Use of Sodium Dodecyl Sulfate, Alone, to Separate Chromatin Proteins from Deoxyribonucleoprotein of *Arbacia punctulata* Sperm Chromatin*

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ABSTRACT: A method using sodium dodecyl sulfate to extract about 90% of the chromatin protein from a remaining deoxyribonucleoprotein of *Arbacia punctulata* sperm chromatin is described. More than 92% of the extracted protein could be concentrated extensively by lyophilization in the presence of sodium dodecyl sulfate and reduced to a sodium dodecyl sulfate to protein weight ratio of 0.1000 *via* a urea dialysis–$BaCl_2$ precipitation procedure. This protein was readily separated into six distinct fractions in the presence of $BaCl_2$ using acrylamide disc gel electrophoresis. Five of the fractions were histone while a sixth contained acidic protein. The remaining 8% of the extracted protein could be redissolved in Lowry C reagent from a precipitate induced by $BaCl_2$ for the elimination of sodium dodecyl sulfate. Precipitated protein, after redissolving in this reagent, behaved electrophoretically like nonprecipitated protein. Once electrophoretically fractionated, the sodium dodecyl sulfate to protein ratio of the protein components could be further reduced by dialysis. The method is capable of yielding 100% of the fractionated chromatin protein in soluble form. It may be used to analyze chromatin into its component parts for their further chemical or physical probing or for reconstitution studies. Sodium dodecyl sulfate also provides a way to examine the deoxyribonucleic acid melting profile of chromatin or nucleoprotein preparations without having to first extract the deoxyribonucleic acid from the preparations.

For some time SDS,[1] an anionic detergent, has been used in conjunction with salts or organic chemicals to separate protein from nucleic acids (Screenivasaya and Pirie, 1938; Kay and Dounce, 1953; Crestfield *et al.*, 1955; Brown, 1967; Marko and Butler, 1951; Kay *et al.*, 1952; Marmur, 1961). Since these procedures were directed at recovering purified nucleic acids, no attempt was made to keep dissociable protein soluble.

In order to study structures and functions of the chromatin proteins, the proteins associated with DNA in isolated chromatin, it is necessary to separate them from DNA and from each other. Three conventional ways have been used to accomplish this, each having rendered at least a portion of the chromatin protein soluble. The first way has been to acid-extract protein from chromatin and then to alkali solubilize the remaining protein followed by hydrolysis of DNA. The second method has also used acid extraction initially, but has been followed by an extraction of the acid-insoluble protein with 1.0% SDS and a centrifugation to bring down the DNA. The third method has taken advantage of high salt concentration (2–4 M NaCl or CsCl) which can remove most of the protein from DNA. In the first two methods chromatin proteins suffered strong acid treatment. It is difficult to reduce salt concentration in the third by dialysis in order to concentrate the protein without causing insolubility. Recently $(NH_4)_2SO_4$ precipitation has been used to concentrate the salt-dissociated chromatin protein. However, the recovery of the protein by this method has not been reported (Paul and Gilmour, 1969).

That SDS by itself might be useful as a tool to separate chromatin into many of its components was suggetsed from the fact that it could aid a high salt concentration in dissociating proteins from nucleic acids (Marmur, 1961) and that it could remove acid-unextractable protein from chromatin keeping it soluble (Marushige *et al.*, 1968). Another specific example of the ability of SDS to remove protein from DNA was reported by Jones and Berg (1966) when they found that SDS by itself was able to free RNA polymerase from DNA. That SDS might be capable of keeping the chromatin components soluble was indicated in the studies of Marko and Butler (1951). Using a 0.15% solution of SDS at different NaCl concentrations they found that at very low or at high salt concentrations protein was most soluble; it was completely soluble with no added NaCl.

We have found that 1% SDS (3.47×10^{-2} M), alone, is able to strip 90% of the protein from sea urchin chromatin and to keep it in soluble form. Furthermore, the chromatin proteins, having been released from DNA by SDS and from the majority of the SDS by urea dialysis and $BaCl_2$ precipitation, were separable from each other according to their electrophoretic mobilities. SDS, therefore, acts as a convenient tool for fractionating and concentrating chromatin protein constituents for analytical and possibly for reconstitution studies.

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[1] Abbreviations used are: SDS, sodium dodecyl sulfate; SSC, saline–citrate buffer, 0.15 M NaCl–0.015 M sodium citrate; DSC, dilute saline–citrate buffer, 1–10 dilution of SSC; T_m, temperature at inflection point of nucleic acid melting curve; Lowry C reagent, 48 parts of 2% Na_2CO_3–0.1 N NaOH, 1 part of 0.5% $CuSO_4$, 1 part of 1% potassium tartrate solution.

Materials and Methods

Chromatin Preparation. Arbacia punctulata sperm chromatin was prepared according to the techniques used by Paoletti and Huang (1969). The chromatin had protein to DNA ratios of 1.35–1.43, the protein being determined by the method of Lowry *et al.* (1951) using histones which were extracted with 0.4 N H_2SO_4 from *A. punctulata* sperm chromatin (Paoletti and Huang, 1969) as a standard and the DNA by the method of Giles and Myers (1965) using DNA extracted from the same source by the method of Marmur (1961) as modified by Bonner *et al.* (1968) as a standard. The spectral ratios gave the following ranges: 230 mμ/260 mμ, 0.608–0.862; 280 mμ/260 mμ, 0.570–0.607; and 320 mμ/260 mμ, 0.052–0.101.

Separation of Chromatin Protein from Deoxyribonucleoprotein after SDS Treatment of Chromatin. An initial SDS–chromatin solution was made up by bringing to a 120-ml volume 800 OD_{260} units of chromatin in 1%, ethanol-recrystallized, SDS. This SDS–chromatin solution was centrifuged for 25 hr at 85,500*g* in a 40 head in a Spinco L2-65 ultracentrifuge at 70°F. The supernatant contained chromatin protein with no DNA. The pellet contained DNA in total recovery and some DNA-bound chromatin protein. This fraction was referred to as the deoxyribonucleoprotein resulting from the SDS extraction of chromatin.

Removal of SDS from Chromatin Protein. The chromatin protein supernatant from the 85,500*g* centrifugation was lyophilized. The lyophilizate was then dissolved in 10 ml of 10 M urea. This solution was dialyzed 2 days against 1 l. of 10 M urea, renewing the dialysis medium after 1 day. $BaCl_2$ (0.5 g) was added and the mixture was centrifuged at 12,000*g* in a SS-34 Sorvall rotor head for 15 min at 65–70°F. The pellet was washed once in a similar $BaCl_2$–10 M urea solution. The combined supernatants made up the chromatin protein fraction.

Electrophoresis of the Chromatin Protein. The separation of the chromatin protein constituents was accomplished using disc electrophoresis on acrylamide gels. Preparative disc electrophoresis using Canalco preparative disc electrophoresis equipment was used to isolate the only chromatin protein fraction that migrated rapidly in the direction of the anode. Analytical acrylamide gel electrophoresis was used to separate five fractions that migrated toward the cathode. The five rapidly moving bands were not isolated individually. A nonmigratory band, throught to be caused by protein aggregation, was removed for analysis from analytical gels by 10 M urea.

BY ANALYTICAL DISC ELECTROPHORESIS. For Cathode-Migrating Fractions (Basic Proteins). The electrophoresis tubes were 8.5 × 0.4 cm i.d. glass tubing. The gels were constructed according to Bonner *et al.* (1968) with the exception that an acrylamide stock solution was used which contained 40 g of acrylamide, 0.27 g of bis, and water to 100 ml to give 10% acrylamide gels. Samples containing chromatin protein or H_2SO_4-extracted histone protein in from 10 to 30 μl of 10 M urea were layered on the gels. The remainder of the procedure along with a description of the apparatus is given by Bonner *et al.* The solutions used to stain the acrylamide gels were 1% (w/v) Buffalo Blue-Black, Amido Schwartz, in 40% (v/v) ethanol–7% acetic acid–water and 1% (w/v) acridine orange in 7% (v/v) acetic acid–water. The destaining solution used to wash the excess Buffalo Blue-Black or acridine orange stain from the gels and to store the gels was simply 7% (v/v) acetic acid.

For Anode-Migrating Fraction (Acidic Protein). The procedure used was a modification of those described in articles by Ornstein (1964), Williams and Reisfeld (1964), and Davis (1964). A two-gel system was used. The electrophoresis apparatus was the same one used for the cathode-migrating fractions. The lower separating gel contained one volume of A (48 ml of 1 N HCl, 0.46 ml of *N,N,N′,N′*-tetramethylethylenediamine, 36.3 g of Tris, and water to 200 ml), one volume of C (30 g of acrylamide, 0.8 g of *N,N′*-methylenebisacrylamide, and water to 100 ml), one volume of 10 M urea, and two volumes of lower gel catalyst (0.02 g of ammonium persulfate in 10 ml of 10 M urea). This gel was overlayed with 3 M urea until polymerization had occurred (usually about 30 min) after which time the 3 M urea was removed and the stacking gel was layered on top. The stacking gel represented about one-fifth the volume of gel that the separating gel did in the tube. The stacking gel was composed of one volume of B (25.6 ml of 1.0 M H_3PO_4, prepared by diluting 68.3 ml of the 85% reagent to 1 l.), two volumes of D (10 g of acrylamide, 2.5 g of *N,N′*-methylenebisacrylamide, and water to 100 ml), four volumes of 10 M urea, and two volumes of upper gel catalyst (4.0 mg of riboflavin in 100 ml of 10 M urea), the last being freshly prepared prior to each electrophoresis. The stacking gel was also overlayed with 3 M urea until polymerization had occurred. The samples were in 10 M urea and contained preparative disc purified acidic protein or extracted chromatin protein in volumes of not more than 30 μl. They were run at 1.5 mA/tube for about 20 min. The lower reservoir buffer was 0.05 M Tris adjusted with HCl to pH 8.75–9.5. The upper reservoir buffer was 1 l. of a solution containing 6.0 g of Tris, 28.8 g of glycine, and water and having a pH of 7.7. The gels were stained, destained, and stored like those for the cathode-migrating fractions.

BY PREPARATIVE DISC ELECTROPHORESIS. For Anode Migrating Fraction (Acidic Protein). The Canalco Prep-Disc electrophoresis apparatus, distributed by Canal Industrial Corp. of Rockville, Md., was employed making use of only the separating gel described under analytical disc electrophoresis for the anode migrating fraction (acidic protein). The gel was about 1.5–2 cm in length after polymerization in the upper column. A 5-ml sample of chromatin protein in 10 M urea containing 10–30 mg of protein was run at 2.5 mA anywhere from 2 to 5 hr. The same buffer system that was used for anode-migrating protein in the analytical disc electrophoresis section was used here. The elution buffer was a pH 8 solution of 0.01 M Tris. The eluted material was dialyzed against distilled water and lyophilized.

For Cathode-Migrating Fraction (Basic Proteins). A single 1.5-cm gel, described for cathode-migrating protein in the analytical disc electrophoresis section, was used. The buffer system was also the same. The eluting medium was 0.01 M acetic acid. A sample load of 0.7 ml was electrophoresed for 7 hr at 9 mA or 70 V.

USING A MODIFIED ANALYTICAL DISC ELECTROPHORESIS PROCEDURE FOR THE ISOLATION OF NONMIGRATORY PROTEIN. Eight large analytical gels (tube size 10 × 0.6 cm i.d.) were used to isolate a fraction that remained preferentially at the origin on the gel when electrophoresis was toward the cathode. The gels and the buffer system were the same used for the

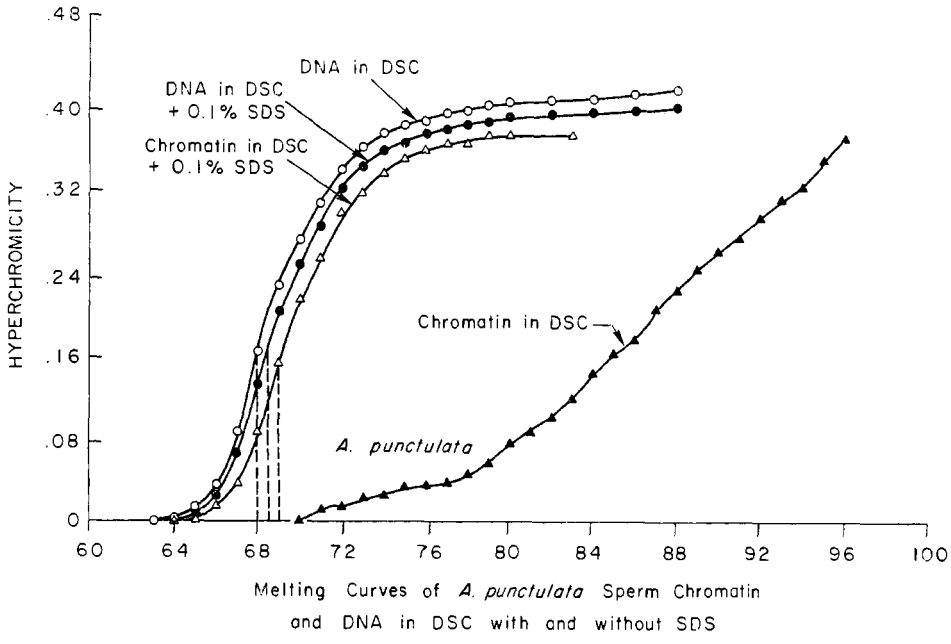

FIGURE 1: Melting curves of chromatin and DNA from sea urchin sperm in DSC with and without the presence of 0.1% SDS. The samples were heated at 1°/min. Hyperchromicity was calculated from the expression: OD_{260} of sample at given temperature minus OD_{260} of sample at 22°/OD_{260} of sample at 22°. (○—○) DNA in DSC; (●—●) DNA in DSC plus 0.1% SDS (0.00347 M SDS); (▲—▲) chromatin in DSC; (△—△) chromatin in DSC plus 0.1% SDS.

analytical electrophoresis of material migrating in the direction of the cathode; 0.1 ml containing from 500 to 900 μg of the chromatin protein fraction which had been 10 M urea dialyzed 1 day after $BaCl_2$ treatment was loaded onto each gel. The electrophoresis was conducted for 2.5 hr at 4 mA/tube. The nonmigratory band portion of the gels, about 0.3 mm at the loading end of the gel, was homogenized thoroughly in 10 ml of 10 M urea over a 1-hr period. The viscous jelly-like homogenate was centrifuged for 30 min at 26,400*g* in a Spinco L2-65 ultracentrifuge, which had been brought to 25°, to pellet the contaminating gel. The 7.2 ml of recovered supernatant was dialyzed against distilled water and then lyophilized.

Amino Acid Analysis of Anode-Migrating (Acidic Protein) and Nonmigratory Bands. Since these two fractions each stained with acridine orange suggesting possible nucleic acid affiliation, portions of them were alkali hydrolyzed at 37° for 18 hr in 0.3 N KOH. Two dialyses against SSC (0.15 M NaCl and 0.015 M sodium citrate, pH 7.5) and three against water were used to free the proteins from salt, nucleotides, and urea (if present). The protein solutions were lyophilized and 0.2 mg of each were redissolved in 1 ml of 6 N HCl. The proteins were hydrolyzed in a nitrogen atmosphere at 110° for 18 hr. The solutions were 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.

Melting Curves of DNA and Chromatin. Melting curves were taken on a Beckman DU spectrophotometer with a Gilford Model 2000 multiple-absorbance recorder. Samples were heated by a Haake circulating water bath at a rate of 1°/min.

Isolation of DNA. DNA was isolated from *A. punctulata* sperm chromatin using Marmur's method (1961) essentially, but with the modifications used by Bonner *et al.* (1968).

Isolation of Basic Protein. Aside from the isolation of basic protein by preparative disc electrophoresis for the purpose of monitoring its SDS to protein ratio described in the section for the isolation of cathode-migrating chromatin protein using preparative disc electrophoresis, basic protein was also extracted using 0.4 N H_2SO_4 according to the procedure used by Paoletti and Huang (1969).

A-150 Bio-Gel Column to Separate Chromatin Protein from Deoxyribonucleoprotein. The column used was 40 × 2.2 cm i.d. It was charged with about 15 OD_{260} units of either chromatin in SDS or deoxyribonucleoprotein which had been extracted three times with 1.0% SDS. The elution solvent, like the solvent used to equilibrate the column, was 0.1% SDS.

^{35}S-Labeled SDS Purchase and Handling. ^{35}S-Labeled SDS (Nuclear-Chicago Corp., Des Plaines, Ill.) was mixed with ethanol-recrystallized SDS as a carrier to quantitate the removal of SDS from chromatin protein in SDS by its specific radioactivity; 0.1-ml samples of solutions to be counted were added to 10-ml proportions of a fluor made up by combining 333 ml of Triton X-100, 666 ml of toluene, 125 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] (Packard Instrument Co.), and 5.5 g of 2,5-diphenyloxazole (New England Nuclear). Measurements were made by counting duplicate solutions for 1 or 10 min, the latter time being used for samples containing less than 10,000 cpm. The samples were counted on a Packard Model 2002 Tri-Carb liquid scintillation spectrometer.

Results

Effect of SDS on a Chromatin's Melting Profile. Melting curves of *Arbacia punctulata* sperm chromatin and DNA extracted from that chromatin differ (Figure 1). The difference

in the melting curves and the resulting T_m's (melting temperatures) is thought to reflect the stability imparted to the DNA by the associated chromatin components. Making the chromatin solution 0.1% in SDS caused it to melt almost identically as its extracted DNA, the 0.5° difference possibly resulting from a small deviation in ionic strength between the two solutions. This suggests that the chromatin component(s) stabilizing DNA against thermal denaturation has lost its ability in 0.1% SDS to do so. Further inspection of the SDS-treated chromatin revealed that 90% of its protein is dissociated from its DNA (Table I). It is thought that the dissociation of this protein by the SDS may have been responsible for the change in the chromatin melting profile in the presence of SDS. The chromatin of the crabs, *Cancer borealis* and *Cancer antennarius*, in 0.1% SDS gave melting curves identical with their component DNAs (T. L. Shirey and R. C. Huang, 1968, unpublished data). This supports the idea that this change in melting phenomenon was not specific to *A. punctulata* sperm chromatin.

Isolation of Chromatin Protein from SDS-Treated Chromatin. DETERMINATION OF AN ADEQUATE SDS CONCENTRATION TO REMOVE THE MAXIMUM AMOUNT OF SDS-DISSOCIABLE PROTEIN FROM CHROMATIN. Since 0.1% SDS caused a dramatic effect on chromatin melting, a study was made on the dissociation of protein from chromatin using different SDS concentrations. At an SDS concentration of 0.5–1.0% the maximal amount of SDS-extractable protein, about 90%, is removed from the chromatin (Table I). However, multiple extractions with 0.1% SDS yielded the same amount of dissociated chromatin protein. One extraction with 1% SDS was chosen to remove protein from chromatin for further studies.

The dissociable protein, referred to as chromatin protein, could be isolated from the supernatant of a 25-hr, 85,500*g* centrifugation. The quantity of DNA remaining with the protein depended upon the time of the centrifugation. Almost all of the DNA had been removed in 25 hr. Protein to DNA ratios taken from 18- and 25-hr centrifugations were 88.0 and 157.5, respectively.

EXAMINATION OF THE 85,500*g* PELLET, DEOXYRIBONUCLEOPROTEIN, RESULTING FROM THE CENTRIFUGATION OF SDS-TREATED CHROMATIN. In order to determine whether the 10% SDS-nonextractable chromatin protein was mechanically trapped, whether it was an aggregate of denatured protein, or whether it was a part of a soluble nucleoprotein which failed to be dissociated from DNA by SDS, the 85,500*g* pellet of chromatin extracted three times in 1% SDS was dissolved in 0.1% SDS and passed through an A-150 Bio-Gel column which had been equilibrated in SDS. The elution profile in Figure 2 resulted, the single peak having a protein to DNA ratio of 0.103. Therefore, the protein appeared to be adhering to the DNA. To show that the A-150 Bio-Gel column would indeed separate dissociated chromatin protein from undissociated nucleoprotein, some initial SDS–chromatin solution was put through the same column. The resulting elution pattern (Figure 2) illustrates chromatin protein separation from nucleoprotein as seen from the protein to DNA ratios of the two eluted peaks, 38.2 and 0.137, respectively. The protein to DNA ratio of this nucleoprotein peak (0.137) is similar to that of the peak eluted when the nucleoprotein resulting from the SDS extraction of chromatin (0.103) had been passed through the column. Since DNA, purified by the Marmur technique (1961) or by the centrifugation of chromatin in 4 M CsCl, has a protein to DNA ratio varying from about 0.020 to 0.040, a significant amount of additional protein remained with both of the SDS-extracted nucleoprotein peaks. Therefore, the 10% of the chromatin protein which was accounted for in the deoxyribonucleoprotein resulting from the SDS extraction of chromatin appeared to be bound to the DNA in the presence of SDS; hence the name of the fraction.

TABLE I: SDS Extraction of Chromatin Protein from Chromatin.

Concn of SDS Used for Extraction (%)	No. of Times Chromatin Extracted	mg of Protein/mg of DNA in Pellet	% Protein Extracted from Chromatin[a]
0.1	1	0.502	63.9
		0.472	70.1
		0.633	72.8
		0.322	81.3
	3	0.191	91.0
		0.109	89.5
0.5	1	0.288	86.7
		0.227	87.1
1.0	1	0.222	87.9
	2	0.174	89.5
	3	0.134	91.5
2.0	1	0.222	86.5
3.0	1	0.190	89.0
5.0	1	0.197	88.8

[a] Calculated from (mg of protein in 85,500*g* supernatant)·(100)/mg of protein in supernatant plus pellet.

Separation of SDS from Chromatin Protein. The chromatin protein in SDS representing about 90% of the chromatin protein was lyophilized to dryness and was totally redissolved into a small volume of 10 M urea, a process which permittted great concentration of chromatin protein with no loss of it. It was then separated from most of its associated SDS. This was done by dialyzing the concentrated chromatin protein against 10 M urea for 2 days followed by subjecting the dialyzed material to a 0.2 M $BaCl_2$ treatment. This approach reduced the SDS to protein ratio greatly, maintained most of the protein in a soluble state, and permitted a fairly clean separation of chromatin protein components electrophoretically.

The employment of 10 M urea dialysis rather than immediately attempting to rid the concentrated chromatin protein of SDS with $BaCl_2$ was prompted for two reasons. The first was that the precipitate resulting from the addition of $BaCl_2$ to the concentrated chromatin protein in SDS solution, containing more than 10% SDS by weight, was large and trapped a lot of protein. The second was that the $BaCl_2$ was incapable of removing all of the SDS from the concentrated chromatin protein solution, and predialysis would aid in its elimination.

The effectiveness of 10 M urea dialysis in separating SDS from chromatin protein is illustrated in Table II. Putting

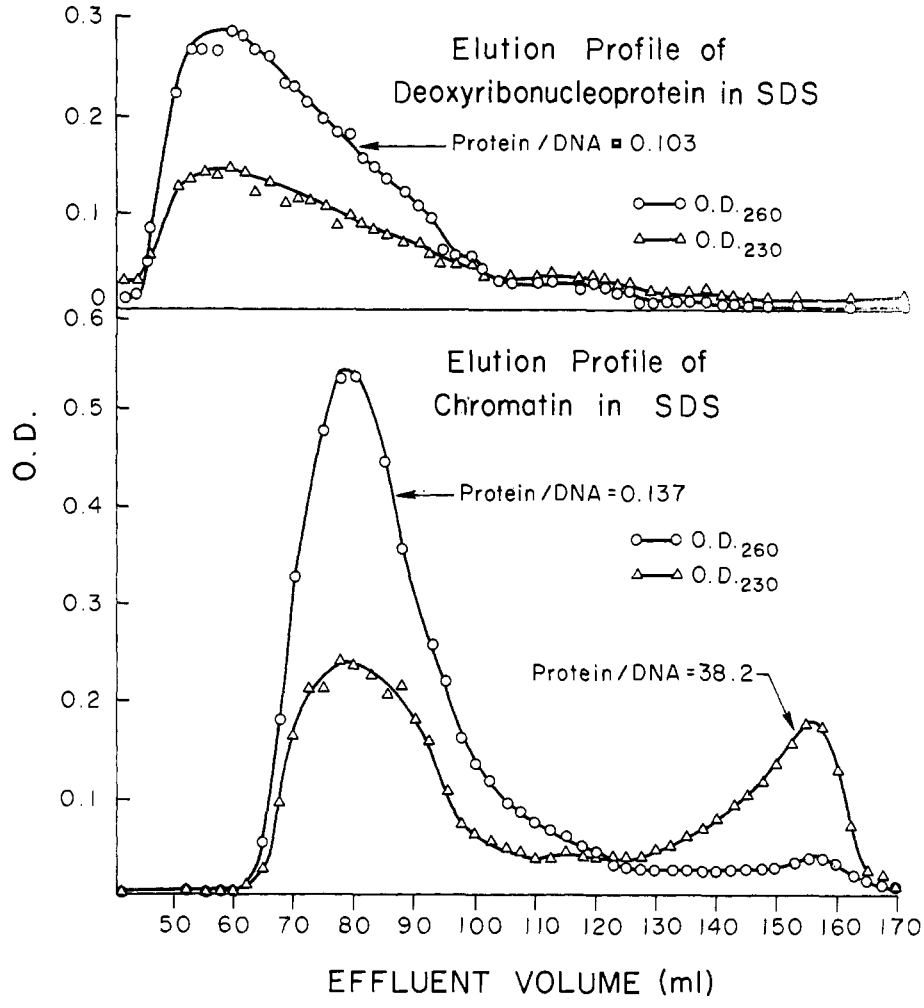

FIGURE 2: Elution profiles of deoxyribonucleoprotein (top), the 85,500 *g* pellet resulting from the third 1% SDS extraction of sea urchin chromatin, and unextracted chromatin (bottom) from an A-150 Bio-Gel column equilibrated in 0.1% SDS (described in Methods). Each sample loaded on the column was in 1.0% SDS and contained 15 OD_{260} units of material in a volume of 5 ml. The protein and DNA components of the peaks were determined by the methods of Lowry *et al.* (1951) and Giles and Myers (1965), respectively. (○—○) OD_{260} and (△—△) OD_{230}.

Dowex 1-acetate into the 10 M urea dialysis medium enhanced the speed at which SDS was removed. The addition of a high salt concentration to the dialysis medium, NH_4Cl in this case, slowed down the rate of SDS dialysis extensively. It was found that it took about 1 day for equilibrium to take place between the SDS entering the dialysis medium and that within the dialysis sac. A greater concentration of protein in the dialysis sac (not shown in Table II) did not affect the rate or quantity of SDS dialysis.

Precipitation was noted in the dialysis sac when the SDS to protein weight ratio was permitted to decrease to a value between 0.35 and 0.43 (just below a calculated SDS anionic charge to chromatin protein cationic charge ratio of 1). Extensive precipitation was noted with further dialysis. No lowest limit was reached for an SDS to protein ratio over a 3-day span of dialysis changing the dialysis medium daily, the lowest ratio recorded being 0.0059. The precipitate was not soluble in alkali, water, acid, or high salt, but it was soluble in the Lowry C reagent. In order to prevent precipitation of 1% SDS-extracted protein, but to eliminate the bulk of the SDS from the protein, the protein solution was dialyzed 2 days with a change of medium after 1 day against 10 M urea. This brought the SDS to protein ratio to about 0.5.

Acrylamide disc electrophoresis of a urea solution of 0.1% SDS-extracted chromatin protein (protein stains with Buffalo Blue-Black) suggested that all of the protein was tied up with the SDS (SDS and nucleic acids stain with acridine orange) in what migrated like a negatively charged micelle (sample 1, Table III). Electrophoresis of 1% SDS-extracted chromatin protein in urea after it was dialyzed 2 days against 10 M urea (protein all soluble, SDS to protein ratio about 0.5) suggested that the negatively charged micelle was still the predominant material, but that a little of the protein was possibly breaking loose from the micelle (sample 2, Table III). Electrophoresis of the supernatant of a chromatin protein solution, which had been dialyzed against 10 M urea until precipitation occurred, and the precipitate, which had been redissolved in Lowry C reagent, yielded migratory patterns that were similar to each other (samples 3 and 4, Table III). Each sample indicated that since its SDS to protein ratio had been further lowered from that of sample 2 (determined by the

reduction in [^{35}S]SDS relative to protein), its protein had been liberated to a greater extent from SDS and had been permitted to migrate more according to its charge.

To remove further SDS from a 1.0% chromatin protein solution after it had been dialyzed 2 days against 10 M urea, and to prevent protein from precipitating, salt was employed. To portions of such a solution were added the following: $BaCl_2$ to concentrations of 0.01 and 0.2 M, KCl to concentrations of 0.04 and 1.3 M, and NH_4Cl to concentrations of 0.04 and 0.80 M. Following centrifugation of the $BaCl_2$ and KCl solutions in which precipitation had occurred, the supernatants and the NH_4Cl solution were all divided in half. The first half was subjected to electrophoresis immediately and the second half was dialyzed against 10 M urea 1 day and then electrophoresed. NH_4Cl liberated no basic protein, the electrophoretic pattern of the solution resembling that of sample 2 in Table III. However, both $BaCl_2$- and KCl-treated solutions yielded distinct bands of basic protein, the $BaCl_2$ solution's electrophoretic profile being illustrated as sample A in Table IV. In both the cases of the $BaCl_2$ and KCl treatment the higher salt concentration permitted much more Buffalo Blue-Black stainable material to migrate toward the cathode. Dialysis of the 0.2 M $BaCl_2$- and KCl-treated supernatants against 10 M urea for 1 day caused a reduction in the intensity of, and the blurring of, the cathode migrating bands (sample B, Table IV). It also caused the nonmigratory band in the cathode-oriented electrophoretic gel to increase markedly in size. These results suggested that the ionic strength of the protein solution has to be fairly high to prevent aggregation of the protein. Because KCl (and NaCl) presented technical difficulty when trying to separate the precipitated material from the supernatant after adding solid KCl (or NaCl) to 2 day, 10 M urea dialyzed chromatin protein in SDS, and because the electrophoretic bands were less distinct than those liberated by $BaCl_2$, further work was carried out with $BaCl_2$ only.

Table V suggests that a 0.2 M $BaCl_2$ concentration not only provided for good separation of chromatin protein electrophoretically, but also yielded the maximal recovery of soluble protein. It is uncertain from this table whether the increased $BaCl_2$ to SDS equivalence ratio or the higher ionic strength imparted by increasing the $BaCl_2$ concentration was responsible for releasing more protein from the precipitate. The 0.2 M $BaCl_2$ concentration decreased the SDS to protein weight ratio from 0.508 to 0.100 of a 1.0% SDS-extracted chromatin protein solution which had been dialyzed for 2 days against 10 M urea. Further 10 M urea dialysis of this $BaCl_2$-treated solution caused the decrease in intensity, and the blurring of the electrophoretic bands mentioned earlier led to eventual protein precipitation, but continued to lower the SDS to protein ratio to 0.007 and probably lower.

Chromatin Protein Isolation Procedure Yielding Maximum of Soluble, Electrophoretically Separable Protein with a Minimum of SDS Contamination (a Summary of the Procedure). Table VI and sample A in Table IV describe the quantity and nature of the chromatin protein which was isolated by the SDS–urea dialysis–$BaCl_2$ precipitation procedure described above. The procedure was capable of yielding about 81% of the chromatin protein in solution (barring loss from mechanical procedures), DNA free, and relatively free of SDS; 7% of the extracted protein remained with the precipitate which developed on the addition of $BaCl_2$ to chromatin protein in SDS. It was soluble in Lowry C reagent and could be electrophoretically separated into its components directly from the reagent. The remaining 12% of the chromatin protein remained with the DNA which pelleted at 85,500*g*. Of this, at least 3.5% was extractable with SDS rinsing and about 8–9% remained with the DNA as a soluble deoxyribonucleoprotein complex which may be dissociated with high salt.

TABLE II: Elimination of SDS from Chromatin Protein by 10 M Urea Dialysis.

Chromatin Protein[a] in SDS Sample	μg of SDS Remaining in 0.1 ml of Sample after Dialysis for			
	0 Time	6 hr	1 Day	3 Days
A. Chromatin extracted with 0.1% SDS[b]	761	68.0	9.0	
B. Dowex 1-acetate in dialysis medium of sample A	761	35.2	4.5	
C. Chromatin extracted with 1.0% SDS[b]	5550			7.1
D. 1 M NH_4Cl in dialysis medium of sample C	5550			2900

[a] Each sample contained 200 μg of protein in 0.1 ml. [b] Samples A and B initially had a much lower SDS to protein ratio than samples C and D. The quantity of SDS in any given fraction was determined by [^{35}S]SDS counting and calculated according to its specific radioactivity. Protein determination was carried out by the method of Lowry *et al.* (1951) (see Methods).

The combination of 10 M urea dialysis and $BaCl_2$ precipitation greatly reduced the SDS concentration in the extracted chromatin protein (Table VI). The SDS to protein ratio of the chromatin protein product, 0.100, may be further reduced by urea dialysis at the expense of being able to electrophoretically separate the chromatin proteins and, eventually, to keep them in solution. However, after electrophoretic separation of the chromatin protein components, further dialysis may be used to reduce the SDS to protein ratios for the individual component proteins.

Nature of the Electrophoretically Separated Chromatin Proteins. Five cathode-moving protein bands (samples A and B, Table IV) and one anode-moving protein band (samples A, B, and D, Table IV) were separated from the SDS-extracted chromatin protein following SDS removal. The cathode-oriented bands migrated identically as the bands of 0.4 N H_2SO_4-extracted chromatin protein (sample C, Table IV) which were shown by Paoletti and Huang (1969) to be histones. The SDS to protein ratio of the basic protein from peak 2 of Figure 3 was 0.017.

The elution profile of a peak containing protein which was recovered from an anode-oriented preparative disc electrophoretic run is shown in Figure 4. After concentrating the peak by lyophilization, it gave the analytical electrophoretic band in sample D in Table IV, and the amino acid composi-

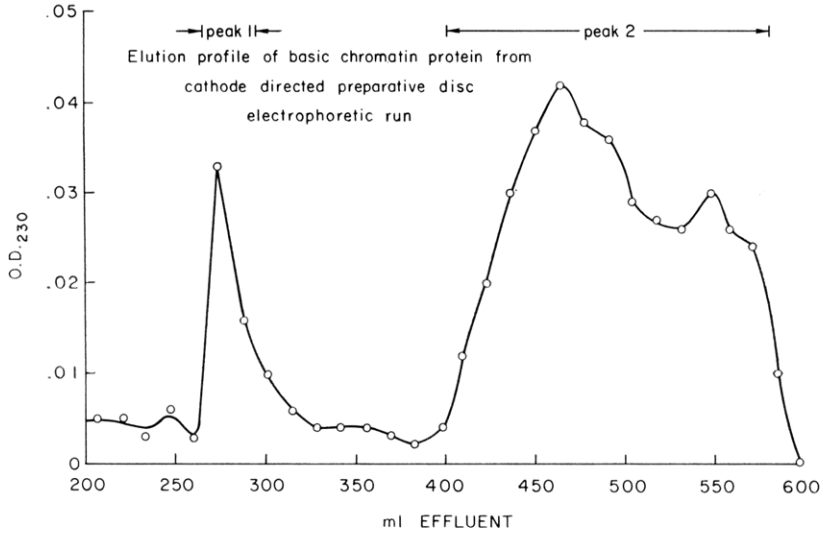

FIGURE 3: Elution profile of basic chromatin protein from a cathode-directed preparative disc electrophoretic run. The sample loaded on the gel contained 5.3 mg of protein retrieved from the upper tray buffer of the anode-directed preparative disc electrophoretic run described in Figure 4. The sample was dialyzed against H_2O, lyophilized, and redissolved in 10 M urea prior to loading. The conditions for the electrophoresis are described in the Methods section. No attempt was made to further fractionate the basic proteins from this run.

TABLE III: Analytical Disc Electrophoresis of Nondialyzed and 10 M Urea Dialyzed Chromatin Protein in SDS.

Sample[a]	Stain Used	Direction of Electrophoresis − ← +	Direction of Electrophoresis − → +
1. Nondialyzed 0.1% SDS extracted protein	Buffalo Blue-Black	No band	
	Acridine orange[b]	No band	
2. 1% SDS extracted protein (I) dialyzed 2 days against 10 M urea	Buffalo Blue-Black		
	Acridine orange[b]	No band	
3. Supernatant of I dialyzed until precipitate noted	Buffalo Blue-Black		
	Acridine orange[b]	No band	
4. Precipitate from sample 3 dissolved in Lowry C reagent	Buffalo Blue-Black		
	Acridine orange[b]		

[a] The quantity of protein loaded on each gel containing sample 1 was 425 μg; sample 2, 57.6 μg; sample 3, 50 μg; and sample 4, 345 μg. [b] The sharp acridine orange bands always appeared to correspond with Buffalo Blue-Black bands except in the case of sample 1 where the more rapidly migrating acridine orange staining band is thought to contain excess SDS.

tion in Table VII. The possibility that the acridine orange staining component of this protein fraction was nucleic acid was not disproven, although 45 μg of SDS was present with the fraction's 76 μg of protein. Dialysis of the fraction for 3 days against water with no change in dialysis medium decreased the SDS content of the fraction to 4 μg giving a SDS to protein ratio of 0.053.

Samples A and B in Table IV show a proteinaceous region, a nonmigratory band, which failed to migrate rapidly to either electrode when chromatin protein was subjected to electrophoresis, but which remained at the origin of the cathode-directed gel. Its protein had a slightly basic composition (Table VII). The band was small in the presence of 0.2 M $BaCl_2$. However, it became more prominent when the ionic

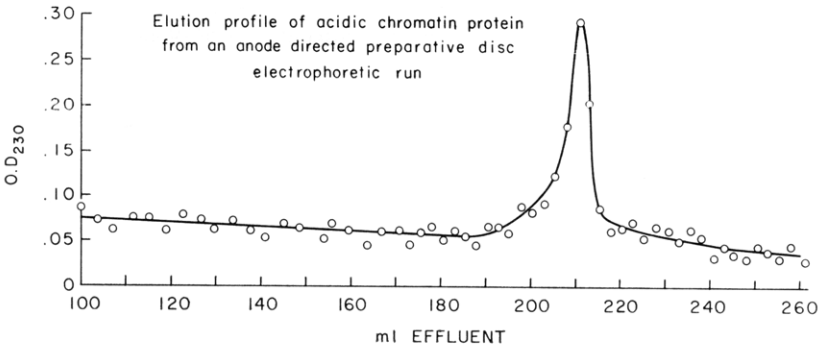

FIGURE 4: Elution profile of acidic chromatin protein from an anode-directed preparative disc electrophoretic run. The sample loaded on the gel contained 11.88 mg of chromatin protein in 10 M urea. The conditions for the electrophoresis are described in the Methods section.

TABLE IV: Analytical Disc Electrophoresis of $BaCl_2$-Treated Chromatin Protein in SDS.

Sample[a]	Stain Used	Direction of Electrophoresis − ← +	− → +
A. Chromatin protein fraction after urea dialysis and $BaCl_2$ precipitation (I)[c]	Buffalo Blue-Black	[f]	
	Acridine orange	[f]	
B. I dialyzed 1 day against 10 M urea[d]	Buffalo Blue-Black		
	Acridine orange		
C. Histones extracted by 0.4 N H_2SO_4	Buffalo Blue-Black		Not run
	Acridine orange	No band	Not run
D. Concentrated anode migratory (acidic protein) band[e]	Buffalo Blue-Black	Not run	
	Acridine orange	Not run	

[a] The quantity of protein loaded on each gel from sample A was 47.7 μg; on the gels run in the cathode direction from sample B, 85 μg; on the gels run in the anode direction from sample B, 585 μg; on the gels from sample C, 56 μg; and on the gels from sample D, 40 μg. [b] The acridine orange bands always appeared to correspond with Buffalo Blue-Black bands. [c] Chromatin protein extracted from chromatin with 1.0% SDS, concentrated by lyophilizing and redissolving in a small volume of 10 M urea, dialyzed 2 days against 10 M urea, and left in resulting solution after 0.2 M $BaCl_2$ treatment. The electrophoresis was run in the presence of $BaCl_2$. [d] The fourth band seen in sample B, but not in sample A, is thought to be the result of underloading sample A on the electrophoretic gel. [e] Anode migratory protein band concentrated *via* preparative disc electrophoresis of a large sample similar to sample B. [f] The dark background in the right half of these two gels was a white granular material which is related to the fact that the samples undergoing electrophoresis still had a 0.2 M $BaCl_2$ concentration.

strength of the chromatin protein was reduced by the 1 day 10 M urea dialysis. The band did have an acridine orange staining component which also was not positively identified. The acridine orange staining component was smaller in size with respect to its Buffalo Blue-Black staining counterpart when compared with the anode-moving band. It is thought that this band was an aggregate of basic proteins with acidic protein.

TABLE V: Recovery of Chromatin Protein after Treatment of the Protein in SDS with $BaCl_2$.

	Mixture[a]				
	1	2	3	4	5
Protein concentration in mixture in milligrams per milliliter	1.03	0.026	1.8	1.6	1.6
Molarity of $BaCl_2$ in mixture	0.027	0.027	0.186	0.205	0.410
Equivalents of $BaCl_2$/equivalents of SDS in mixture	4.94	4.94	13.1	19.7	39.4
% chromatin protein remaining with the precipitate induced by the $BaCl_2$[b]	33.9	37.2	6.1	6.0	5.8

[a] The mixtures were made by adding $BaCl_2$ to a solution of chromatin protein in SDS. The solutions of chromatin protein in SDS resulted from 0.1% SDS extraction of chromatin rather than 1% SDS extraction. The initial SDS to protein ratio was about 4.0. [b] Lowry C reagent has the ability to redissolve precipitated chromatin protein. The $BaCl_2$-precipitated material was centrifuged from the mixture, and resuspended in 10 M urea, and protein determinations of this suspension were made directly, in triplicate. The $BaCl_2$-precipitated material had been washed twice with the appropriate $BaCl_2$ concentration in 10 M urea.

Discussion

SDS Effect of Chromatin. SDS has the capacity to denature, precipitate, and form complexes with protein (Putnam, 1948). The interaction between SDS and protein is both ionic and hydrophobic in nature (Ray *et al.*, 1966). When added to chromatin, SDS was capable of removing more than 90% of the chromatin protein. In contrast to Dounce's conclusions (1965), it is thought that SDS rather than enzymes removed the protein from the DNA in our system because chromatin was capable of giving a typical chromatin melting profile several days after its isolation (indicating that chromatin protein was still associated with DNA), but immediately gave a DNA melting profile on addition of SDS. It may be questioned whether the proteolytic enzymes noted by Dounce in his nuclear preparations were even present in our chromatin. The SDS concentration (1.0% or 3.47×10^{-2} M) used to treat the chromatin (800 OD_{260} units in 120 ml) theoretically provided enough anions to exceed the quantity of cationic sites on the protein by a factor of 40 (assuming that 800 OD_{260} units of chromatin had 50 mg of chromatin protein, that the average molecular weight of an amino acid residue

TABLE VI: Resume of the Isolation of Chromatin Protein by the SDS–Urea Dialysis–$BaCl_2$-Precipitation Procedure.

	% of Chromatin Protein Accounted for in Supernatant	Mg of SDS/mg of Protein
Chromatin in 1% SDS, centrifuged at 85,500*g* for 25 hr (I)	87.9[a]	27.8
2-day 10 M urea-dialyzed supernatant of I in 0.2 M $BaCl_2$, centrifuged at 12,000*g* for 30 min	81.2[b]	0.100

[a] This value reached 91.5% when the pellet was further extracted with 1% SDS. [b] The 6.7% (87.9% minus 81.2%) of the chromatin protein which remained with the precipitate induced by $BaCl_2$ was soluble in Lowry C reagent.

TABLE VII: Mole Per Cent Amino Acid Compositions of the Proteins Present in the Anode-Migratory (Acidic Protein) and Nonmigratory Disc Electrophoresis Bands.

Amino Acid	Histones[a]					Anode Migratory Band	Nonmigratory Band
	α	β	γ	δ	ϵ		
Lys	10.6	6.7	27.5	11.9	14.3	6.5	15.9
His	tr[b]	tr	0.3	1.2	1.7	1.6	4.5
Arg	14.4	15.5	10.3	11.6	10.7	3.9	9.2
Asp	7.6	4.3	2.8	5.7	5.3	10.8	7.2
Thr	4.6	5.8	2.4	4.9	4.2	2.9	2.7
Ser	7.2	6.7	5.7	6.4	7.4	5.4	4.0
Glu	11.8	11.1	3.1	8.4	6.4	16.1	12.9
Pro	tr	2.0	8.4	4.1	4.0	1.8	2.4
Gly	8.9	12.3	7.1	10.1	11.8	12.5	14.4
Ala	13.1	12.4	19.3	11.8	13.6	9.7	10.2
CySH	tr	1.5	tr	2.0	1.0	0	0
Val	5.9	4.0	3.3	6.3	5.5	5.1	4.2
Met	5.1	6.0	1.5	1.3	4.1	4.1	0.5
Ile	3.4	3.8	3.0	3.6	3.0	3.9	3.4
Leu	7.2	8.4	1.9	7.5	4.7	8.8	5.0
Tyr	tr	0.7	0.7	1.4	0.9	2.0	1.4
Phe	tr	0.7	0.6	1.9	0.9	3.9	2.0

[a] The amino acid compositions of the histones were determined by Paoletti and Huang (1969). [b] tr, trace quantity.

for chromatin protein is 120, and that 30% of the amino acid residues in chromatin protein are cationic). This should have provided the detergent excess required to dissociate all of the protein from the DNA and capable of putting the protein into solution as a negatively charged micelle (Putnam, 1948). However, 10% of the chromatin protein remained with the DNA. In view of the fact that 2–4 M NaCl or CsCl is capable of extracting another 6–8% of the protein bound to DNA when combined with SDS as in the Marmur method (1961) for DNA extraction it is thought that the ionic strength of the SDS medium alone may have been insufficient to remove the 10% of the chromatin protein remaining bound to the DNA even though the anionic charges of the detergent were well in excess of the cationic charges of the protein.

Removal of SDS from Chromatin Protein. Dialysis against Tris buffer in which was suspended ion-exchange resin (Utsumi and Karush, 1964), anion-exchange chromatography (Cebra, 1964) and salts with Ba and K ions (McMeekin *et al.*, 1949; Marushige *et al.*, 1968) have been used to separate SDS from protein with varying degrees of success. The urea dialysis–$BaCl_2$ precipitation procedure used in this work was successful in separating most of the SDS from the chromatin protein resulting in the freeing of the protein for electrophoretic separation. About 92% of the chromatin protein remained in solution following this procedure and the remaining 8% could be redissolved in Lowry C reagent from which it also could be electrophoretically separated. The data from the procedure suggested that at a high enough SDS to protein ratio, SDS not only maintained chromatin protein in a soluble complex, but it also shielded the charged interaction of the chromatin proteins. As SDS was removed, the positively and negatively charged chromatin proteins interacted forming insoluble aggregates. The addition of the proper quantities of salts like $BaCl_2$, KCl, and NaCl, having cations which are effective in removing detergent from protein solutions, not only caused the precipitation of the protecting SDS molecules, but also increased the ionic strength of the solution sufficiently to minimize the formation of aggregates between the acidic and basic proteins as they were liberated from the SDS. NH_4Cl exemplified a salt which failed to liberate electrophoretically detectable basic proteins. As an additional piece of information it was interesting to note that SDS would not replace the sulfate anion from H_2SO_4-extracted histones, the histone–SO_4 being able to migrate typically toward the cathode in the presence of 0.1% SDS. Once the chromatin proteins are separated electrophoretically, further dialysis of the component proteins against 10 M urea continues to reduce their SDS content.

No studies have yet been undertaken to ascertain whether chromatin protein, once it has been released from SDS, has been structurally modified. The fact that SDS denatures protein has been reported by several authors, *e.g.*, Putnam (1948), Gubert *et al.* (1966), and Ishikawa and Maruyama (1966). However, SDS at the 1% concentration employed in this work may not necessarily denature all enzymes, at least to the point of total inactivation, as demonstrated by Dounce (1965) using the proteolytic activities of liver nuclei cathepsins in the presence of various SDS concentrations. Other observations also suggest that SDS may not severely damage the structure of protein. For example, Cebra (1964) reported the restoration of specific precipitating activity and complement fixation ability of an SDS-treated globulin after having removed the SDS. Also a virus which had been inactivated by a cationic detergent was reactivated upon the removal of the detergent (Pfankuch and Kausche, 1942).

The Nonmigratory Band, an Acidic Protein–Basic Protein Complex. Three pieces of evidence support the contention that the nonmigratory band seen in samples A and B in Table IV is an acidic protein–basic protein complex and not a separate protein fraction. The first, mentioned in the Results section, is that a fairly high concentration of salt almost completely prevented the formation of the band. Secondly, the increased Buffalo Blue-Black to acridine orange staining ratio seen in the nonmigratory band compared to that of the acidic band suggests that such a complexing could have taken place. And finally, no amino acid in the band appeared to distinguish the protein as being unique. In fact the mole per cent of each amino acid of this protein reflected a somewhat quantitative average of that in the acidic band and the combined histone fraction (Table VII). In other words, the protein in the nonmigratory band probably does not exist in this form in chromatin but is an artifact of the preparation, its presence depending on how well the charges on the acidic and basic proteins were shielded from each other. This suggests that the 90% of the chromatin protein extracted from chromatin by SDS is either basic or acidic in nature. Therefore, if a residual protein exists in *A. punctulata* sperm chromatin, it remains as part of the 10% protein bound to DNA after SDS treatment. The postulated histone–acidic protein complex with its dependence on salt concentration may be chemically similar to such a complex that might occur *in vivo* and which may be significant in regard to the regulation of gene activity.

Advantages of Using SDS by Itself to Separate Chromatin Components Over Other Methods Currently Employed. SDS has the ability to separate 90% of the chromatin protein from chromatin and to virtually keep all 100% of the chromatin protein soluble. It facilitates the concentration of the chromatin protein. The component proteins may readily be resolved electrophoretically once most of the SDS is removed.

An added advantage of the SDS–chromatin interaction is the ability of SDS at low ionic strength (0.00347 M, 0.1%) to permit chromatin to melt like its component DNA at that ionic strength. A check on the chromatin–DNA melting profile may be made without having to first isolate the DNA. This should prove convenient for describing the nature of the DNA in any given deoxyribonucleoprotein or chromatin preparation. For example, it would provide a fast check for relative G-C composition of DNA, satellite DNAs, and the degree of denaturation of DNA if physical or chemical probing had been used on the deoxyribonucleoprotein or the chromatin.

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