22.4	23.2
Equine cytochrome <i>c</i>	17.4, 16.4	12.4
Bovine ribonuclease	19.2	13.7

Elution volumes of a number of proteins and of the histone fractions were measured in solutions containing 0.01M-, 0.10M- and 1.00M-NaCl buffered in the range of either pH3.0–4.0 (sodium acetate-HCl) or pH6.7–7.1 (Na_2HPO_4 - KH_2PO_4). The buffer concentration did not exceed one-tenth of the NaCl concentration. The relevant properties of the proteins used in this work are listed in Table 1.

Theoretical studies on the mechanism of gel filtration (see, e.g., Anderson & Stoddart, 1967) suggest that the elution characteristics of a solute can be primarily related to the effective hydrodynamic volume of the solute particles. Other relationships involving the molecular weight of the solute have been proposed, but these presuppose a knowledge of the shape and degree of hydration of the molecules. Particle volume can be most simply characterized by the Stokes radius. Several equations have been proposed that relate Stokes radius to elution volume or distribution coefficient (Porath, 1963; Ackers, 1964; Laurent & Killander, 1964; Squire, 1964).

Calibration curves were drawn for each of the above relationships and it was found that the Stokes radius determined for a histone fraction by using each of the curves differed by only 3–5%. Data presented in this paper have been obtained from a calibration curve calculated by using the equation of Porath (1963):

$$K_D^{\frac{1}{3}} = A + B \cdot a$$

where *a* is the Stokes radius and *A* and *B* are constants determined from data obtained by using the standard proteins.

RESULTS AND DISCUSSION

The first experiments were performed with fraction F2(b) with a column buffered in the pH range 3–4. Fig. 1 shows the elution profiles recorded. Compared with profiles obtained with the proteins used as standards the elution profiles of histone fraction F2(b) exhibited no significant asymmetry. Although the elution volume of histone fraction F2(b) in 1.00M-sodium chloride was

Fig. 1. Elution diagrams of histone fraction F2(b) with Sephadex G-200 in various concentrations of NaCl buffered at pH3–4: —, 0.01M-NaCl; ----, 0.10M-NaCl; ·····, 1.00M-NaCl.

obviously less than in either 0.01M- or 0.10M-sodium chloride, this difference was due to changes in the characteristics of the gel bed rather than to changes in the shape or size of the protein particles. In 1.00M-sodium chloride, for example, the elution volumes of the proteins used as standards decreased and the void volume of the column was also decreased (Table 2). The results obtained with the standard proteins were used to construct a calibra-

Table 2. *Elution volumes of Blue Dextran 2000, proteins used as standards and histone fraction F2(b) with Sephadex G-200 at different salt concentrations buffered in the range pH 3-4*

The elution volumes of standard proteins, Blue Dextran 2000 and histone fraction F2(b) are given. The interpolated values of the Stokes radius of histone fraction F2(b) obtained from Fig. 1 are quoted at the bottom of the table.

Macromolecule	Elution volume (ml)		
	0.01 M-NaCl	0.10 M-NaCl	1.00 M-NaCl
Blue Dextran 2000	163	159	135
γ -Globulin	224	223	212
Albumin	264	264	257
Peroxidase	314	315	297
Chymotrypsinogen	—	380	367
Ribonuclease	371	431	400
Cytochrome c	395	—	—
Histone fraction F2(b)	304	307	273
Stokes radius of fraction F2(b) (Å)	31.4	31.5	34.5

tion curve (Fig. 2). The changes in the distribution coefficients for the standard proteins at the three different salt concentrations were sufficiently small that a single calibration line could be fitted to all the results. The Stokes radius of the histone fraction interpolated from Fig. 2 is given in Table 2. The error of the determination was estimated to be ± 3 Å (s.d.).

The Stokes radius of the particles of histone fraction F2(b) increased slightly in 1.00 M-sodium chloride, but this was hardly significant in view of the experimental error. At all three salt concentrations the histone eluted at a position between peroxidase (mol.wt. 40 200) and albumin (mol.wt. 66 500). Sedimentation experiments (Edwards & Shooter, 1969) have shown the mean molecular weight in a 1% solution at this pH to be 7800 in 0.01 M-sodium chloride, 31 000 in 0.10 M-sodium chloride and 110 000 in 1.00 M-sodium chloride. It is therefore clear that there is no correlation between elution volume and molecular weight of the histone particles. The results of the gel-filtration experiments suggested that no gross change in the hydrodynamic volume of the histone occurred as the degree of aggregation increased.

To avoid possible difficulties of interpretation arising from the use of the low pH the experiments with histone fraction F2(b) were repeated with a new column of Sephadex G-200 buffered at pH 6.7-7.1. Under these conditions it was again found that the distribution coefficient of the histone was nearly the same at all three salt concentrations, the histone being eluted from the column between chymotrypsinogen (mol.wt. 23 200) and albumin (mol.wt. 66 500). Samples of other histone fractions were also examined at pH 6.7-7.1. Elution profiles for each fraction at the three different salt concentrations are shown in Fig. 3. A calibration curve constructed from the results obtained with the

Fig. 2. Calibration curve obtained by plotting $K_D^{\dagger}$ versus Stokes radius for a series of proteins at different salt concentrations buffered at pH 3-4: $\square$, 0.01 M-NaCl; $\circ$, 0.10 M-NaCl; $\triangle$, 1.00 M-NaCl. The straight line is a least-squares fit obtained by using the data from each salt concentration. The error in the Stokes radius was calculated to be ± 3 Å (s.d.).

standard proteins is shown in Fig. 4. The Stokes radius of each histone fraction determined from Fig. 4 is given in Table 3. From Fig. 3 and Table 3 it is clear that the elution characteristics of the histone fractions show considerable variation. The elution profiles of histone fraction F1 were symmetrical and the Stokes radius decreased slightly with increasing salt concentration. The elution profiles of histone fraction F2(a) indicated that in 0.10 M- and 1.00 M-sodium chloride two components were present. One of these components, accounting for 70-80% of the

Fig. 3. Elution diagrams of histone fractions F1 (a), F2(a) (b), F2(b) (c) and F3 (d) with Sephadex G-200 in various concentrations of NaCl buffered at pH 6.7-7.1: — 0.01 M-NaCl; ----, 0.10 M-NaCl; ·····, 1.00 M-NaCl.

solute, showed a slight increase in Stokes radius with increasing salt concentration from 29 Å to 34 Å, whereas the Stokes radius of the remaining 20-30% of the solute on the other hand increased to 47 Å in 1.00 M-sodium chloride. The elution profile of the sample of histone fraction F2(b) used in these experiments also showed the presence of two components. The proportion of the major component (90%) was unchanged at each salt concentration. The Stokes radius of this component changed little with increase of salt concentration, whereas the Stokes radius of the minor component increased to 77 Å in 1.00 M-sodium chloride. With histone fraction F3 a single peak was observed in 0.01 M-sodium chloride, but in 0.10 M- and 1.00 M-sodium chloride the greater part of the solute (approx. 90%) eluted at the void volume of the column.

Sedimentation experiments (Edwards, 1968) with solutions of histone fractions F2(a) and F3 in 0.10 M-sodium chloride have shown that both these

fractions undergo gross aggregation near neutral pH, the onset of the aggregation being associated with the appearance of heterogeneous components with sedimentation coefficients in the range 7-20 S and a disappearance of the unaggregated material, which, for both fractions, at acid pH, had a sedimentation coefficient of 2.0-2.4 S. It seemed likely, therefore, that in the gel-filtration experiments with fraction F3, in 0.10 M- and 1.00 M-sodium chloride the material eluted at the void volume corresponded to the gross aggregate observed in sedimentation experiments at neutral pH. Similarly the part of fraction F2(a) that exhibited an increasing Stokes radius with increasing salt concentration probably corresponded to the grossly aggregated material seen in sedimentation experiments. Johns (1964) and Phillips & Johns (1965) have shown that fraction F2(a) consists of two subfractions, F2(a)1 and F2(a)2, in equal amounts. The component of histone fraction F2(a) having a large Stokes radius may be an intermolecular aggregate

of subfraction F2(a)2 or an aggregate of either subfraction F2(a)1 or subfraction F2(a)2 singly. The elution profiles of fraction F2(b) show that about 10% of this preparation undergoes gross aggregation as the salt concentration is increased. In sedimentation experiments with other preparations of histone fraction F2(b) no boundaries corresponding to gross aggregates have been seen at sodium chloride concentrations equal to or less than 1.0M. It seemed, therefore, that the aggregated material observed with the present preparation might be an impurity. Gel electrophoresis experiments (E. W. Johns, personal communication) established that the preparation contained 5–10% of subfraction F2(a)2. The 10% of histone fraction

F2(b) with the larger Stokes radius could be either a self-aggregated form of subfraction F2(a)2 or an intermolecular aggregate of fraction F2(b) and subfraction F2(a)2. The specificity of histone–histone interactions has been relatively little studied. The results presented above suggest that gel filtration might be a useful tool in such studies.

As mentioned above, measurements of the molecular weight of histone fraction F2(b) suggest that it exists as single molecules in 0.01M-sodium chloride, as aggregates of, on average, three molecules in 0.10M-sodium chloride and of about ten molecules in 1.00M-sodium chloride. The gel-filtration experiments indicate that this aggregation process is accompanied by only small, hardly significant, increases in the hydrodynamic volumes of the particles, the Stokes radius increasing from 30 Å to 34 Å when measured at pH 7 or from 31 Å to 35 Å when measured at pH 3–4. These observed increases in radius, if significant, would be equivalent to an increase in volume of about 45%. It is conceivable that an increase in volume resulting from aggregation might be offset by a decrease in volume due to a more compact folding of the histone chains as the increasing salt concentration decreases the electrostatic repulsion of the charged groups on the molecules. Since the molecules of histone fraction F1₁ do not aggregate with each other (Teller, Kinkade & Cole, 1965; Haydon & Peacocke, 1968; Edwards & Shooter, 1969) the results obtained with this fraction can be used to assess the extent of the decrease in hydrodynamic volume as the salt concentration is increased. The results in Table 3 show that the effect is small, the Stokes radius decreasing from 47 Å in 0.01M-sodium chloride to 44 Å in 1.00M-sodium chloride, i.e. a decrease in hydrodynamic volume of about 20%. The changes in volume in going from single molecules of histone

Fig. 4. Calibration curve obtained by plotting $K_D^{1/2}$ versus Stokes radius for a series of proteins at different salt concentrations buffered at pH 6.7–7.1: □, 0.01M-NaCl; ○, 0.10M-NaCl; △, 1.00M-NaCl. The straight line is a least-squares fit obtained by using the data from each salt concentration. The error in the Stokes radius was calculated to be ± 3 Å (s.d.).

Table 3. Stokes radii of histone fractions obtained by gel-filtration with Sephadex G-200 at different salt concentrations buffered in the range pH 6.7–7.1

Two values of the Stokes radii of histone fractions F2(a) and F2(b) are quoted corresponding to the two elution peaks observed, the relative proportions being indicated by the adjacent values in parentheses. The Stokes radius of histone fraction F3 in 0.10M- and 1.00M-NaCl is not indicated since approx. 90% of this fraction was aggregated and eluted at the void volume of the column. For further details refer to the text.

Concn. of NaCl (M)	Histone fraction ...	Stokes radius (Å)			
		F1	F2(a)	F2(b)	F3
0.01		47	29	30 (90%)	34
				40 (10%)	
0.10		46	32 (70%)	32 (90%)	—
			41 (30%)	51 (10%)	
1.00		44	34 (80%)	34 (90%)	—
			47 (20%)	77 (10%)	

fraction F2(b) to aggregates containing, on average, ten molecules, is therefore small.

Studies of the amino acid sequence of histone subfraction F2(a)1 (DeLange, Fambrough, Smith & Bonner, 1969; Ogawa *et al.* 1969) and of fraction F2(b) (Hnilica, Kappler & Jordan, 1970) indicate that the hydrophobic amino acid residues occur in a large cluster rather than being randomly distributed along the chain of the molecule. It is possible that self-association of histone fraction F2(b) results from a salting out of the hydrophobic regions of the chains. The associated hydrophobic regions of several molecules could form a compact core. The hydrophilic parts of the molecule would protrude from the central core and, because these parts of the molecule carry most of the charged groups, electrostatic repulsion would keep individual chains apart giving the total complex an approximation to spherical symmetry, i.e. the diameter of the aggregate would not be much different from the length of a single molecule.

In conclusion it may be pointed out that, in the discussion on the charge effects on the sedimentation velocity of histone fraction F2(b) (Edwards & Shooter, 1969), it was assumed that aggregation did not affect the contribution to the concentration-dependence term of the physical interaction of the histone particles, i.e. it was assumed that aggregation had little effect on the shape or hydrodynamic volume of the particle. The results of the present work lend support to the above assumption.

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