Characterization of Sea Urchin Sperm Chromatin and Its Basic Proteins*

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ABSTRACT: There are five basic proteins which are associated with deoxyribonucleic acid in the mature sperm chromatin of the sea urchin, *Arbacia punctulata*. They are clearly histone-like in contrast to the protamines or protamine-like proteins found in many sperm. They differ, however, from calf thymus or pea bud histones in that they possess higher total basicity, higher cysteine content, and a substantial (36%) content of a particular protein, γ. Protein γ represents a new type of basic protein. Its high content of lysine, proline, and alanine reflects its similarity to lysine-rich histones; its high content of arginine and lysine/arginine ratio are similar to slightly lysine-rich histones. The dissociation of histones from isolated chromatin by increasing concentrations of NaCl was studied. In the salt concentration range 0.6–1.5 M, protein γ and a slightly lysine-rich histone (δ) are dissociated; from 1.5 to 4.0 M sodium chloride, the arginine-rich histones (α, β) and another slightly lysine-rich histone (ϵ) are dissociated. The dissociation of basic protein alters the melting profile of the chromatin and its ability to serve as template for *in vitro* ribonucleic acid synthesis. Isolated chromatin displays a biphasic melting curve and is nearly inert as a template for *in vitro* ribonucleic acid synthesis. As increasing amounts of protein are dissociated by salt, the melting profile and template activity of the salt-treated chromatin approach those of deoxyribonucleic acid.

As an initial step in elucidating the mechanism of differential gene activity, isolation and characterization of the genetic material (chromatin) are essential to understanding its role in the process and its relationships with other cellular macromolecules. The ability of isolated chromatin to serve as template, *in vitro*, for RNA synthesis under the auspices of endogenous or exogenous RNA polymerase, indicates that this most important function of chromatin is retained during isolation. Furthermore, the template activities of isolated chromatin appear to reflect the genetic activities of the cells from which it is derived (Bonner *et al.*, 1963; Paul and Gilmour, 1966a,b; Huang, 1968; Huang and Huang, 1969; K. Smith, R. Church, and B. J. McCarthy, 1968, unpublished data).

The main feature of all chromatins thus far investigated is their restricted genetic activity relative to DNA from the same cell. Progressive removal of histones from chromatin resulted in corresponding increases in its template activity in supporting RNA synthesis (Huang and Bonner, 1962; Allfrey *et al.*, 1963; Marushige and Bonner, 1966). Apparently, the binding of histone to DNA provides the physical basis for such restriction. Although experiments on histone chemistry from many laboratories indicate the lack of cell or species specificity (Hnilica *et al.*, 1962; Lindsay, 1964; Neidle and Waelsch, 1964; Fambrough and Bonner, 1966; Dingman and Sporn, 1964; Kischer *et al.*, 1966; Marushige and Ozaki, 1967; Comings, 1967), there are exceptions to the rule (Neelin *et al.*, 1964; Hnilica, 1967; Bellair and Mauritzen, 1967; Bustin and Cole, 1968).

The basic protein which Neelin and Hnilica have found in avian erythrocyte nuclei and sperm of some species is different from any of the familiar histone fractions: its basicity and content of lysine, proline, and alanine are similar to lysine-rich histones; its arginine content and the lysine/arginine ratio are similar to slightly lysine-rich histones. This fraction therefore represents a new class of histone.

The nuclear basic protein of mature sperm cells is varied. Many fish sperm contain a true protamine (Ando *et al.*, 1962). Sperm of other species, such as the snail, squid, and grasshopper contain a protamine-like basic protein (Bloch and Hew, 1960a,b; Bloch and Brack, 1964; Bloch, 1962). Finally, basic protein very similar to typical somatic histone (calf thymus) is found in the sperm of the sea urchin.

Although chick erythrocytes and sea urchin sperm cells are highly differentiated cells and each has its unique properties, they bear many common characteristics: mitotic activity is suspended, genetic activity (measured as ability to support RNA synthesis) is reduced, the chromatin is highly condensed, and each contains a new class of basic protein (histone).

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In an attempt to elucidate the relationship between the properties of chromatin and the properties of the cells which contain such chromatin, we have studied the chromatin of erythrocytes and sperm. In the present paper we shall report our studies on the chemical analysis of histones in chromatin of sperm from the sea urchin, *Arbacia punctulata*, and the nature of the binding between histones and DNA in the chromatin. These results may help to elucidate the basis for restriction of template activity in a highly repressed chromatin, and the role of the basic protein in condensation of chromatin.

Materials and Methods

A. Obtaining Animals. *Arbacia punctulata* from the North Carolina coast were obtained from Norris Hill, Beaufort, N. C. The animals were maintained in the laboratory at 15–18° in the Instant Ocean circulating artificial sea water aquarium system (Aquarium Systems Inc., Wickliffe, Ohio). Ripe gametes were available year round with greatest abundance in spring months (see also Ellis, 1966).

B. Obtaining Mature Sperm. Mature sperm were obtained by methods discussed in Costello *et al.* (1957). The quantity of sperm obtained from each animal was usually from 2 to 4 ml of sedimented cells.

C. Preparation of Sperm Chromatin. Chromatin was prepared from mature sperm by the methods of Bonner *et al.* (1968), with the following modification. The pellet from 0.01 M Tris (pH 8.0) is resuspended and centrifuged successively in 0.005 and 0.001 M Tris (pH 8.0) at 1500*g*, 15 min. The pellet is then centrifuged once at 5000*g*, 30 min in 0.001 M Tris (pH 8.0). This pellet is dispersed in ten volumes of glass-distilled water (pH 8.0) by stirring for several hours or overnight in the cold. Chromatin in the final solution is not pelleted by centrifugation at 10,000*g* for 30 min. Recovery of DNA from the original homogenate was at least 90%.

D. Chemical Composition of Chromatin. 1. DNA. DNA was estimated spectrophotometrically using the relationship 1 mg/ml of DNA solution equals 20.0 OD at 260 mμ. DNA was also determined colorimetrically by the method of Burton (1956) calibrated against sea urchin sperm DNA prepared by the method of Marmur (1961) or by equilibrium centrifugation of chromatin in 4 M cesium chloride. DNA prepared by either method gave similar spectra and melting profiles and contained 4% protein.

2. RNA. Chromatin was precipitated with 10% trichloroacetic acid and washed successively with 10% trichloroacetic acid, 80% ethanol, and ethanol–ether (3:1). The pellet was hydrolyzed in 0.3 N KOH for 18 hr at 37°. Nonhydrolyzed nucleic acid and protein were precipitated with acid and the pellet was washed with acid. The supernatant was analyzed for RNA by the method of Dische (1955), calibrated against yeast total RNA (Sigma).

3. BASIC PROTEIN. A solution of chromatin at a concentration of less than 500 μg/ml was made 0.4 N in H_2SO_4 and stirred 30 min at 0°. The solution was centrifuged (clinical) and the pellet was extracted again for 10 min. The solution was centrifuged as before; the supernatants were combined, neutralized with NaOH, and analyzed for protein by the method of Lowry *et al.* (1951) calibrated against sea urchin sperm total basic protein. The protein of the supernatant may also be precipitated with 20% trichloroacetic acid, washed with ethanol and ethanol–ether (3:1), dissolved in 0.1 M acetic acid, neutralized with NaOH, and determined as above.

4. NONBASIC PROTEIN. Basic proteins were extracted as described in part 3 above. The final acid-insoluble pellet was made 5% in trichloroacetic acid and heated 30 min at 100° to remove nucleic acids. The solution was centrifuged and the pellet was washed with 5% trichloroacetic acid, then ethanol, and ethanol–ether (3:1). The nucleic acid free precipitate contains the residue protein. Protein soluble in 0.5 N NaOH was determined by the method of Lowry *et al.* (1951) calibrated against bovine serum albumin.

E. Preparative Isolation of Basic Protein by Acid Extraction. Isolation was according to Bonner *et al.* (1968) except that 0.4 N H_2SO_4 was utilized and the initial extraction was for 1 hr.

F. Disc Electrophoresis of Basic Proteins. Electrophoresis of basic protein was performed according to the methods outlined in Bonner *et al.* (1968). Electrophoresis tubes were of two sizes: small gels 8.5 × 0.4 cm i.d. and large gels 9.0 × 0.7 cm i.d., 10% gels (pH 4.3) 6 M in urea were routinely used, but 7.5 and 15% gels gave the same banding pattern and relative electrophoretic migration of each of the bands.

For the smaller gels, the sample load was from 10 to 40 μg of whole protein, and electrophoresis was for 60 min for 4 mA/tube. For the larger gels the sample load was from 50 to 100 μg of whole protein, and electrophoresis was for 140 min at 4 mA/tube.

The relative concentration of each protein band was determined by the method of Johns (1967), which consists of cutting out individual stained bands from the gels, extracting the color with concentrated dimethyl sulfoxide, and determining the amount of color by optical density at 600 mμ. The values reported here are only approximate, since the staining standard for each individual band protein was not determined.

G. Preparation of Basic Protein Fractions from Sperm Chromatin. Fractions were prepared by three methods: (1) elution of each protein band following disc electrophoresis, (2) fractionation by the method of Johns (1964), and (3) Bio-Gel column chromatography.

1. ELUTION FOLLOWING ELECTROPHORESIS. Eight large gels were electrophoresed as described earlier. Seven were frozen immediately on Dry Ice; the other was stained, destained, and used as a template to cut disks corresponding to single bands from the frozen gels. The disks corresponding to single bands were pooled, crushed by homogenization, and extracted for several hours with 10 M urea. The gels were then centrifuged down, and the supernatants were exhaustively dialyzed against 0.1 M acetic acid and lyophilized. A portion of each fraction was taken for disk electrophoresis to determine purity, the remainder was processed for amino acid analysis.

2. FRACTIONATION BY THE METHOD OF JOHNS (1964). Only two pure fractions were obtainable by this extraction method. The new histone, γ, is extracted by 5% perchloric acid and thus resembles the lysine-rich histone fraction, F_1, in its extraction behavior. Fraction δ (slightly lysine-rich histone) behaves during fractionation as does fraction F_2b (also slightly lysine rich). Other fractions obtained by this extraction method are contaminated and of little use for the purposes of this paper.

3. BIO-GEL COLUMN CHROMATOGRAPHY. Chromatin was first extracted with 5% perchloric acid to release fraction γ. The resulting chromatin was then extracted with 0.25 N HCl or H_2SO_4 and the extracted protein was precipitated with 80% ethanol; 10–15 mg of the protein in 1 ml of 0.01 N HCl was applied to a Bio-Gel P-60 column (1 × 50 cm) and eluted with 0.01 N HCl. The initial peak contained the arginine-rich histones α and β. The trailing peak (II) contained a mixture of histones and was not used further.

The amino acid compositions of the proteins were determined by dissolving in boiling 6 N HCl and hydrolyzing at 105° for 18–20 hr. The solution was diluted to 2 N HCl, lyophilized, taken up in 0.2 N sodium citrate (pH 2.2), and chromatographed on a Beckman Spinco amino acid analyzer, Model 120.

H. Selective Dissociation of Basic Protein from Sperm Chromatin by Sodium Chloride. The techniques used were those discussed in Olivera (1966), with the following modifications. If the type 30 rotor was utilized for centrifugation, sedimentation was at 29,000 rpm for 48 hr; if the type 40 rotor was used, sedimentation was at 36,000 rpm for 24 hr.

I. Melting Profiles of DNA and Chromatin. Melting profiles of DNA or chromatin were carried out in a solution of 2.5×10^{-4} M EDTA (pH 8.0) utilizing a Gilford Model 2000 multiple-sample absorbance recorder to follow changes in absorbance during heating at a constant rate of 1°/min with a Haake circulating-water bath.

J. Template Activities of DNA and Chromatin. The method of determining template activity was that described in Bonner *et al.* (1968). Each determination of template activity included a blank (incubation mixture and enzyme but no template). The amount of incorporation in the blank (10 $\mu\mu$moles or less) was subtracted from the incorporation of all other mixtures.

Results

A. Properties of Isolated Chromatin. 1. ULTRAVIOLET ABSORPTION SPECTRA AND CHEMICAL COMPOSITION. The absorption spectra of sperm chromatin and of DNA prepared from such chromatin are given in Figure 1. The spectra are typical of nucleic acid; the chromatin, however, possesses a higher absorbance at 230 mμ than does DNA, reflecting its higher complement of protein. The absence of aggregation is reflected in the low absorbance of chromatin at wavelengths over 300 mμ.

The chemical composition of sperm chromatin is given in Table I. Of particular note are the small amounts of nonbasic protein and RNA.

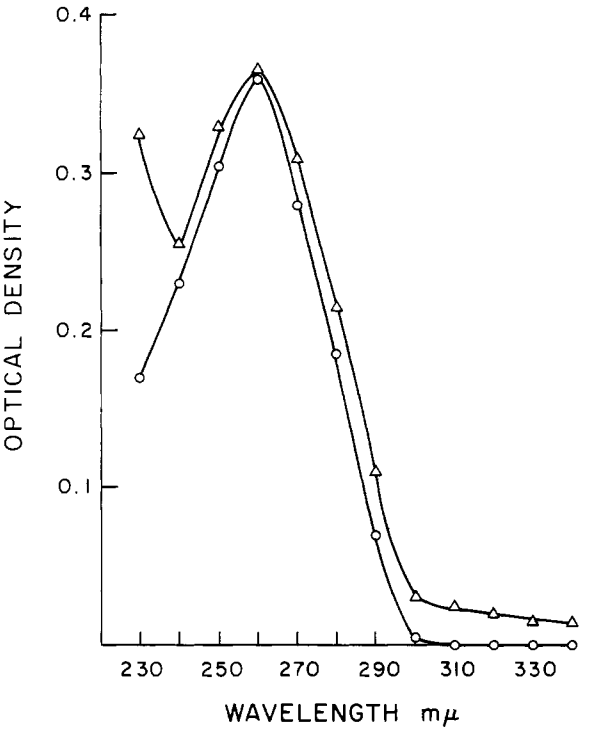

FIGURE 1: Absorption spectra of sea urchin sperm chromatin and DNA prepared from chromatin by deproteinization. (△—△) Sperm chromatin and (○—○) sperm DNA.

TABLE I: Chemical Composition Sea Urchin Sperm Chromatin.

Component	% Total Mass	Mass Ratio
DNA	41.0	1.0
RNA	0.4	0.01
Basic protein	49.0	1.2
Nonbasic protein	7.0	0.2

2. MELTING PROFILES OF DNA AND CHROMATIN. The proteins associated with DNA in chromatin of sperm stabilize the DNA double helix against thermal denaturation (Figure 2). Not only is the melting profile of sperm chromatin shifted to higher temperatures relative to DNA, but the profile shows a two-step melting behavior, T_{m_1} 66°, T_{m_2} 84°; DNA T_m 47°.

The first step of the profile accounts for 35% of the total hyperchromicity, the second step for 65%.

3. TEMPLATE ACTIVITIES OF DNA AND CHROMATIN. Differences between sperm chromatin and DNA prepared from chromatin are manifested in their abilities to serve as template for the *in vitro* synthesis of RNA (Figure 3). At saturation concentrations sperm chromatin has but 2% the priming ability of DNA.

B. Biochemical Analysis of Chromosomal Proteins. 1. AMINO ACID ANALYSIS. Amino acid analysis of total basic protein and nonbasic protein from sea urchin sperm chromatin (Table II) reveals that, on the basis of numbers, kinds, and relative amounts of amino acids

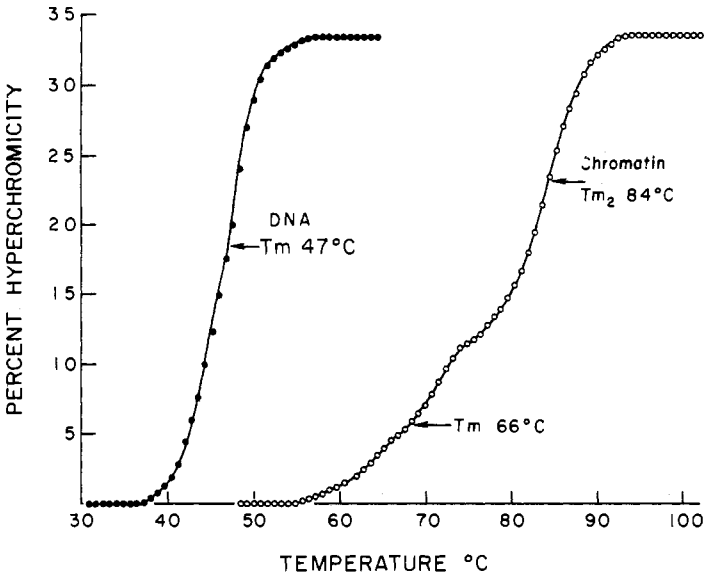

FIGURE 2: Melting profiles of sea urchin sperm chromatin and DNA prepared from chromatin by deproteinization. Solvent 2.5×10^{-4} M EDTA (pH 8.0); heating rate 1°/min. (●—●) DNA, T_m 47°; (○—○) chromatin, T_{m_1}66 °, T_{m_2} 84°.

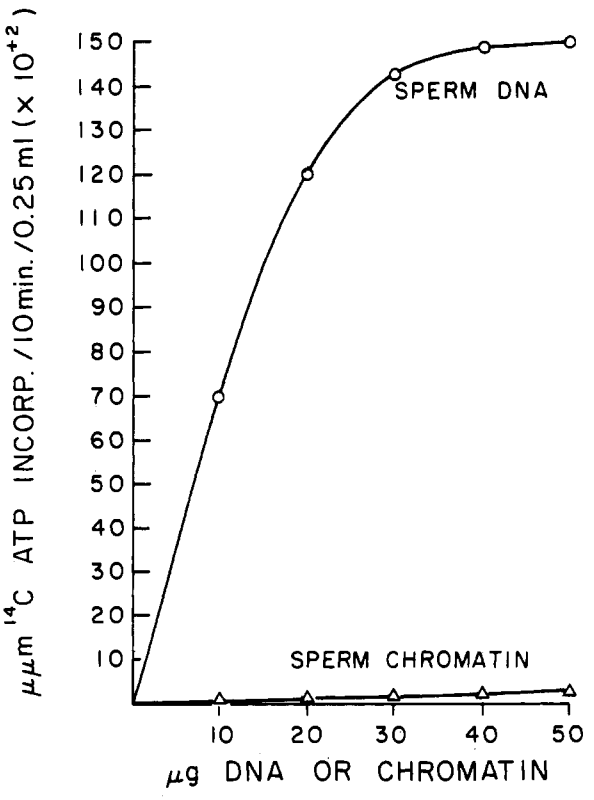

FIGURE 3: Template activities of sea urchin sperm chromatin and DNA prepared from chromatin by deproteinization. The indicated amounts of chromatin or DNA were added to the incubation mixture and incubated at 37° for 10 min. The RNA was then precipitated with 10% trichloroacetic acid, washed, and counted by gas flow. RNA synthesis expressed as μμmoles of labeled nucleotide incorporated into trichloroacetic acid insoluble material per 10 min per volume of incubation mixture (0.25 ml). (△—△) Chromatin and (○—○) DNA.

TABLE II: Amino Acid Composition of Sea Urchin Sperm Chromatin Basic and Nonbasic Proteins.

	Moles % of Amino Acid Recovd	
Amino Acid	Total Basic Protein	Total Nonbasic Protein
Lysine	18.9	5.9
Histidine	0.8	2.3
Arginine	11.4	8.0
Aspartic	4.4	9.7
Threonine	4.0	5.2
Serine	4.8	7.4
Glutamic	5.5	12.2
Proline	6.4	3.0
Glycine	8.2	9.0
Alanine	16.4	9.0
Cystine	1.4	0.3
Valine	4.2	3.8
Methionine	0.8	2.7
Isoleucine	4.0	3.3
Leucine	6.0	8.8
Tyrosine	1.6	3.9
Phenylalanine	1.0	4.5

present, these proteins are clearly of somatic histone type and bear little resemblance to protamine-like or true protamine-type proteins.

2. DISC ELECTROPHORESIS. The basic proteins of sperm chromatin may be resolved into several components by polyacrylamide gel electrophoresis (Figure 4). Five bands, designated as protein α, β, γ, δ, and ε beginning at the origin (anode), are observed.

The relative concentration of each of the bands is shown in Table III.

3. AMINO ACID COMPOSITION OF FRACTIONATED SPERM

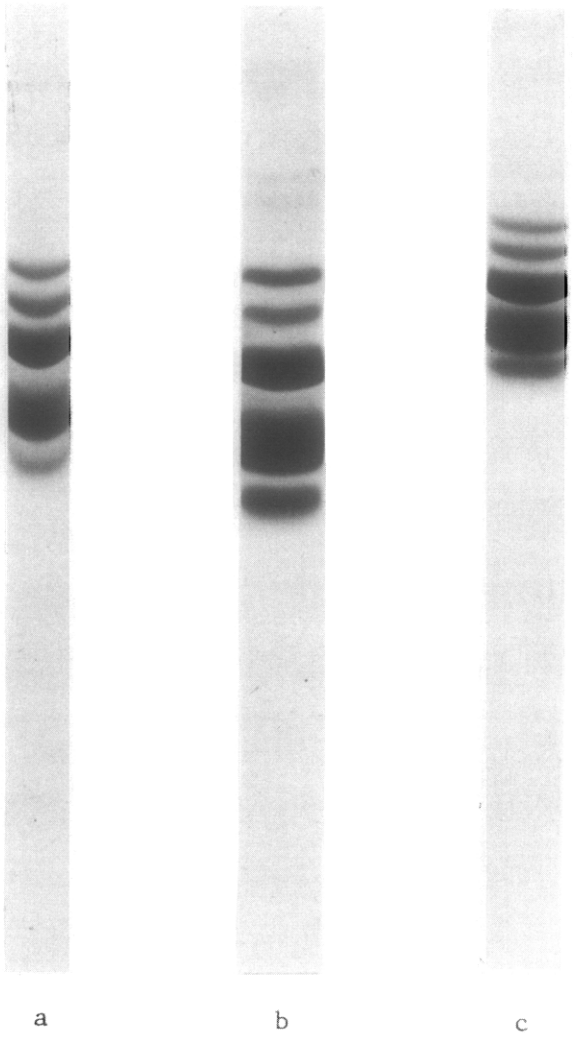

FIGURE 4: Photograph of electrophoretic separation pattern of sea urchin sperm basic proteins. Direction of migration is from top to bottom. (a) Total basic protein, 10% polyacrylamide, 6 M in urea, tube size 0.4 × 8.5 cm. (b) Total basic protein, 10% polyacrylamide gel, 6 M in urea, tube size 0.7 × 9.0 cm. (c) Total basic protein, 15% polyacrylamide gel, 6 M in urea, tube size 0.7 × 9.0 cm.

BASIC PROTEIN. The degree of separation achieved by the fractionation procedures outlined above is shown in Figure 5. Isolation of electrophoretically pure fractions β, γ, and δ was achieved, while procedures for purification of fractions α and ϵ remain to be improved. Comparison of fractions γ and δ prepared by electrophoresis to those same fractions prepared by the method of Johns (1964) showed no difference in electrophoretic purity or in amino acid analysis (see below). Bio-Gel column chromatography is a useful method for preparation of arginine-rich histones (α, β) in appreciable quantity (Figure 6).

Amino acid analysis of the isolated fractions is shown in Table IV. From the compositions shown, three types of histones based on relative lysine and arginine contents may be discerned: the lysine-rich, slightly lysine-rich, and argine-rich histones. The three types of fractions are similar to the same three types of fractions derived from calf thymus histone but with one major difference. Protein γ of sperm protein is related to the lysine-rich histones of calf thymus (F 1) in that they contain high lysine, proline, and alanine, and have similar basicity. However, protein γ has a much higher content of arginine than does calf thymus lysine-rich histone and, on the basis of the lysine to arginine ratio (Phillips and Johns, 1965), is related to the slightly lysine-rich histone. The high lysine, alanine, and proline contents, characteristic of lysine-rich histones, merits its placement in that category, recognizing its distinctiveness. Protein γ accounts for 36% of the total basic protein of sperm chromatin. Histidine, cysteine, and methionine, absent in calf thymus lysine-rich histine, are present in protein γ of sperm basic protein.

TABLE III: Relative Concentration of Histone Band Proteins.

Electrophoresis Band	Amount (% of total 660 mμ OD)
α	4
β	8
γ	36
δ	42
ϵ	8

Proteins δ and ϵ of sperm basic protein are related to the slightly lysine-rich fractions of calf thymus (FIIb1 and FIIb2). Fraction FIIb2 of calf thymus has a higher lysine to arginine ratio, a higher serine but lower leucine content than fraction FIIb1. The same distinctions are evident in the sperm fractions; fractions δ and ϵ are related to calf thymus fraction FIIb1. The slightly lysine-rich histones (fractions δ and ϵ) account for 50% of the total basic protein in sperm chromatin.

Fractions α and β in sperm are related to calf thymus arginine-rich histones. Fraction β of sperm basic protein has a greater proportion of arginine than any other arginine-rich histone fraction. Note also the high glutamic acid content of arginine-rich basic proteins. Principal differences between thymus and sperm fractions are in the contents of histidine, serine, proline, methionine, tyrosine, and phenylalanine. ϵ-*N*-Methyllysine has been detected in arginine-rich histones of calf thymus (Murray, 1964). The arginine-rich protein fractions α and β account for 12% of the total basic protein in sperm chromatin. Protein γ, whether isolated by electrophoresis or by the method of Johns (1964), gives the same amino acid composition. Similar results apply to protein δ.

C. Selective Dissociation of Basic Proteins from Sperm Chromatin. As a step toward elucidating the distribution of histones along the DNA helix and their relative strengths of association with the DNA, salt extraction procedures, which selectively and progressively remove histones from the DNA, have been utilized (Marushige

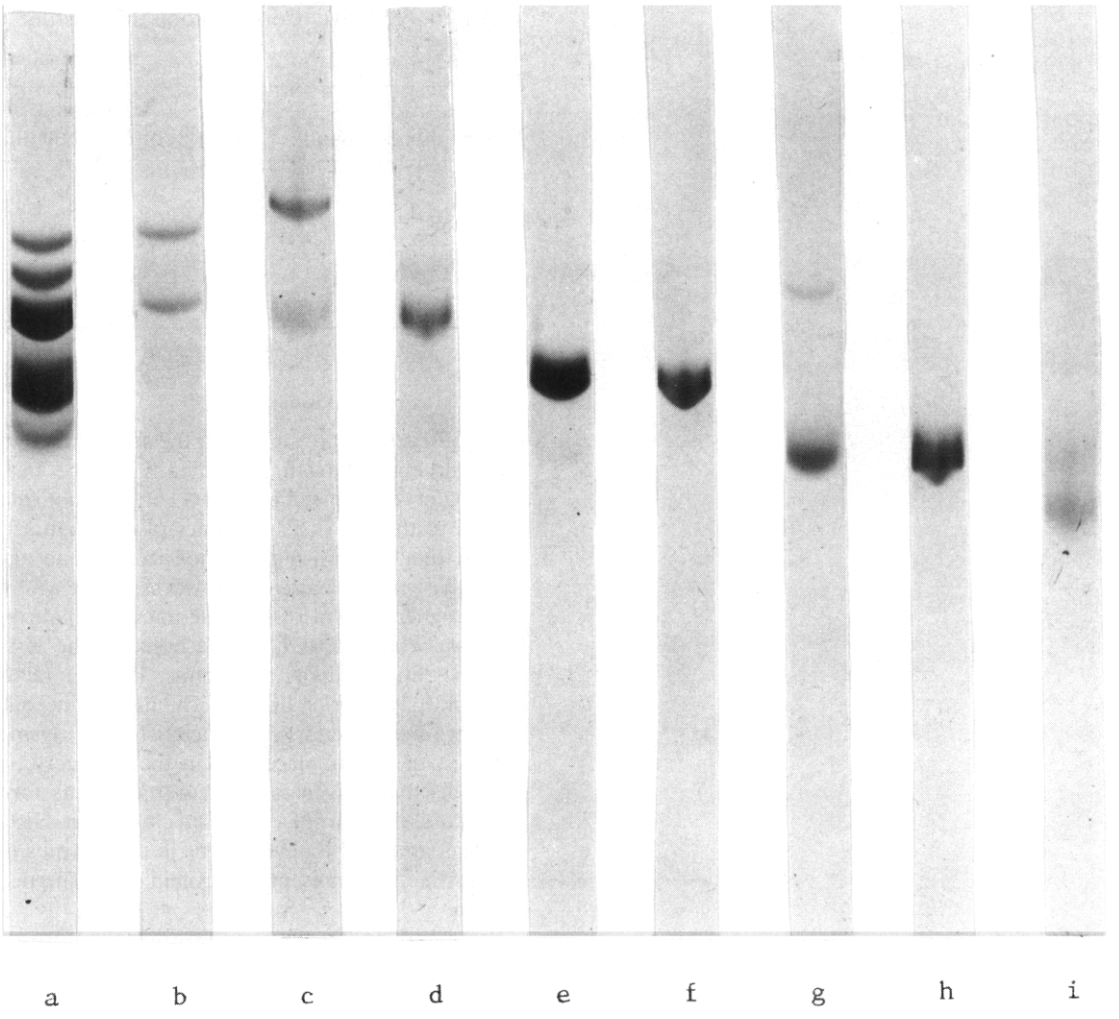

FIGURE 5: Photograph of electrophoretic purity of isolated basic protein fractions from sea urchin sperm. All gels are 10% polyacrylamide, migration is from top to bottom. (a) Whole sample basic protein α, β, γ, δ, and ϵ. (b) Fractions α and β, arginine rich; separated by Bio-Gel. (c) Fraction α, arginine rich; separated by disc electrophoresis. (d) Fraction β, arginine rich; separated by disc electrophoresis. (e) Fraction γ, new histone; separated by method of Johns (1964). (f) Fraction γ, new histone; separated by disc electrophoresis. (g) Fraction δ, slightly lysine rich; separated by the method of Johns (1964). (h) Fraction δ, slightly lysine rich; separated by disc electrophoresis. (i) Fraction ϵ, slightly lysine rich; separated by disc electrophoresis.

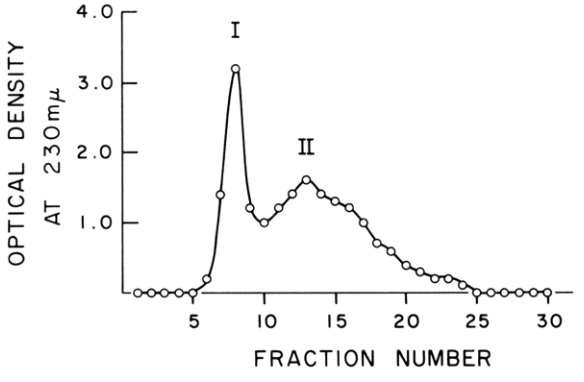

FIGURE 6: Elution profile of sea urchin sperm basic protein from Bio-Gel P-60. Acid-extracted histone (10–15 mg) was applied to a 1 × 50 cm column and eluted with 0.01 N HCl. The protein concentration in the effluent fractions was determined by optical density at 230 mμ. Peak I contains the arginine-rich histones α and β; peak II contains a mixture of histones.

and Bonner, 1966; Olivera, 1966; Ohlenbusch *et al.*, 1967).

Polyacrylamide gel electrophoresis analysis of dissociated (supernatant) basic proteins at each salt concentration are shown in Figure 7.

It can be seen from the gel patterns that from 0.6 to 1.5 M NaCl, only two proteins are extracted, fractions γ and δ. Their extent of dissociation increases throughout the salt concentration range but their extent of dissociation relative to each other remains fairly constant. Fractions γ and δ are totally dissociated by 1.5 M NaCl. Between 1.5 and 2.0 M NaCl fractions α, β, and ϵ are almost completely dissociated, being completely dissociated by 4.0 M NaCl. Thus, fractions γ and δ and α, β, and ϵ have completely different dissociation ranges, but within the ranges the degree of dissociation increases with salt concentration.

Removal of basic proteins from chromatin has profound effects on the structure of chromatin also.

TABLE IV: Amino Acid Composition of Histone Fractions from *Arbacia punctulata* Sperm Chromatin.

Amino Acid	Mole % of Amino Acids Recovd					
	$(\alpha + \beta)^a$	α^b	β^c	γ^d	δ^e	ϵ^f
Lysine	13.0	10.6	6.7	27.5	11.9	14.3
Histidine	Tr	Tr	Tr	0.3	1.2	1.7
Arginine	18.8	14.4	15.3	10.3	11.6	10.7
Aspartic	8.1	7.6	4.3	2.8	5.7	5.3
Threonine	2.7	4.6	5.8	2.4	4.9	4.2
Serine	5.4	7.2	6.7	5.7	6.4	7.4
Glutamic	8.1	11.8	11.1	3.1	8.4	6.4
Proline	Tr	Tr	2.0	8.4	4.1	4.0
Glycine	10.8	8.9	12.3	7.1	10.1	11.8
Alanine	11.0	13.1	12.4	19.3	11.8	13.6
Cystine	2.5	Tr	1.5	Tr	2.0	1.0
Valine	5.2	5.9	4.0	3.3	6.3	5.5
Methionine	2.6	5.1	6.0	1.5	1.3	4.1
Isoleucine	5.2	3.4	3.8	3.0	3.6	3.0
Leucine	5.3	7.2	8.4	1.9	7.5	4.7
Tyrosine	0.5	Tr	0.7	0.7	1.4	0.9
Phenylalanine	0.5	Tr	0.7	0.6	1.9	0.9
Basic aa/acidic aa	1.9	1.2	1.3	6.4	1.8	2.3
Lys/Arg	0.7	0.7	0.4	2.7	1.0	1.3

[a] Fractions α and β isolated by column chromatography on Bio-Gel P-60, peak I (see Figure 6). Electrophoretic purity of this fraction indicated in Figure 5. [b] Fraction α isolated by disc electrophoresis. Electrophoretic purity of this fraction indicated in Figure 5. [c] Fraction β isolated by disc electrophoresis. Electrophoretic purity indicated in Figure 5. [d] Fraction γ isolated by disc electrophoresis or by method of Johns (1964). Either method of isolation results in a fraction bearing the amino acid composition shown. Electrophoretic purity of fraction γ indicated in Figure 5. [e] Fraction δ isolated by disc electrophoresis or by method of Johns (1964). Either method of isolation yields a fraction having the amino acid composition indicated. Electrophoretic purity of fraction δ indicated in Figure 5. [f] Fraction ϵ isolated by disc electrophoresis. Electrophoretic purity indicated in Figure 5.

Figure 8 shows the melting profiles of the extracted chromatin at each salt concentration used. Throughout the salt concentration range 0.6–1.5 M NaCl, where two proteins (protein γ and δ) are progressively dissociated, the first step of the profile is shifted to lower temperatures and has a more DNA-like melting profile. Salt concentrations below 1.5 M affect only the first step of the melting profile although this step now includes 95% of the hyperchromicity. Only at 2 M NaCl does the second step disappear. This would seem to indicate that fractions α, β, and ϵ are clustered in certain regions of the DNA in chromatin. Since the association of these proteins alone with DNA accounts for only 5% of the total hyperchromicity in extracted chromatin, fractions γ and δ must also contribute to the second step of the melting profile in native chromatin since, originally, the second step accounts for 65% of the total hyperchromicity. At 2.0 M NaCl all two-step character is lost, and by 4.0 M NaCl the melting profile is indistinguishable from the profile of deproteinized DNA.

Parallel to the structural effect on chromatin, removal of histones from chromatin also caused significant effect on its template activity in supporting RNA synthesis (Table V). As expected, the template activity increases as more histone is dissociated and approaches that of deproteinized DNA. The increase in template activity parallels the shift in the melting profile from native chromatin to deproteinized DNA. At 0.6 M NaCl, where only small amounts of fractions γ and δ are dissociated, there is only a small change in the melting profile and only a small increase in template activity. As more and more of fractions γ and δ are dissociated, the melting profile shifts to an increasing extent toward that of DNA as does the template activity. However, a small portion of the melting profile (5% of the total hyperchromicity) remains very near the native profile of chromatin and a considerable amount of template activity (35% that of DNA) is held in abeyance until fractions α, β, and ϵ are dissociated. DNA–RNA hybridization and competition studies between RNA primed by chromatin after extraction by various salt concentrations are now in progress. This result may help to determine whether different regions of DNA are in fact masked by different kinds of histones.

Discussion

A. Nature of Chromosomal Proteins. The amino acid composition of total basic protein from sperm chro-

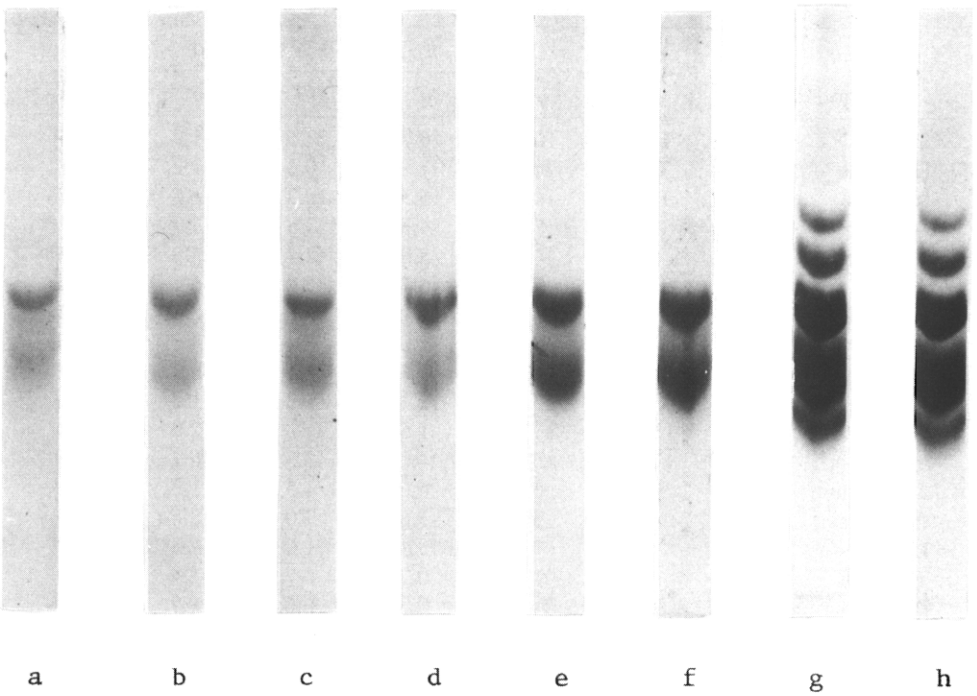

FIGURE 7: Photograph of electrophoretic identification of dissociated histones from salt extraction of chromatin. Chromatin was dialyzed against the desired concentration of sodium chloride, then centrifuged to separate dissociated protein (supernatant) from the resultant chromatin (pellet). The supernatant proteins were then examined by analytical disc electrophoresis to determine the numbers and kinds of basic proteins dissociated at each salt concentration. All gels are 10% polyacrylamide, 6 M in urea; migration is from top to bottom. (a) 0.6 M NaCl supernatant; fractions γ and δ. (b) 0.7 M NaCl supernatant; fractions γ and δ. (c) 0.8 M NaCl supernatant; fractions γ and δ. (d) 0.9 M NaCl supernatant; fractions γ and δ. (e) 1.0 M NaCl supernatant; fractions γ and δ. (f) 1.5 M NaCl supernatant; fractions γ and δ. (g) 2.0 and 4.0 M NaCl supernatant; fractions α, β, γ, δ, and ϵ. (h) Whole histone; fractions α, β, γ, δ, and ϵ.

matin is in accord with that of Hamer (1955), insofar as the basic protein from *Arbacia punctulata* sperm is histonelike. The lower lysine to arginine ratio, lower basicity, and the lower alanine content which he reported are the main differences when compared with this work. His use of dilute HCl for extraction of whole sperm heads which had been washed with dilute citric acid prior to extraction may account for some of the discrepancy.

The amino acid composition of total basic protein from sperm chromatin is clearly histonelike and aside from minor variations already noted resembles calf thymus histone. The slightly lysine-rich fractions (proteins δ and ϵ) and arginine-rich fractions (α, β) of sea urchin sperm are very closely related to these corresponding fractions in calf thymus histone. Sea urchin sperm protein fraction γ, classified as lysine-rich, does not belong exclusively in that category; its high arginine content, and its Lys/Arg ratio clearly relate it to the slightly lysine-rich histones. Further comparisons with other types of basic proteins showed near exact identity with a group of proteins which have been found in nucleated erythrocytes of the chick, fish, and frog, and were very similar to a protein fraction found in the sperm of another urchin (Hnilica, 1967). This type of protein appears to be associated with cells having a condensed nucleus, which have ceased mitotic activity and are inactive in supporting RNA synthesis. It appears significant that this protein type accounts for 35% of the total basic protein in the mature sperm of *Arbacia punctulata*.

B. Stabilization of DNA by Chromosomal Protein. The displacement of the melting curves of DNA to higher temperatures and their melting over a broader temperature range on combination with protein as nucleohistone, but retention of the shape of the curve, mimics the effect of salt on the melting curve of DNA and has led to the interpretation that the stabilization afforded DNA by protein is primarily shielding of the negative charges on the phosphate groups leading to a more stable helical structure. The similar shapes of the melting profiles of DNA and nucleohistone (although nucleohistone has a broader melting range) implies that the stabilization afforded DNA by the histones is nearly equal throughout the molecules and consequently the histones themselves are heterogeneously situated along the DNA molecules (Lee *et al.*, 1963).

This is supported by the facts that: (1) reconstituted nucleohistones show varying degrees of stabilization depending upon the histone fraction used for reconstitution. Stabilization increases from arginine rich to slightly lysine rich to lysine rich (Huang *et al.*, 1964);

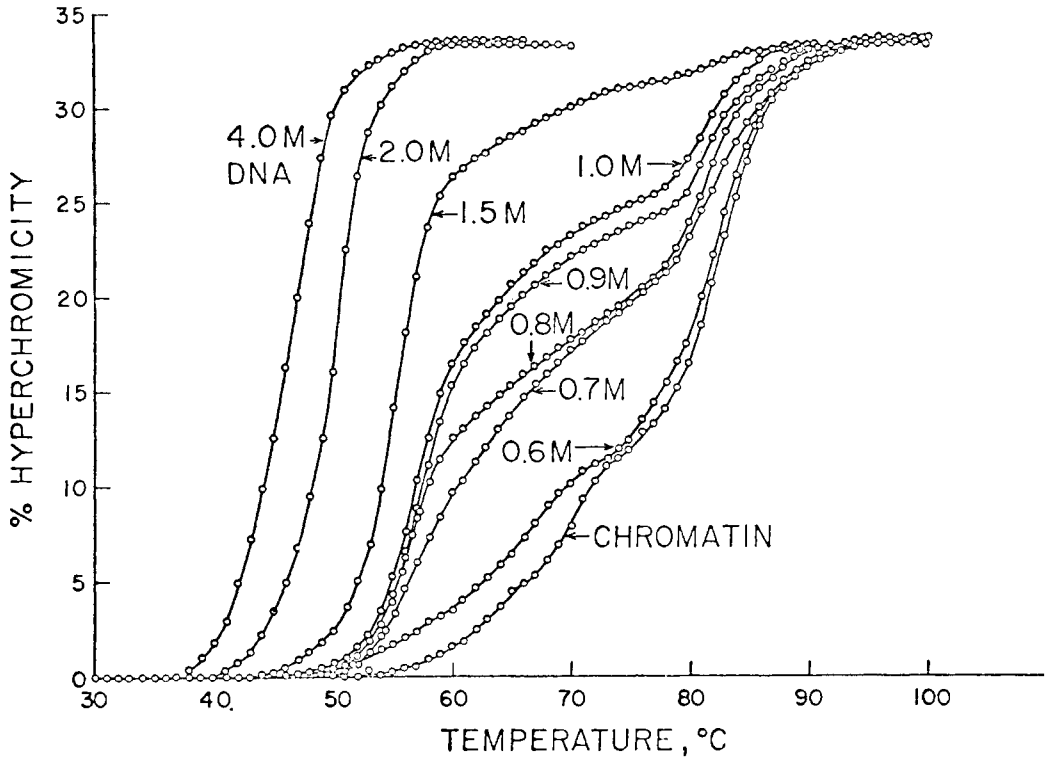

FIGURE 8: Melting profiles of sea urchin sperm chromatin following partial dissociation of basic proteins by sodium chloride. After dialysis against the desired salt concentration, the chromatin was centrifuged to separate dissociated protein (supernatant) from the resultant chromatin (pellet). The resultant chromatin pellets, depleted of certain histone fractions, were dissolved in water and dialyzed against 2.5×10^{-4} M EDTA (pH 8.0). The melting profile of each was then determined.

(2) salt extraction experiments have shown only broad one-step, melting profiles after selective dissociation of any of the histone fractions (Ohlenbusch *et al.*, 1967).

The two-step melting profile of sperm chromatin suggests that different regions of the DNA of the chromatin are stabilized to different extents by the constituent proteins associated with those regions. This implies differential binding of proteins at specific sites along DNA and/or grouping of similar types of protein at specific sites along DNA. The results of salt extraction experiments supports this view also. The dissociation behavior of the proteins of sea urchin sperm chromatin is different from the behavior of calf thymus histones (Olivera, 1966; Ohlenbusch *et al.*, 1967).

To summarize their data: lysine-rich histones are dissociated completely by 0.6 M NaCl; slightly lysine-rich histones are dissociated throughout the range 0.8–1.2 M NaCl; arginine-rich histones are dissociated in the range 0.9–2.0 M NaCl. Compared with the dissociation behavior of calf thymus histones from DNA, fractions γ and δ begin to dissociate before slightly lysine-rich histones normally do and extend beyond the usual dissociation range of slightly lysine-rich histones. It is interesting that fraction γ, having some typical features of lysine-rich histone, is not completely dissociated by 0.6 M NaCl. Fraction δ, slightly lysine rich, begins to dissociate at 0.6 M, before the usual range of typical slightly lysine-rich histone dissociation. The fact that both proteins dissociate together in the same relative proportions over the entire salt concentration range suggests that they may be interacting with each other in the chromatin complex and hence dissociate together.

The dissociation of arginine-rich fractions α and β does not begin until a slightly higher salt concentration than does calf thymus arginine-rich histone. No obvious reason for this behavior comes from the amino acid compositions unless the Lys/Arg ratio is most important. Fraction α has a Lys/Arg ratio very similar to calf thymus arginine-rich histone. Fraction β, however, has a much higher relative amount of arginine (Lys/Arg ratio much lower). This is due to a reduction in the amount of lysine present (see Table IV). The behavior of fraction ϵ, a slightly lysine-rich histone, is aberrant. Its behavior (not dissociating until very high salt concentrations and only when arginine-rich histones dissociate) suggests that for protein fractions α, β, and ϵ also, some association may be present in chromatin leading to combined dissociation.

C. Template Activity of DNA and Chromatin. Functionally, the stabilization of DNA by chromosomal protein affects the template activity of DNA. It is interesting that sperm chromatin, possessing histones of composition similar to somatic cells, is almost genetically silent. Clearly, the relative amounts of histone fractions and their disposition along the DNA are of utmost importance in determining the functional state of chromatin. The presence of a peculiar histone

TABLE V: Template Activity of Sea Urchin Sperm Chromatin after Extraction with Sodium Chloride Solutions.[a]

NaCl (M)	μμmoles of [^{14}C]ATP/10 min per 0.25 ml	% Template Act. Compared with DNA
0, chromatin	250	2.2
0.6	750	7.2
0.7	1,650	15.7
0.8	2,000	19.0
0.9	2,900	27.6
1.0	4,700	44.7
1.5	6,900	65.7
2.0	9,000	85.8
4.0	9,900	94.3
DNA	10,500	100.0

[a] Template activity of sea urchin sperm chromatin without treatment or after salt extraction as indicated. Chromatin was dialyzed against the desired concentration of sodium chloride, then centrifuged to separate dissociated protein (supernatant) from the resultant chromatin (pellet). The pellets were then redissolved in water and dialyzed against water. The pellets were then used as templates for *in vitro* RNA synthesis. After salt extraction the template activity may be compared to the template activity of DNA as an indication of the restrictive role played by the proteins that have been dissociated. RNA synthesis is expressed as μμmoles of [^{14}C]ATP incorporated into 10% trichloroacetic acid insoluble material per 10-min incubation at 37°, in a total reaction mixture of 0.25 ml.

fraction ($\gamma = F_2c$, 5) in the chromatin of repressed cell types warrants further investigation as to its role.

Not to be neglected is the role of nonhistone protein: evidence from other systems indicates that the basic proteins alone may not account for differences in template activity (Dingman and Sporn, 1964; Swift, 1964; Frenster, 1965; Marushige and Bonner, 1966; Marushige and Ozaki, 1967). The bulk of this evidence relates a greater amount of nonhistone protein present in chromatin with a more active (capable of a greater amount of RNA synthesis) genome, and reinforces the notion that histone plays an inhibitory role in gene activity and nonhistone protein alters the inhibitory capacity of histones. The small amount of nonhistone protein present in sperm chromatin and its genetic silence seems to support this view. A detailed report concerning interactions between RNA, nonhistone protein, and histones will be described in a subsequent paper.

It seems quite significant that there is no true lysine-rich histone in sea urchin sperm. Instead, it is replaced by a protein (γ) which has some properties of the slightly lysine-rich histones and others similar to the lysine-rich histones. The slightly lysine-rich and, to a lesser extent, the arginine-rich histones play the most important role in inhibiting the ability of DNA to serve as template for RNA synthesis and also seem to play a most important role in the coiling and condensation of chromatin (Zubay and Doty, 1959; Izawa *et al.*, 1963; Huang *et al.*, 1964; Bonner *et al.*, 1968). In sea urchin sperm chromatin, nearly 90% of the histone may be classed as slightly lysine rich, the remainder arginine rich. The condensation of the sperm chromatin and its low template activity may be the result of disposition of these particular classes of histones along the DNA molecules.

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Determination of the Equilibrium Constants of Associating Protein Systems. III. Evaluation of the Weight Fraction of Monomer from the Weight-Average Partition Coefficient (Application to Bovine Liver Glutamate Dehydrogenase)*

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ABSTRACT: Procedures for evaluation of weight fraction monomer from experimental weight-average partition coefficients have been derived for several important cases of association. Since the partition coefficients are sensitive to molecular asymmetry, the molecular sieve data can be combined with molecular weight data to provide inferences regarding the mode of aggregation for an associating system. These procedures have been applied to bovine liver L-glutamate dehydrogenase. For this enzyme the correlation of molecular weight and partition coefficient data indicated a linear aggregation of subunits. The subunit association was of the "indefinite" type in which a single equilibrium constant pertains to subunit addition for all species.

In recent years the experimental study of associating protein systems has depended largely upon development of useful theories for interpretation of data that can be obtained by a variety of physical techniques such as osmotic pressure, light scattering, molecular sieve chromatography, and sedimentation equilibrium (Steiner, 1952; Squire and Li, 1961; Rao and Kegeles, 1958; Gilbert and Jenkins, 1963; Jeffrey and Coates, 1966; Adams and Filmer, 1966; Van Holde and Rossetti, 1967; Albright and Williams, 1968; Chun and Fried, 1967; Adams and Lewis, 1968; Chun *et al.*, 1968).

Recently it has been demonstrated (Ackers and Thompson, 1965; Ackers, 1967a; Chiancone *et al.*, 1968) that molecular sieve chromatography may be used for quantitative studies which give results analogous to those of previous transport boundary analysis (Gilbert, 1955; Gilbert and Jenkins, 1963). It is clear from these studies that in order to determine the mode of association in experimental systems by molecular sieve chromatography, it is necessary to carry out an evaluation of the weight fraction of monomer from the

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