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Chromatographic Separation of Chick Brain Chromatin Proteins Using a SP-Sephadex Column*

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ABSTRACT: Salt-dissociated chromatin proteins isolated from 11-day-old chick embryo brain tissue were fractionated by chromatography on SP-Sephadex columns using a step sodium chloride gradient. The column was equilibrated in 7 M urea–0.23 M NaCl–0.01 M NaAc buffer at pH 5.2. Total recovery of protein from the column averaged approximately 90%. At least 90% of the total nonhistone proteins of chromatin was eluted from the column at 0.23 M NaCl; histone IIb_1 at 0.3 M NaCl; histones IIb_2, III, and IV at 0.4 M NaCl; and histone I at 0.8 M NaCl. The 10% or so of the nonhistone proteins which were not eluted by 0.23 M NaCl were recovered partially at 0.3 M NaCl and partially at 0.4 M NaCl. These nonhistone proteins eluted at 0.3 and 0.4 M NaCl were also present in the group of proteins eluted at 0.23 M NaCl. Thus the latter is a representative population of all nonhistone proteins. The molecular weight of the smallest nonhistone protein was found to be *ca.* 10,000. The largest nonhistone protein had molecular weight greater than *ca.* 200,000.

Chromatin isolated from higher organisms is composed of DNA, RNA, and protein, the protein complement consisting of histones and nonhistones. The amount of nonhistone protein found in the chromatin varies with the organism (Bonner *et al.*, 1968). The amount of chromosomal nonhistone protein found also varies within tissues of the same organism (Dingman and Sporn, 1964) and with the developmental stage of the organism (Marushige and Ozaki, 1967). In general, chromatin obtained from metabolically active tissues contains a greater quantity of nonhistone protein than chromatin obtained from inactive tissues (Seligy and Miyagi, 1969; Paoletti and Huang, 1969). The functional importance of these proteins in controlling the template activity of chromatin has been indicated in several studies (Wang, 1969; Gilmour and Paul, 1970; Spelsberg and Hnilica, 1969; Teng *et al.*, 1971). There are, however, no conclusive studies in regard to the role of these proteins in chromatin structure. Attempts to understand the role of the nonhistone proteins in the organization of the genome will depend largely upon the successful isolation of these proteins, free of histones and in good yield.

There are two principle approaches to the isolation of nonhistones from chromatin. One method employs an initial extraction of histones by strong acids (HCl or H_2SO_4), followed by solubilization of the nucleic acid and nonhistones in detergent (Marushige *et al.*, 1968), phenol (Shelton and Allfrey, 1970; Shelton and Neelin, 1971; Teng *et al.*, 1971), or concentrated salt solution of high pH (Benjamin and Gelhorn, 1968). Subsequently, the nucleic acid and nonhistone are separated by centrifugation according to their difference in molecular weight (Marushige *et al.*, 1968) or density (Benjamin and Gelhorn, 1968). Alternatively, the nucleoprotein complex can be partitioned between aqueous buffers and phenol (Shelton and Neelin, 1971; Teng *et al.*, 1971). Using

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this approach, the recovery of both histone and nonhistone protein is good. The major drawback of this method lies in the initial strong acid extraction step. We believe that acid treatment affects the properties of the nonhistone proteins, making further structural studies of these proteins difficult, if not impossible.

The second approach to the isolation of the nonhistone proteins avoids acidic extraction of chromatin. Histones are separated from nonhistone protein by electrophoretic methods or by ion-exchange resins. Shaw and Huang (1970) have reported the resolution of histone and nonhistone proteins using the polyacrylamide gel system of Panyim and Chalkley (1969). While a useful analytical tool, this method is not easily performed on a preparative scale. Langan (1966) and Kleinsmith and Allfrey (1969) have prepared a class of nonhistone proteins, the nuclear phosphoproteins, using a weakly acidic cation-exchange resin (Bio-Rex 70) to remove histones and calcium phosphate gel to fractionate the phosphoproteins. Wang (1967) has prepared a group of chromosomal acidic proteins which can be further fractionated by DEAE-cellulose chromatography (Wang and Johns, 1968). Gilmour and Paul (1970) have used a QAE-Sephadex batch procedure to separate histones and nonhistones. And more recently, MacGillivray *et al.* (1971) reported the use of hydroxylapatite in high salt and urea to separate nonhistones from histones and DNA. This method, while not completely eliminating interaction between histones and nonhistones, permits the authors to recover approximately 50% of the total nonhistone proteins from chromatin.

Because of the high affinity of the basic amino groups of lysine and arginine for sulfurous ions, we have employed a strong sulfo ion ($C_3H_6SO_3^-$) exchanger for the separation of chromatin proteins. Using a SP-Sephadex column, at least 90% of the total nonhistone proteins of chick brain chromatin has been separated from histones. By the use of a step salt gradient in the presence of urea, complete separation of histone I and partial separation of histone IIb_1 from the rest of the chromosomal proteins are made possible.

This communication describes the entire procedure for the isolation of these chromatin proteins.

Methods

Isolation of Nuclei from Chick Embryo Brain. Nuclei were prepared from 11-day-old chick embryo brain tissue using essentially the method of Shaw and Huang (1970). The brains of 11-day-old chick embryos were dissected out, weighed, and homogenized in nine volumes of 0.25 M sucrose–0.0033 M calcium acetate–0.5% Triton X-100. The homogenate was filtered through four layers of cheesecloth and then spun at 3500*g* for 20 min in a SS-34 rotor, Sorvall centrifuge at 4°. The crude nuclear pellets were suspended in 2 M sucrose–0.0033 M calcium acetate–0.5% Triton X-100 using approximately 40 ml/g of brain tissue. Aliquots of 25 ml of the suspension were layered on 5-ml aliquots of 2 M sucrose in cellulose nitrate tubes. The tubes were placed in buckets of a SW 25.1 rotor and centrifuged at 40,000*g* for 60 min. The resultant pellet consisted of nuclei, as determined microscopically.

Preparation of Chromatin. Chromatin was prepared from the brain nuclei using the procedure of Paoletti and Huang (1969). All operations were performed in the cold. The nuclear pellets were suspended in saline–EDTA (0.075 M NaCl–0.024 M EDTA, pH 7.9) and centrifuged at 7700*g* for 20 min. The pellets were washed three more times in saline–EDTA, centrifuging at 7700*g* for 10 min each time. The pellets were then washed twice in 0.05 M Tris, twice in 0.01 M Tris, and twice in 0.002 M Tris, all at pH 7.9. The chromatin was then dissolved by stirring in distilled water for 60 min. The dissolved chromatin was sheared at 80 V for 90 sec in an OmniMixer. The concentration of the sheared chromatin was 11–13 $OD_{260\ m\mu}$/ml.

Extraction of Chromatin with 3 M NaCl on a Bio-Gel A-50 Column. The method described by Shaw and Huang (1970) was used. The sheared chromatin was made 3 M in NaCl by adding solid NaCl and stirring in the cold for 12–16 hr. The salt-dissociated chromatin was centrifuged at 12,100*g* for 20 min. The clarified solution, which contained more than 93% of the original chromatin as judged by optical density at 230 and 260 mμ, was then applied to a Bio-Gel A-50 column equilibrated in 3 M NaCl. Approximately 500 $OD_{260\ m\mu}$ of the dissociated chromatin were applied to a 4.0 × 50 cm column. The column fractions were combined into a nucleic acid pool and a protein pool.

This protein pool contains the total protein complement of chromatin (Shaw and Huang, 1970). A portion of this pool was dialyzed aginst 0.05 N acetic acid, lyophilized, and reserved for protein determination and gel electrophoresis. The remainder of the protein pool was then treated as described below.

Dialysis of the Protein Pool. The buffer used for the dialysis of the protein pool was 7 M urea–0.23 M NaCl–0.01 M NaAc (pH 5.2). The urea used throughout this study was prepared as follows. A freshly prepared solution of 10 M urea was deionized on an Amberlite MB-3 column. The deionized 10 M urea was then used to prepare the 7 M urea–0.23 M NaCl–0.01 M NaAc buffer. The prepared buffer was stored in the cold and used within 2 days of preparation. Approximately 200 ml of the Bio-Gel A-50 protein pool containing 75–125 μg of protein/ml were dialyzed against five 2-l. changes of 7 M urea–0.23 M NaCl–0.01 M NaAc buffer (pH 5.2).

Fractionation of the Chromatin Proteins on a SP-Sephadex C-25 Column. The dialyzed protein pool was applied to a SP-Sephadex C-25 column (sulfopropyl-Sephadex C-25, Pharmacia Fine Chemicals, Piscataway, N. J.) equilibrated in 7 M urea–0.23 M NaCl–0.01 M NaAc buffer (pH 5.2). The dialyzed protein pool containing 15–25 mg of protein was applied to a 2 × 29 cm column. Following application of the sample, the column was washed with four 7 M urea–0.01 M NaAc buffers containing 0.23, 0.3, 0.4, and 0.8 M NaCl, respectively. All buffers were pH 5.2. Fractions collected from the column were combined into four pools. The pools were dialyzed extensively against either distilled water or 0.05 N acetic acid. The protein content of each dialyzed pool was determined by the method of Lowry *et al.* (1951). The dialyzed pools were lyophilized to dryness and analyzed by polyacrylamide gel electrophoresis.

Analytical Gel Electrophoresis. The gel system of Panyim and Chalkley (1969) was used. The gel composition was 15% acrylamide, 0.1% *N,N'*-bismethyleneacrylamide, 2.5 M urea, 0.5% *N,N,N',N'*-tetramethylenediamine, 0.125% ammonium persulfate, and 0.9 N acetic acid. The electrophoresis buffer was 0.9 N acetic acid. The gels were preelectrophoresed at 2 mA/gel for 3–5 hr in the cathode direction. The gels were stained by immersing them in 1% Amido-Schwarz–7% acetic acid for 1 hr. Destaining was done in 7% acetic acid.

Sodium Dodecyl Sulfate Gel Electrophoresis. Two sodium dodecyl sulfate gel systems were used. Using the method of Shapiro *et al.* (1967), the final gel composition was 5% acrylamide, 0.13% *N,N'*-bismethyleneacrylamide, 0.1% sodium dodecyl sulfate, 0.1 M sodium phosphate buffer (pH 7.1), 0.05% *N,N,N',N'*-tetramethylenediamine, and 0.075% ammonium persulfate. The electrophoresis buffer was 0.1 M phos-

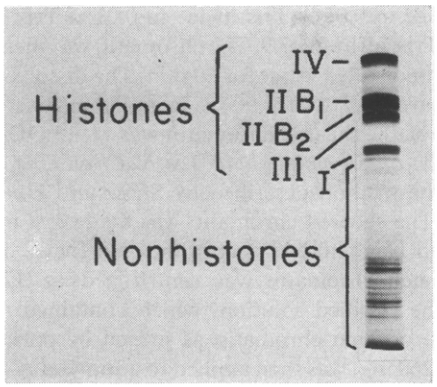

FIGURE 1: Urea–polyacrylamide gel electrophoresis of total chromatin proteins from 11-day-old chick embryo brain tissue. Sample prepared as described in Methods. Sample size, 83 μg. Histone bands are designated according to the nomenclature of Luck *et al.* (1958). Gels run from bottom (anode) to top (cathode) for 3 hr at 150 V.

phate (pH 7.1) containing 0.1% sodium dodecyl sulfate. Protein samples were dissolved in 1% sodium dodecyl sulfate–1% β-mercaptoethanol in 0.01 M sodium phosphate buffer (pH 7.1) and incubated at 37° for 2 hr. The samples were then dialyzed overnight against 0.1% sodium dodecyl sulfate–0.1% β-mercaptoethanol–10% glycerol in 0.01 M sodium phosphate buffer (pH 7.1). Electrophoresis was conducted at 8 mA/gel for 4 hr. The gels were stained in 0.25% coomassie brilliant blue in 50% methanol–7% acetic acid and destained electrophoretically in 5% methanol–10% acetic acid.

For estimation of the molecular weight range found in the nonhistone proteins, the method of Weber and Osborn (1969) was used. The final gel composition was 10% acrylamide, 0.27% *N,N'*-bismethyleneacrylamide, 0.1% sodium dodecyl sulfate, 0.1 M sodium phosphate buffer (pH 7.0), 0.15% *N,N,N',N'*-tetramethylenediamine, and 0.075% ammonium persulfate. The electrophoresis buffer was 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulfate. Protein samples were dissolved in 1% sodium dodecyl sulfate–1% β-mercaptoethanol in 0.01 M sodium phosphate buffer (pH 7.0) and incubated at 37° for 2 hr. The protein samples were then dialyzed for several hours at room temperature against 0.1% sodium dodecyl sulfate–0.1% β-mercaptoethanol–0.01 M sodium phosphate buffer (pH 7.0). An aliquot of the protein sample was added to a solution containing 3 μl of tracking dye (0.05% bromophenol blue in water), 1 drop of glycerol, 5 μl of β-mercaptoethanol, and 50 μl of buffer (0.1% sodium dodecyl sulfate–0.1% β-mercaptoethanol–0.01 M sodium phosphate, pH 7.0). The protein samples were then applied to the gels and electrophoresed until the tracking dye had migrated three-fourths the length of the gel. The gels were stained in 0.25% coomassie brilliant blue in 50% methanol–7% acetic acid for 2 hr, then electrolytically destained in 5% methanol–7.5% acetic acid. The proteins used as markers were bovine serum albumin (Sigma, fraction V), glyceraldehyde phosphate dehydrogenase (Calbiochem), aldolase (C. F. Boehringer Mannheim), paramyosin, and myosin (a gift from Mr. J. Halsey of Dr. W. Harrington's laboratory). A standard curve of molecular weight *vs.* mobility was constructed and the molecular weight range of the nonhistone proteins was determined using this curve.

Quantitation of the Amount of Nonhistone Protein in Chromatin Protein. The procedure of Johns (1967) was used to determine the amount of nonhistone protein in total chromatin protein. Gels of total chromatin protein were run on urea–polyacrylamide gels, stained in 1% Amido-Schwarz–7% acetic acid, and then destained in 7% acetic acid. The gels were then immersed in an acetone–Dry Ice bath for 15 sec and cut into sections which contained nonhistone and histone bands. The gel sections were broken into small pieces and 1–3 ml of dimethyl sulfoxide added to each. This mixture was incubated at 56° for 24 hr. After centrifuging the pieces of gel down, the color intensity was measured at 600 mμ. The amount of protein found in the histone and nonhistone regions was expressed as a per cent of the total color eluted from the gel. The staining standard for histone and nonhistone proteins was not determined and therefore the values reported here are only approximate.

The procedure described by Paoletti and Huang (1969) was also used to quantitate the amount of nonhistone protein found in total chromatin protein. Total chromatin protein was electrophoresed on urea–polyacrylamide gels. After electrophoresis, one gel was stained and destained. The other gels were frozen on Dry Ice. The stained gel was used as a template to cut the unstained gels into histone and nonhistone regions. These gel sections were homogenized and extracted for several hours in 10 M urea. The gels pieces were removed by centrifugation and the supernatants exhaustively dialyzed against 0.05 N acetic acid. The supernatants were then lyophilized and analyzed for protein content by the method of Lowry *et al.* (1951).

General Methods. DNA was assayed by the method of Burton (1956), using calf thymus DNA (Sigma) as a standard. Protein was assayed by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin (Sigma, fraction V) as a standard. All samples which were analyzed for protein content were dialyzed extensively against distilled water or 0.05 N acetic acid to remove salt and urea before the assay was run. Samples for amino acid analysis were dissolved in 6 N HCl, placed in vials which were flushed with N_2, and incubated at 110° for 18 hr. The hydrolysates were diluted to 1 N HCl with distilled water, lyophilized, dissolved in 0.2 N sodium citrate (pH 2.2), and chromatographed on a Beckman Spinco amino acid analyzer, Model 120.

Results

Chemical Composition of Chromatin. Chromatin prepared from 11-day-old chick embryo brain nuclei by the methods described contains approximately 66% of the total nuclear protein and 100% of the nuclear DNA. Using a value of 1.0 for the DNA content of the chromatin, the average protein content was 3.45. In this paper, the term nonhistone protein refers to that portion of the nuclear acidic proteins which are present in the isolated chromatin.

Quantitation of Nonhistone Protein in Chromatin. The two procedures employed to determine the amount of nonhistone protein found in total chromatin protein measure the quantity of nonhistone protein which enters the urea–polyacrylamide gel system. Shaw and Huang (1970) have reported that 96% of the applied protein enters such gels. Figure 1 shows the banding pattern of total chromatin proteins in this gel system. Using the method of Johns (1967), an average of 41.5% of the total chromatin protein was found in the nonhistone region of five gels (see Table I). A similar value of 43.7% nonhistone protein was found using the 10 M urea gel extraction method of Paoletti and Huang (1969).

3 M NaCl Extraction of Chromatin on a Bio-Gel A-50 Column. Chromatin is dissociated into DNA, RNA, and protein by

TABLE I: Percentage of Nonhistone Protein in Chick Brain Chromatin.

Gel No.	% Protein Stain Obtained in Nonhistone Region of Gel[a]
1	39.2
2	40.7
3	45.6
4	42.6
5	39.6
	% Protein Eluted from Nonhistone Region of Gel[b]
6	43.7

[a] The per cent protein stain on the gel (see Figure 1) is the per cent of dimethyl sulfoxide extracted Amido-Schwarz stain in the nonhistone protein region of the urea–polyacrylamide gel. These percentages are obtained by dividing the total optical density at 600 mμ eluted from the nonhistone protein region of the gel by the total color (optical density at 600 mμ) eluted from all of the bands in the gel. The protein samples used for this determination were the 3 M NaCl-dissociated chromatin proteins. The electrophoresis of these proteins and the procedure for elution of Amido-Schwarz from the gels are described in the Methods section. [b] Per cent protein eluted from gel refers to the quantity of protein eluted from the nonhistone region of urea–polyacrylamide gels with 10 M urea divided by the total quantity of protein applied to the gels × 100. The protein samples used for this determination were the 3 M NaCl-dissociated proteins of chromatin. Additional details are given in the Methods section.

3 M NaCl and these components can be separated by gel filtration on a Bio-Gel A-50 column (Shaw and Huang, 1970). A typical separation and the pools made are shown in Figure 2. In 12 preparations, the total DNA and protein recovered in these pools averaged 72.4 and 85.3%, respectively. Because the pools made do not include the entire peak, the actual recovery of DNA and protein is in fact higher. The nucleic acid pool (excluded peak) contained an average of 66.6% of the applied DNA and 13.2% of the applied protein. The protein pool (included peak) contained an average of 72.1% of the applied protein and an average of 5.8% of the applied DNA. The protein pool, which contains the total protein complement of chromatin, was the one used for subsequent separation of the histone and nonhistone proteins.

Fractionation of Chromatin Proteins on SP-Sephadex C-25. After dialysis of the protein pool into 7 M urea–0.23 M NaCl–0.01 M NaAc buffer (pH 5.2) the pool was run onto a SP-Sephadex C-25 column equilibrated in the same buffer. Preliminary experiments using various concentrations of salt established that an initial salt concentration of 0.23 M was optimal for the separation of histone and nonhistone protein. Step elution with buffers of increasing salt concentrations produced a profile as shown in Figure 3. The column fractions were combined into four pools as indicated in Figure 3, dialyzed against distilled water or 0.05 N acetic acid, and lyophilized.

The original sample applied to the column and each of the four column pools were analyzed for protein content to determine the recovery from the column. As shown in Table II, the average recovery from two typical columns was 89.8%.

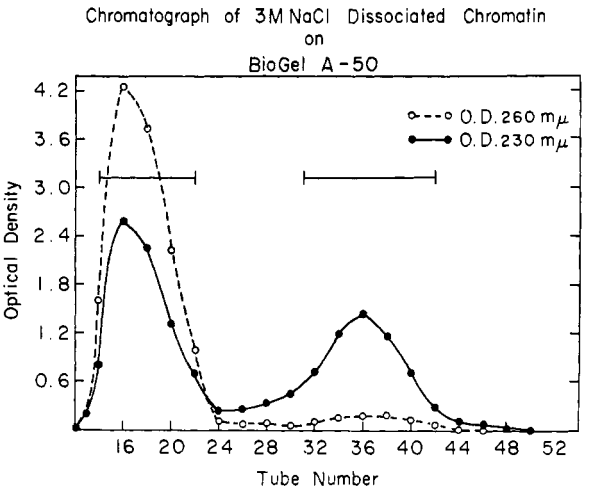

FIGURE 2: Chromatographic fractionation of 11-day-old chick embryo brain chromatin in 3 M NaCl on a Bio-Gel A-50 column. The 3 M NaCl-dissociated chromatin was fractionated on the column as described in Methods. Bars across top of graph indicate which fractions were combined to give the nucleic acid and protein pools.

Analysis of the SP-Sephadex C-25 Column Pools. The pools obtained from the SP-Sephadex column were analyzed in two gel systems—the urea–polyacrylamide system of Panyim and Chalkley (1969) and the sodium dodecyl sulfate gel system of Shapiro *et al.* (1967). Figure 4 shows the banding patterns of total chromatin proteins and the SP-Sephadex column pools in the urea–polyacrylamide gel system and Figure 5 shows sodium dodecyl sulfate gel electrophoresis of these proteins.

The SP-Sephadex pool 1 contains chromatin nonhistone proteins. Initially, this protein pool was assumed to contain nonhistone proteins because it was not retained on the column at the initial salt concentration used. Subsequent electrophoresis of the proteins of this pool at three different concentrations indicated that histones were absent (Figure 6). Amino acid analysis (Table III) of this pool confirmed that the pool con-

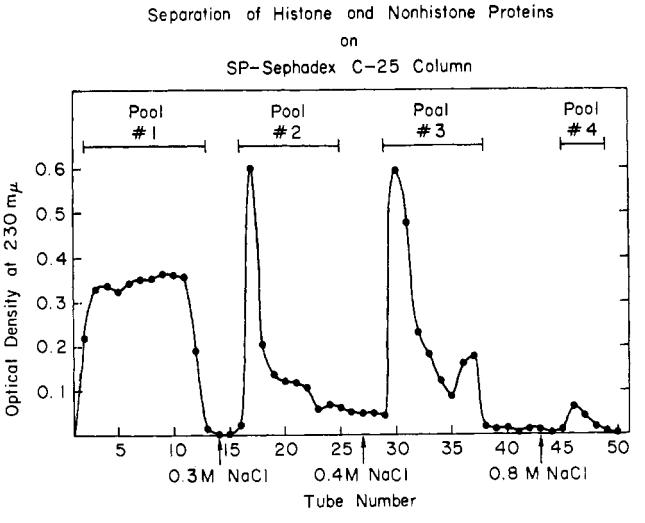

FIGURE 3: SP-Sephadex C-25 column profile of total chromatin proteins in 7 M urea–0.23 M NaCl–0.01 M NaAc (pH 5.2). Before application to the column, the Bio-Gel A-50 column protein pool was treated as described in Methods. The arrows indicate where the different salt concentrations were applied to the column. The fractions combined into pools 1–4 are indicated by bars above the column profile.

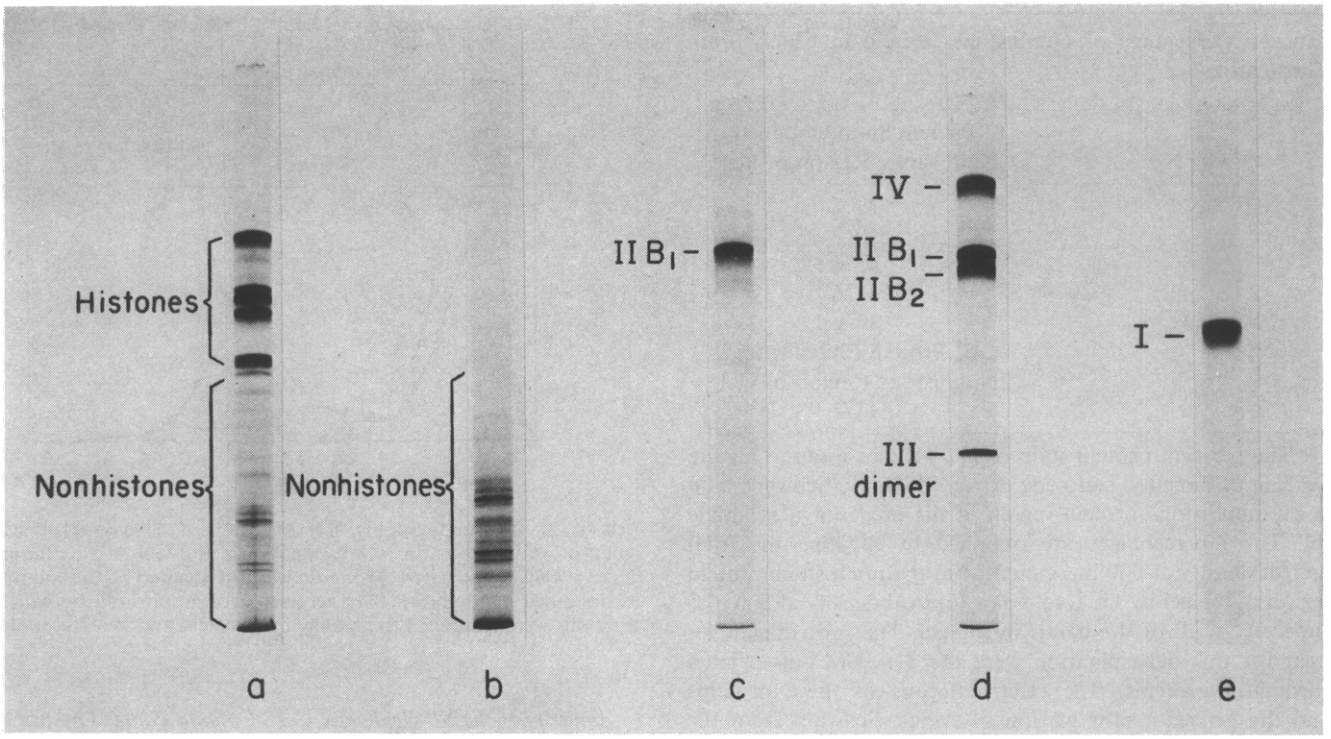

FIGURE 4: Urea–polyacrylamide gel electrophoresis of total chromatin proteins and SP-Sephadex C-25 column pools. Samples prepared as described in Methods. Gels run from bottom (anode) to top (cathode) for 5 hr at 150 V. Left to right: (a) total chromatin protein; (b) SP-Sephadex column pool 1; (c) SP-Sephadex column pool 2; (d) SP-Sephadex column pool 3; (e) SP-Sephadex column pool 4. Sample size, 80–100 μg on all gels.

TABLE II: Protein Recovery from SP-Sephadex C-25 Column.

	Total Protein Applied		Protein Recovered in Column Pools								
			Pool 1		Pool 2		Pool 3		Pool 4		Total
Column	mg	%	mg	%	mg	%	mg	%	mg	%	Recov(%)
1	22.8	100	10.1	44.4	5.1	22.3	3.4	15.1	0.9	4.1	86.0
2	16.9	100	8.2	48.5	3.6	21.3	3.0	17.7	1.0	6.0	93.6

tained proteins with a high acidic amino acid content. Comparison of the gels for total chromatin protein and SP-Sephadex pool 1 indicates that all nonhistone protein bands present in the total chromatin are present in SP-Sephadex pool 1 (see Figure 4a,b). This can also be seen in the 5% polyacrylamide–sodium dodecyl sulfate gels (see Figure 5a,b). As shown in Table II, an average of 46.5% of the total protein applied to the column is recovered in SP-Sephadex pool 1. This value correlates well with the amount of nonhistone protein found in total chromatin protein (Table I) as determined by dimethyl sulfoxide or urea extraction of gels (41.5 and 43.7%, respectively).

To determine the molecular weight range found in the nonhistone proteins, sodium dodecyl sulfate gels were run in the Weber–Osborn system (1969). Gels of SP-Sephadex pool 1 protein and protein standards run in this sytem are shown in Figure 7. The smallest nonhistone proteins were found to have a molecular weight of *ca.* 10,000. The largest nonhistone proteins had molecular weights in excess of *ca.* 200,000. Although Elgin and Bonner (1970) have reported finding chromatin nonhistone proteins of molecular weight as low as 5000, we have not detected any proteins of such small size. However, we have not employed the same gel system as they did.

The second pool, which was eluted from the column at 0.3 M NaCl, contains a few nonhistone proteins in low concentration and histone IIb_1 (see Figures 4 and 5). The nonhistone proteins present in this pool are also present in the first pool eluted from the column (compare Figure 4b,c; Figure 5b,c). This second pool has been rerun over another SP-Sephadex C-25 column, however it was still not possible to completely separate the nonhistones of this pool from histone IIb_1. Because the nonhistones in this pool are in low concentration, it is necessary to apply large amounts of this protein pool on gels in order to detect the nonhistone bands.

The third pool, which was eluted from the column at 0.4 M NaCl, contains histones IIb_2, III, and IV and even fewer nonhistone proteins. Again these nonhistone proteins are also present in SP-Sephadex pool 1 (compare Figure 4b and d; Figure 5b and d). The gels of this protein pool shown in Figures 4 and 5 show a heavy band in the nonhistone region. This heavy band is a dimer of histone III and thus not a nonhistone protein. When the proteins of this pool are reduced in β-mer-

TABLE III: Amino Acid Composition of SP-Sephadex Column Pool 1 and 4.[a]

Amino Acid	SP-Sephadex Column Pool 1	SP-Sephadex Column Pool 4
Lysine	5.6	23.4
Histidine	1.3	0.8
Arginine	4.5	3.5
Aspartic	8.9	3.3
Threonine	5.4	4.5
Serine	7.0	7.6
Glutamic	13.0	5.7
Proline	6.4	9.1
Glycine	11.6	8.1
Alanine	8.4	21.7
Half-cystine	0.8	
Valine	5.3	3.6
Methionine	2.2	0.9
Isoleucine	3.9	1.4
Leucine	9.0	4.6
Tyrosine	3.2	0.7
Phenylalanine	3.5	1.1
A:B[b]	1.9	0.32

[a] Moles per 100 moles of recovered amino acids, uncorrected for hydrolytic loss. [b] A:B is the ratio of acidic to basic amino acids.

captoethanol and run on urea–polyacrylamide gels, the heavy band in the nonhistone region disappears.

Because the second and third column pools contain 10% or less nonhistone protein, the SP-Sephadex C-25 column does not separate histone and nonhistone proteins completely. However, it should be emphasized that all nonhistones present in these two pools are also present in the first column pool. Therefore we are confident that the first pool eluted from the column is representative of all nonhistone proteins found in chromatin.

The last pool which was eluted from the column at 0.8 M NaCl contains histone I (see Figures 4 and 5). This histone, being the largest and most basic of the histones, would be expected to elute from the cation-exchange column last. An amino acid analysis of this protein pool confirming its identity as histone I is shown in Table III.

It is interesting to note that the order in which histones are eluted from the SP-Sephadex column using a step salt–urea gradient is essentially the same as the order in which histones are dissociated from native chromatin (Kleiman, 1971; Senshu, 1971).

Discussion

The choice of SP-Sephadex C-25 as a suitable resin to separate histones from nonhistones was based on two distinct properties of these two groups of proteins: large molecular weight (*ca.* 10,000 to greater than 200,000) and high acidity (acidic: basic amino acid ratio $\cong$ 2) for nonhistones and lower molecular weight 11,000–19,000) and high basicity (basic: acidic amino acid ratio $\cong$ 2.7) for histones. The successful separation of these proteins as described in this paper is also dependent upon several other factors.

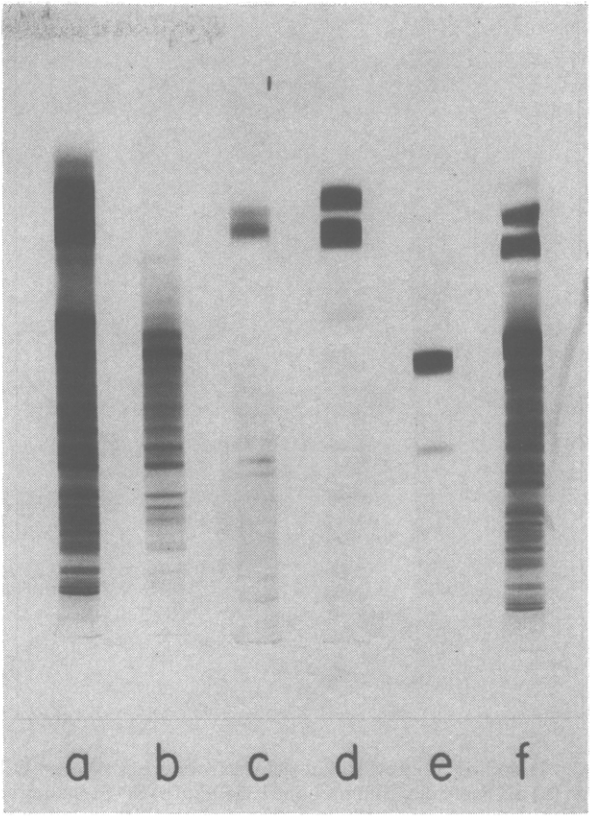

FIGURE 5: Sodium dodecyl sulfate gel electrophoresis (Shapiro system) of total chromatin proteins and SP-Sephadex C-25 column pools. Samples prepared as described in Methods. Gels run from bottom (cathode) to top (anode) at 40 V for 4 hr. Left to right: (a) total chromatin proteins; (b) SP-Sephadex column pool 1; (c) SP-Sephadex column pool 2; (d) SP-Sephadex column pool 3; (e) SP-Sephadex column pool 4; (f) total chromatin proteins. Sample size, 50–80 μg on all gels.

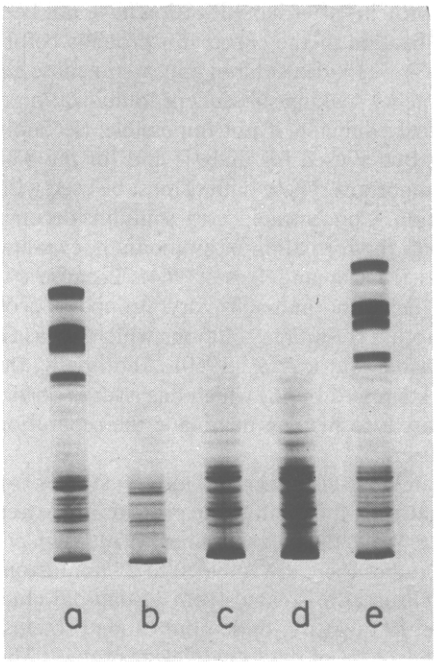

FIGURE 6: Urea–polyacrylamide gel electrophoresis of total chromatin proteins and SP-Sephadex column pool 1 at three different concentrations. Left to right: (a) total chromatin protein, 83 μg; (b) SP-Sephadex column pool 1, 50 μg; (c) SP-Sephadex column pool 1, 83 μg; (d) SP-Sephadex column pool 1, 124 μg; (e) total chromatin proteins, 72 μg. Gels run from bottom (anode) to top (cathode) at 150 V for 3 hr.

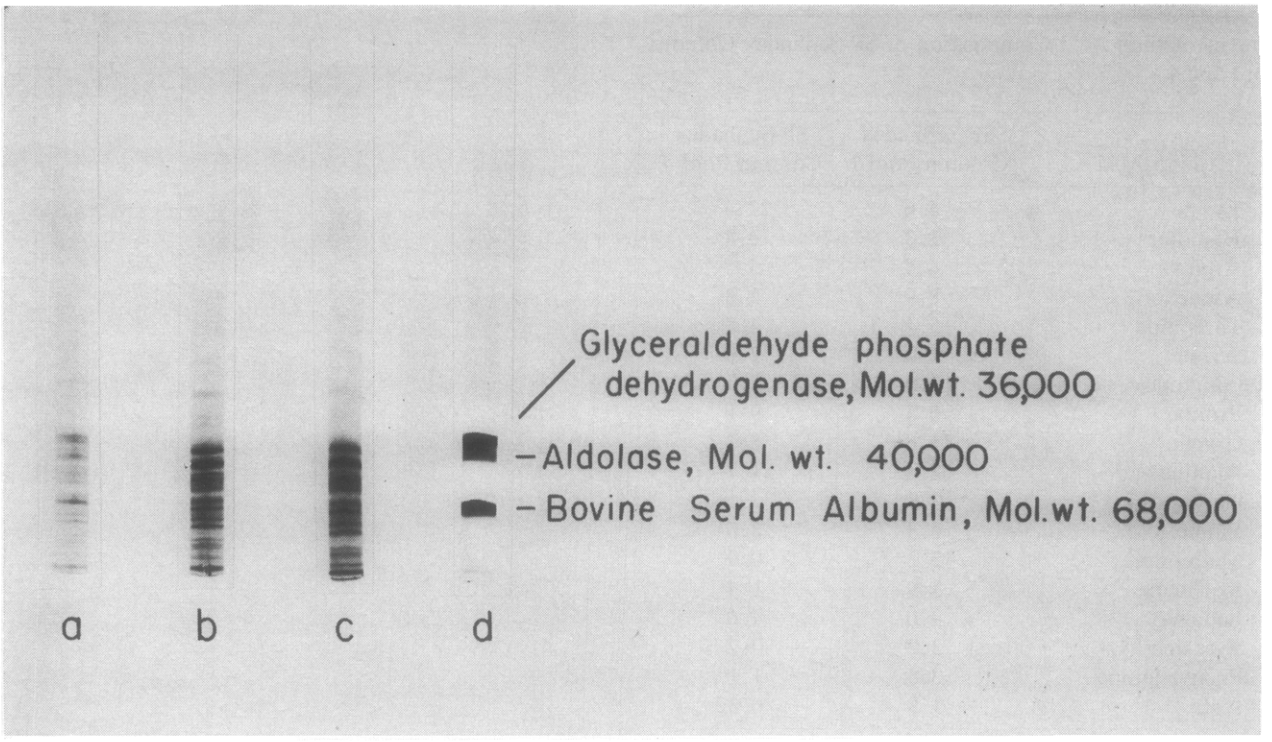

FIGURE 7: Sodium dodecyl sulfate gel electrophoresis (Weber–Osborn system) of SP-Sephadex column pool 1 and protein standards. Left to right: (a) SP-Sephadex column pool 1, 34 μg; (b) SP-Sephadex column pool 1, 100 μg; (c) SP-Sephadex column pool, 1 167 μg; (d) marker proteins — bovine serum albumin, 20 μg, glyceraldehyde phosphate dehydrogenase, 30 μg, and aldolase, 30 μg. Gels were run from bottom (cathode) to top (anode) as described in Methods.

First, the Bio-Gel A-50 column protein pool is never concentrated and never lyophilized before application to the ion-exchange column. The protein complement of chromatin is thus always in solution. Previous attempts to fractionate the chromatin proteins after lyophilization have not been successful. Lyophilization of the chromatin proteins before application to the SP-Sephadex column appears to cause aggregation of these proteins, making subsequent solubilization of the proteins extremely difficult, if not impossible. Secondly, the age of the urea buffer used for dialysis and for the SP-Sephadex column is important. These buffers must be used within 2 days of preparation. Concentrated urea solutions decompose upon standing with the formation of ammonium, cyanate, and carbonate ions (Marier and Rose, 1964). Because cyanate can react with the amino and sulfhydryl groups of proteins, it is important to use fresh urea solutions which are as free of cyanate as possible (Stark *et al.*, 1960). And finally, the use of a tissue (chick embryo brain) which has a large portion of nonhistone relative to histone has made the separation of these proteins easier.

As indicated by this paper and also by studies from several other laboratories, the nonhistone proteins are extremely complex, (Wang, 1967; Elgin and Bonner, 1970; Teng *et al.*, 1971). From our studies there are some 20 to 25 nonhistone proteins present in chromatin isolated from 11-day-old chick embryo brain tissue. In quantity, these nonhistone proteins represent approximately 30% of the total nuclear protein. The presence of this group of proteins in chromatin and their association with the other components of chromatin (DNA, histone, and RNA) undoubtedly influences chromatin structure. Therefore the separation of each protein from the others in this group will be an important task for the future in order to assess their individual roles. We are presently developing procedures which avoid the use of concentration and lyophilization for the isolation of each individual nonhistone protein.

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Incorporation of Molecular Oxygen into Glycine and Serine during Photorespiration in Spinach Leaves*

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ABSTRACT: When spinach leaves were exposed to an atmosphere of [^{18}O]oxygen the label was rapidly incorporated into the carboxyl groups of glycine and serine. This incorporation occurred only in the light. No label was incorporated into the hydroxyl group of serine. Under the same conditions, glycerate and phosphoglycerate did not become labeled. The data are consistent with a two-electron oxidation of a sugar phosphate intermediate of the photosynthetic carbon cycle to form phosphoglycolate or glycolate and an aldose phosphate. A considerable pool of unlabeled erythronic (or threonic) acid was detected by combined gas chromatography–mass spectrometry in phosphatase-treated extracts of spinach leaves.

The inhibition of net photosynthetic carbon dioxide fixation by oxygen, often referred to as the Warburg oxygen effect (Warburg, 1920), has been observed in a wide variety of algae and higher plants (Turner and Brittain, 1962) and isolated chloroplasts (Ellyard and Gibbs, 1969). It has become evident that this phenomenon is due to photorespiration; that is, the light-dependent uptake of oxygen and release of carbon dioxide which is thought to be associated with the glycolate pathway of metabolism (Jackson and Volk, 1970). Photorespiration is especially evident under conditions of high light intensity, limiting carbon dioxide, and high oxygen concentrations. In these circumstances a large part of the total carbon fixed during photosynthesis flows through the glycolate pathway (Tolbert, 1963). Recently, many of the enzymes of the glycolate pathway have been located in the peroxisomal (microbody) fraction, as distinct from both chloroplasts and mitochondria (Tolbert, 1971). The origin of phosphoglycolate and glycolate is one of the most interesting problems in photosynthetic carbon metabolism and one which is of fundamental importance in photorespiration. Glycolate may arise from phosphoglycolate by the action of a specific phosphatase, which is located in the chloroplast (Richardson and Tolbert, 1961), but whether it does so exclusively is not clear. Two radically different mechanisms have been proposed to account for the formation of uniformly labeled glycolate during photosynthetic [^{14}C]CO_2 fixation. One, proposed by Tanner *et al.* (1960), Stiller (1962), and Zelitch (1965), suggests that glycolate arises by means of a hitherto undiscovered reductive condensation of two molecules of carbon dioxide. However, the ^{14}C-labeling experiments of Hess and Tolbert (1966) and Coombs and Whittingham (1966) tend to discount this possibility. The other mechanism, proposed in various forms, suggests that glycolate and/or phosphoglycolate is formed as the result of the oxidation of one or more intermediates of the photosynthetic carbon cycle (Wilson and Calvin, 1955; Bassham and Kirk, 1962; Tolbert, 1963; Coombs and Whittingham, 1966; Gibbs, 1969). If the latter hypothesis is correct the nature of the oxidant is of considerable interest. To investigate the possibility that the oxidant is molecular oxygen, or is derived from it, we performed experiments in which detached spinach leaves were allowed to photorespire in an atmosphere of [^{18}O]oxygen. This report concerns the incorporation of ^{18}O into the products of the glycolate pathway, glycine and serine.

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