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Fractionation of Chromatin Components†

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ABSTRACT: A general method for the isolation and fractionation of the components of chromatin is described. The procedure, based primarily on ion-exchange chromatography in the presence of urea, separates all components of chromatin from a single tissue sample, avoids both the use of acidic or alkaline conditions and the use of ionic detergents, and is capable of preparative scale operation. Dissociation of chromatin in 6 M urea, 0.4 M guanidine hydrochloride, 0.1% β-mercaptoethanol, and 0.1 M sodium phosphate (pH 7.0) leads to solubilization of 90–95% of the proteins of chromatin from DNA. The nucleic acid is isolated by ultracentrifugation. Following sedimentation, the supernatant is applied to a cation-exchange column in the same buffer. Nonhistone proteins pass unretarded through the column, while the histones are quantitatively absorbed. The histones may be eluted as a group with a step of guanidine hydrochloride, or alternatively, be fractionated into subclasses by gradient elution with guanidine hydrochloride. The nonhistones are fractionated by gradient chromatography on DEAE-cellulose in the presence of 3 M urea. This fractionation step reveals several major groups of nonhistones, separated from each other, but within each group, heterogeneity is still present. The overall method discards none of the known components of chromatin, is rapid in performance, and avoids the use of denaturants which are difficult to remove from isolated proteins. The fractionation procedure should allow the isolation of any component of chromatin deemed to be of interest.

Deoxyribonucleic acid in eukaryotic organisms is present in chromatin, a complex containing, in addition, relatively stable amounts of histones, and a variable content of nonhistone proteins (Zubay and Doty, 1959; Bonner *et al.*, 1968a,b). When present in this complex, transcription of DNA appears to be highly limited (Marushige and Bonner, 1966; Bonner *et al.*, 1968a; Paul and Gilmour, 1968; Tan and Miyagi, 1970), presumably providing the basis for the limited gene expression seen in differentiated cells. The exact role of nonhistone proteins in this restriction of transcription has not been clear. The content and nature of nonhistones have been reported to vary from tissue to tissue, and within a single tissue, as a function of developmental or physiological conditions (Bonner *et al.*, 1968a; Elgin and Bonner, 1970; Sadgopal and Bonner, 1970; Rovera and Baserga, 1971). These and similar observations have suggested that the nonhistone proteins might have a role in determining the nature of the RNA transcribed from the chromatin complex. A correlation of phenotypic changes with alterations in chromatin is not feasible without a general method for the fractionation of chromatin and its components, hence such a method was sought.

Ideally, such a method should be capable of retrieving all the known components of chromatin from a single tissue sample. The method should be adaptable to an analytical scale, and also allow scale-up to preparative levels of fractionation.

If biological function is to be ascribed to the isolated entities, it is particularly important that the use of irreversible denaturing agents be avoided. Unfortunately, previously available methods for the preparation of chromatin components have not met all these criteria. Thus, harsh conditions such as acid extraction of histones, alkaline extraction of nonhistone proteins, organic solvent extractions, and ionic detergents have been employed (Bonner *et al.*, 1968b; Benjamin and Gellhorn, 1968; Shirey and Huang, 1969; Elgin and Bonner, 1970; Marushige *et al.*, 1968). Some milder extraction methods that have been used may recover only a portion of the nonhistones (Langan, 1967; Wang, 1967). Other methods have been suitable solely for analytical purposes (Elgin and Bonner, 1970; Shaw and Huang, 1970). Most of the methods have been designed for the preparation or analysis of only one of the components of chromatin, and have included procedures which denature, destroy or discard other components.

In the current communication, we report a method which does meet all the goals set forth above. Chromatin is fractionated primarily by ion-exchange chromatography in the presence of urea. The known chromatin components are all

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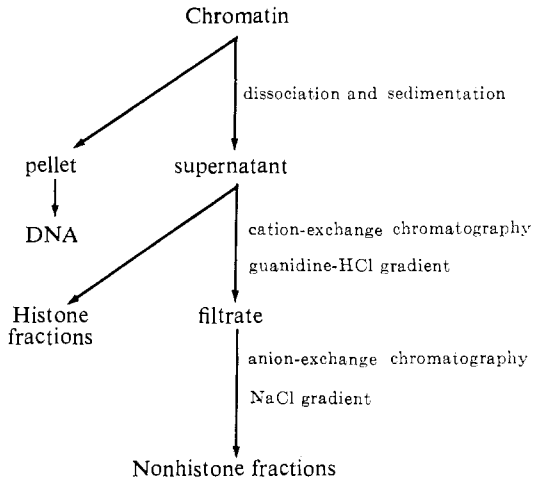

FIGURE 1: Flow sheet for the fractionation of chromatin.

recovered, and the three classes of components are cleanly separated from one another. Further, methods for the preliminary fractionation of the nonhistone proteins of chromatin have been developed.

Experimental Section

Urea and guanidine hydrochloride (Ultra Pure grade) were obtained from Schwarz-Mann Corp. Specially purified sodium dodecyl sulfate was a product of B. D. H. Corp. Bio-Rex 70 (200–400 mesh, sodium form, 10.2 mequiv/g) was obtained from Bio-Rad Laboratories, while DE-52, DEAE-cellulose (1 mequiv/g), was purchased from Whatman Corp.

The Bio-Rex 70 resin was cleaned, precycled, and equilibrated as described by Bonner *et al.* (1968a). The resin was packed under a hydrostatic head of about 40–50 cm and columns were pumped at about 20–30% of the flow rates obtained at this hydrostatic pressure. Cleaning and equilibration of the cellulose was performed as described by Peterson and Chiazze (1962). DEAE-cellulose was then packed to a final pressure of 5–6 psig and equilibrated further by washing with two to three column volumes of starting buffer. These columns were operated at 30–50% of the flow rates achieved at the termination of the packing operation. Effluents were monitored using 2- or 10-mm path-length flow cells in a Beckman DB-G spectrophotometer at either 220 or 230 nm. Conductivity was measured with a Radiometer conductivity meter. Buffers for chromatography were prepared by dilution of stock 0.78 M Na_2HPO_4–0.22 M NaH_2PO_4 or 1.0 M Tris–0.5 M HCl. The pH values listed in the text are the apparent pH values at 20° in the diluted buffer mixture.

Protein concentrations were determined by the Lowry *et al.* (1951) procedure using bovine serum albumin (Armour) as standard. Total nucleic acid was determined by measurement of the absorbance at 260 nm of material solubilized by hydrolysis with 5% perchloric acid for 20 min at 100°. DNA (1 mg/ml) yields an absorbance value of 30 under these conditions. RNA concentrations were obtained by measurements at 260 nm of the material which becomes soluble in cold 5% perchloric acid following alkaline hydrolysis (0.2 N KOH, 20 min, 100°) using 34 as the absorbance obtained for 1 mg/ml of RNA. DNA concentrations were derived from the difference between total nucleic acid and RNA.

Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate was performed on 10% acrylamide–0.27% bisacrylamide gels essentially as described by Weber and Osborn (1969). The running and gel buffer was 0.03 M sodium phosphate and the sample was loaded in 0.001 M sodium phosphate onto a 0.6 × 9 cm gel. Electrophoresis on polyacrylamide gels in the presence of urea at pH 2.7 was performed as described by Panyim and Chalkley (1969), also using 0.6 × 9 cm gels, on samples prepared by the method of Shaw and Huang (1970). All gels were stained for 30–60 min with 1% Amido Black in 7% (v/v) acetic acid, 40% (v/v) ethanol, and destained by soaking in the acetic acid–ethanol solution containing a small amount of Dowex 1-X2. The stained gels were scanned using a Zeiss PMQII spectrophotometer equipped with a transmittance–absorbance converter and the ZK3 densitometric scanner attachment.

Chromatin was isolated from various organs of mature New Zealand White rabbits. The animals were stunned and exanguinated and the liver, kidneys, and spleen removed. Nuclei were isolated from these organs using the Triton X-100 procedure of Hymer and Kuff (1964). Chromatin was prepared from the isolated nuclei by washing in solutions of decreasing ionic strength at pH 8.0 (Huang and Huang, 1969). In some cases, the shearing step (Bonner *et al.*, 1968b) was omitted and the unsheared chromatin utilized directly for the isolation of chromatin constituents (*vide infra*).

Fractionation of the constituents of chromatin was performed following the outline in Figure 1, beginning immediately after the chromatin was prepared. Sheared chromatin at concentrations of up to 1 mg of DNA/ml was adjusted to contain 6 M urea, 0.4 M guanidine hydrochloride, 0.1% β-mercaptoethanol, 0.1 M sodium phosphate (pH 7.0), by addition of solid urea, pure mercaptoethanol, and ten-times-concentrated solutions of guanidine hydrochloride, and phosphate buffer. DNA was then sedimented by centrifugation at 100,000*g* at 4° for 48 hr, using a no. 30 rotor in the Spinco Model L2-50 ultracentrifuge. For larger volumes, the Spinco Ti-15 zonal rotor has been successfully employed as a batch rotor for the removal of DNA.

More than 90% of the proteins of sheared chromatin were present in the supernatant following such sedimentation of DNA. The residual proteins remaining bound to DNA could be further extracted by extensive dialysis of the suspended pellet *vs.* 2% sodium dodecyl sulfate–0.1% β-mercaptoethanol–0.01 M sodium phosphate (pH 7.0) followed by a similar sedimentation at 20°. The pellet, containing DNA which was nearly protein free could be further purified by the method of Marmur (1961), using isoamyl alcohol–chloroform extractions, ribonuclease treatment, and alcohol precipitation.

The supernatant from the initial sedimentation was applied to a Bio-Rex 70 column equilibrated with 6 M urea–0.4 M guanidine hydrochloride–0.1 M phosphate (pH 7.0). A column of dimensions 5.0 × 20 cm was adequate for the separation of the proteins from 200 mg of DNA derived from rabbit liver or kidney chromatin. The initial, nonabsorbed peak from such a column contained the nonhistone proteins (Figure 2). The column was washed with the equilibrating buffer until the absorbance at 230 nm of the eluate returned to base-line values, and then the histones were eluted in one peak with a step of 4 M guanidine hydrochloride in the equilibrating buffer. Alternatively the histones could be fractionated into three subclasses using a linear gradient of guanidine hydrochloride from 0.8 to 1.5 M, in 0.1 M sodium phosphate (pH 7.0), followed by a step of 4 M guanidine hydrochloride, as detailed by Rasmussen *et al.* (1962).

The nonabsorbed peak from the Bio-Rex 70 column

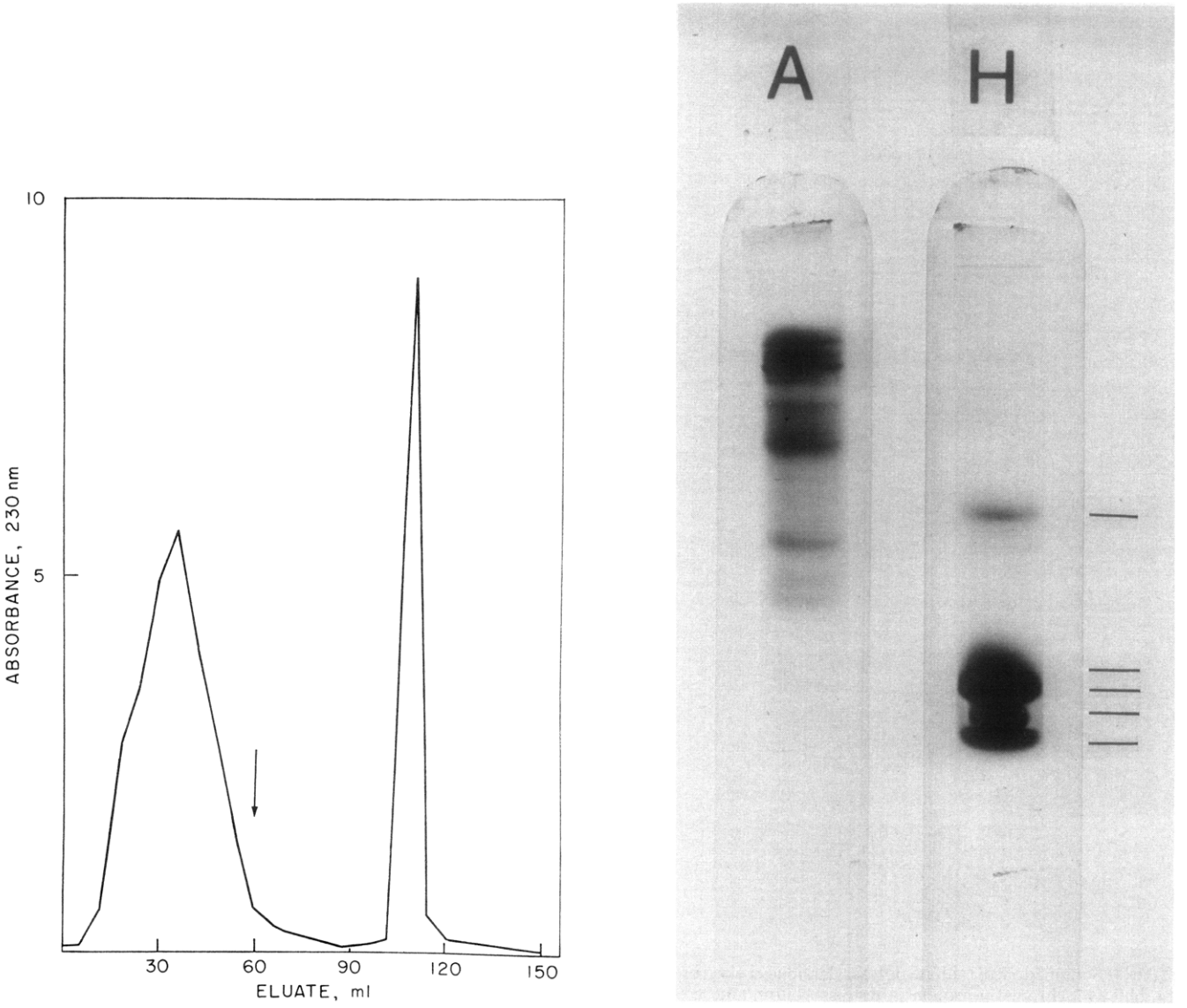

FIGURE 2: (a, left) Bio-Rex 70 chromatography of chromatin proteins from rabbit kidney. A 2.5 × 40 cm column of Bio-Rex 70 was loaded with the supernatant from dissociation and sedimentation of 50 mg of DNA as rabbit kidney chromatin, under the conditions described in in the Experimental Section. After washing with the equilibrating buffer, a step of 4 M guanidine hydrochloride was applied at the arrow. (b, right) Sodium dodecyl sulfate polyacrylamide gel electrophoreses of the two pooled peaks, the acidic nonabsorbed peak (A) and the retained histone peak (H). Equal amounts of proteins (relative to the initial DNA concentration) were applied to the two gels. Electrophoresis was from the top (−) to the bottom (+). Histone fractions F1, F3, F2b, F2a2, and F2a1 are indicated, from top to bottom.

containing the nonhistone proteins was dialyzed to 3 M urea–0.01 M Tris-hydrochloride (pH 8.5) and further fractionated on DEAE-cellulose. In the case of sheared chromatin, this peak contained both nonhistone proteins and a small portion of low molecular weight nucleic acids. These two components were separated by absorbing the dialyzed fraction to a 2.5 × 30 cm column of DE-52 equilibrated with 3 M urea–0.01 M Tris-hydrochloride (pH 8.5). The nonhistone proteins were eluted with a step of 0.3 M NaCl in the equilibrating buffer, while the nucleic acid components were eluted with a step of 0.6 M NaCl in this buffer. After dialysis of the nonhistone proteins back to the starting buffer, they were fractionated on another DEAE-cellulose column. Alternatively, when the eluate stream from the Bio-Rex 70 column was concentrated in line with an Amicon CEC-1 concentrator and PM-30 filter, most of the low molecular weight nucleic acid contaminants passed through the membrane, allowing elimination of the preliminary step fractionation of these moieties from the nonhistone proteins.

A 0.9 × 25 cm column of DE-52 was found to be adequate for the further fractionation of about 10 mg of purified nonhistone proteins. After loading the sample and washing the column with the equilibrating buffer, a linear gradient of 0–0.3 M NaCl in 3 M urea–0.01 M Tris-hydrochloride (pH 8.5) was developed. A complex gradient of 0–0.25 M NaCl in this buffer was designed with an Ultragrad gradient mixer (LKB Instruments, Inc.). Generally, a total gradient volume of 10–20 ml/ml of bed volume was utilized.

Certain modifications in this procedure were possible when nonsheared chromatin preparations were employed. Thus, while more protein remained bound to DNA after dissociation and sedimentation in the case of nonsheared chromatin, the supernatant was nearly totally devoid of nucleic acid contaminants. Hence, the nonadsorbed eluate from the Bio-Rex

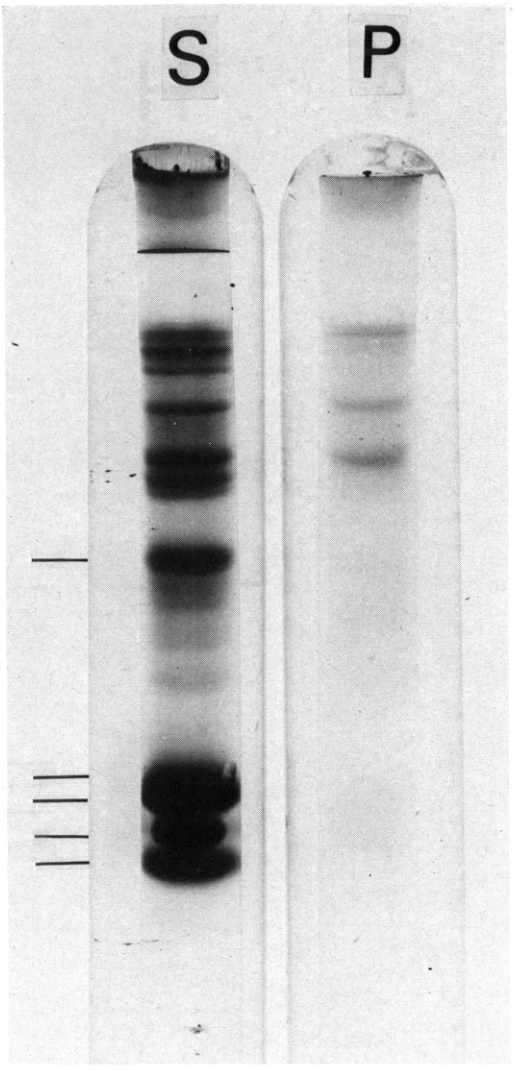

FIGURE 3: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of rabbit liver chromatin proteins after dissociation and sedimentation. Rabbit liver chromatin (unsheared) was dissociated and DNA sedimented as described in the Experimental Section. Residual proteins in the pellet were isolated by dialysis against 2% sodium dodecyl sulfate and sedimentation of DNA. Both fractions, corresponding to the initial supernatant (S) and pellet (P) were analyzed. The direction of migration is from the top (−) to the bottom (+). Histone fractions are indicated from top to bottom, F1, F3, F2b, F2a2, and F2a1.

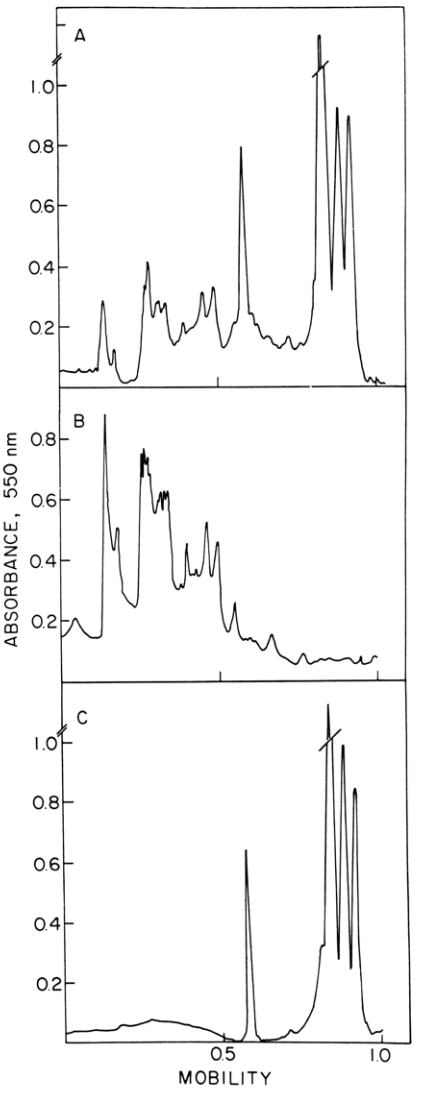

FIGURE 4: Densitometric scans of stained sodium dodecyl sulfate electrophorograms of total chromatin proteins (A), nonhistone proteins (B), and histones (C), all obtained from rabbit liver. The two fractions in parts B and C were obtained from Bio-Rex 70 as in Figure 2a. Electrophoresis was from left (−) to right (+) with loads of 200 μg of protein (A and B) and 100 μg of protein (C). Mobilities are expressed relative to the mobility of bromophenol blue tracking dye.

70 column could, after dialysis, be utilized directly for the gradient DEAE-cellulose chromatographic step.

Results

In initial attempts to achieve maximal dissociation of chromatin proteins from nucleic acids, a number of solutions were employed. The relative effectiveness of those tested was 6 M urea < 4 M NaCl < 6 M urea–0.4 M guanidine hydrochloride < 5 M guanidine hydrochloride < 2% sodium dodecyl sulfate. The latter three systems removed, respectively, 90–95, 95–97, and 96–99% of the proteins of sheared chromatin. Since the urea–guanidine mixture was nearly as effective as any system tested, and in addition, was the buffer to be employed for loading the first chromatography column, it was selected for further use. The addition of β-mercaptoethanol was found to reduce aggregation between histone and nonhistone proteins, and thereby facilitated the subsequent separation of these two groups of proteins.

When sheared chromatin was employed for a starting material, up to 5% of the total nucleic acid content might not sediment after 48-hr centrifugation at 100,000*g*. These low molecular weight nucleic acid fragments (estimated to be of the order of 50–100 nucleotides from their chromatographic behavior) cochromatographed with the nonhistone proteins in the first column procedure, and did not interfere with the separation of the histones from the nonhistone proteins. However, removal of the nucleic acids, by a preliminary DEAE-cellulose step, was essential for adequate resolution of the nonhistone proteins.

Following dissociation and sedimentation of nonsheared chromatin, a greater proportion of the proteins of the complex remained bound to the nucleic acid. Thus, when dissociated and separated at a DNA concentration of 0.5 mg/ml, the supernatant contained 85% of the proteins while the residual

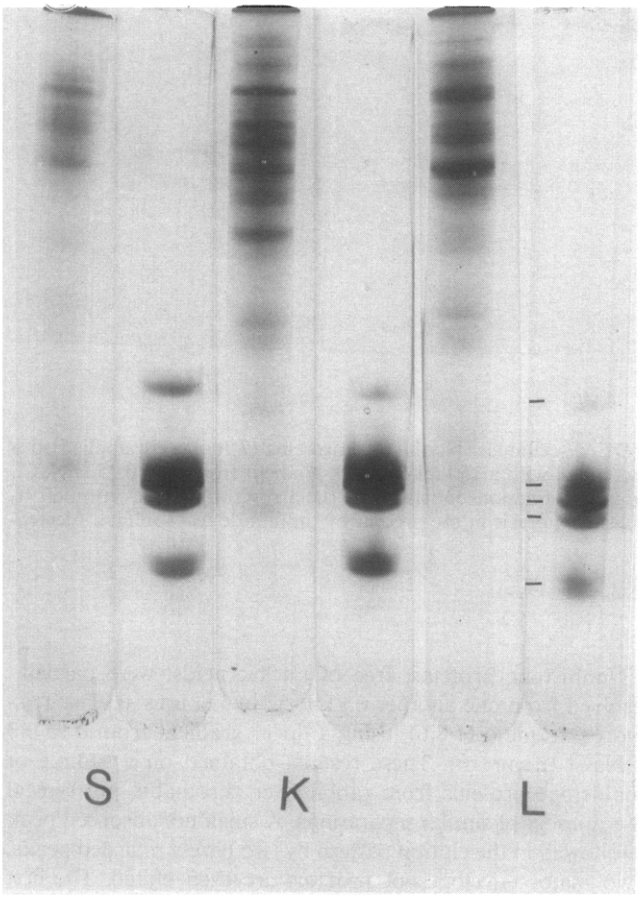

FIGURE 5: Polyacrylamide gel electrophoresis at pH 2.7 in the presence of urea, of the nonhistone (left gel in each pair) and histone (right gel in each pair) proteins of rabbit spleen (S), kidney (K), and liver (L). The protein fractions were obtained from Bio-Rex 70 chromatography as in Figure 2a, and analyzed as described in the Experimental Section. Electrophoresis was from the top (+) to the bottom (−). Histone fractions F1, F3, F2b, F2a2, and F2a1 are indicated from top to bottom.

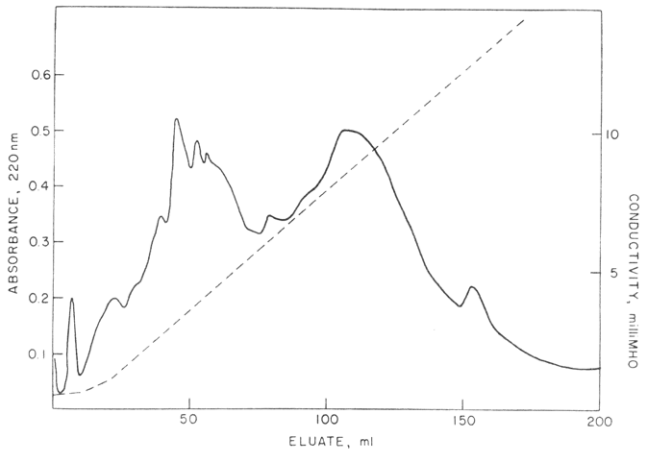

FIGURE 6: Fractionation of nonhistone proteins of rabbit liver on DEAE-cellulose. Nonhistone proteins (10 mg) were applied to a 0.9 × 25 cm column of DEAE-cellulose as in the Experimental Section and eluted with a 200-ml linear gradient from 0 to 0.3 M NaCl. Absorbance (——) and conductivity (- - -) are plotted.

15% remained with DNA in the pellet. Even though less protein was dissociated, avoidance of shearing also led to the near abolition of the small molecular weight nucleic acids found in sheared samples. The supernatant from dissociated unsheared chromatin contained less than 1% of the total nucleic acid.

Since omission of the shearing step allowed the omission of a column procedure at a later stage of the fractionation scheme, it would seem to be the method of choice. This is true only if it is known that all protein fractions are at least partially dissociated when the separation of nucleic acid and protein is performed with unsheared chromatin. This situation was therefore evaluated. After dissociation and sedimentation of DNA from unsheared chromatin, the residual proteins were dissociated further using sodium dodecyl sulfate and compared electrophoretically to the proteins solubilized by the initial dissociation step (Figure 3). No protein bands appeared in the sodium dodecyl sulfate supernatant that were not also present in the supernatant fraction from the initial dissociation. Thus, all proteins were at least partially dissociated, although the dissociation is somewhat selective. While the histones were totally dissociated from the DNA, traces of some nonhistones fractions still remained bound to DNA after urea–guanidine hydrochloride treatment.

Separation of histones from nonhistone proteins was achieved by chromatography of the supernatant fraction from sedimentation on the cation-exchange resin Bio-Rex 70 (equivalent to IRC-50). The column, equilibrated with 6 M urea–0.4 M guanidine hydrochloride–0.1 M sodium phosphate (pH 7.0), was loaded with the supernatant fraction directly and washed with the equilibrating buffer until base-line absorbance was reestablished for the eluate stream (Figure 2a). The nonhistone proteins, together with any nucleic acid still present, passed unabsorbed through the column under these conditions, while the histones were tightly absorbed. The histones were later eluted as a group with a step of 4 M guanidine hydrochloride in the equilibrating buffer. Alternatively, urea may be washed out of the column, and a previously described gradient elution program of guanidine hydrochloride in phosphate buffer carried out in order to fractionate the various histone classes (*cf.* Rasmussen *et al.*, 1962; Bonner *et al.*, 1968a).

The efficacy of this procedure in the separation of nonhistone from histone proteins was indicated by the results of polyacrylamide gel electrophoresis of the two peaks in sodium dodecyl sulfate (Figure 2b). The five histone fractions identifiable on these gels were present in the fractions eluted with the guanidine hydrochloride step, and no bands of molecular weight greater than that of F_1 histone (*ca.* 22,000) were present in the histone fraction. Similarly, the nonhistone proteins, generally of higher molecular weight than the histones, were free of contamination with the basic, smaller histones.

Densitometric scans of polyacrylamide gel electrophoreses of total chromatin proteins, histones, and nonhistones, allow more careful comparison of the distributions of the proteins in the two fractions obtained by this chromatographic step (Figure 4). The clear separation of the two classes of proteins by the cation-exchange step is apparent. We estimate that less than 1% cross contamination between histone and nonhistone proteins occurs after this separation. The total chromatin proteins are completely accounted for by the two subfractions, histone and nonhistone proteins.

While our separation methods give a clear separation of histone from nonhistone when these fractions are examined on a size basis, the results of others have suggested that there may be nonhistone proteins with the same electrophoretic mobility on sodium dodecyl sulfate electrophoresis as the

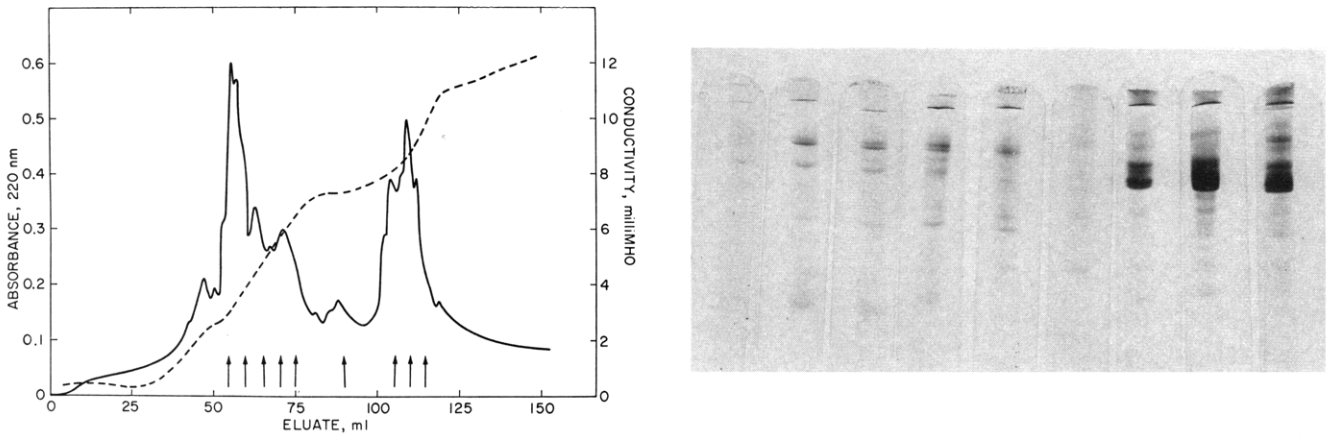

FIGURE 7: (a, left) Fractionation of nonhistone proteins of rabbit liver on DEAE-cellulose. Nonhistone proteins (7 mg) were applied to a 0.9 × 25 cm column of DEAE-cellulose as in the Experimental Section and eluted with a 150-ml complex gradient from 0 to 0.25 M NaCl. Absorbance (——) and conductivity (- - -) are plotted. (b, right) Individual 2.5-ml fractions indicated by the arrows in part A, from left to right were dialyzed, dried, and subjected to analytical polyacrylamide gel electrophoresis in the present of sodium dodecyl sulfate. Electrophoresis was from top (−) to bottom (+).

histones (Elgin and Bonner, 1970). Our present results would suggest that their finding probably is explained by the incomplete separation of the two classes of proteins. To further verify the complete separation of the basic histones from the acidic (or less basic) nonhistones, the two fractions from the Bio-Rex 70 column were examined by analytical electrophoresis at pH 2.7 in the presence of urea.

Histones from the three tissues examined, liver, kidney, and spleen, are seen to yield electrophoretic patterns which are quite similar to one another and to those previously obtained by others (Panyim and Chalkley, 1969; Shaw and Huang, 1970) (Figure 5). No contamination of the histones by acidic proteins is detectable. Similarly, the nonhistones, the filtered peak on Bio-Rex 70 chromatography, are acidic in nature, and are uncontaminated by highly basic proteins (Figure 5). The nonhistones vary both quantitatively and qualitatively in the three tissues examined.

The complete separation of the nonhistone proteins from histones reduced the tendency of the nonhistones to aggregate upon reduction of ionic strength, and allowed the fractionation of the nonhistone proteins by ion-exchange chromatography in relatively low concentrations of urea in the absence of any other disaggregating solvent.

Fractionation of the nonhistone proteins was performed by gradient elution from DEAE-cellulose. As previously noted, if sheared chromatin were employed as the starting material for fractionation, the "nonhistone proteins" at this stage might contain up to 5% of the total nucleic acid as low molecular weight contaminants. By binding to the anion exchanger, the nucleic acids can markedly reduce the degree of resolution obtained on chromatography of the nonhistone proteins, and hence their removal was required. This removal was accomplished by absorption of the mixture in 3 M urea–0.01 M Tris-hydrochloride (pH 8.5) to a column of DEAE-cellulose in the same solvent, and elution of the proteins with a step of 0.3 M NaCl in the equilibrating buffer. Alternatively, the sample eluted from the Bio-Rex 70 column could be concentrated in line using a PM-30 membrane and an Amicon CEC-1 concentrator. The nucleic acid components are largely filtered through the membrane, leaving the eluate nearly free of contaminants, and allowing the direct chromatography of the nonhistone proteins.

Nonhistone proteins, free of nucleic acids, were partiall resolved from one another on DE-52 in 3 M urea–0.01 M Tris-hydrochloride (pH 8.6), using a linear gradient from 0 to 0.3 M NaCl (Figure 6). These results, obtained on a sample of nonhistone proteins from rabbit liver chromatin, are typical of a number of similar separations. A small nonabsorbed peak is followed in the elution pattern by two barely retarded peaks. Two major envelopes of proteins are then eluted. The first contains several species of protein which are partially resolved, while the second is broader, and appears to be largely unresolved. A final small peak is eluted on the trailing edge of the second major peak.

While maintaining the same absorbent and equilibrating buffer, a number of parameters were varied in order to evaluate the effect of other column parameters on the separation achieved. Doubling column length, halving gradient steepness, and halving the sample load did not lead to marked increases in the obtained resolution. Doubling the sample load led to a diminution of resolution. In this case, only two broad peaks were observed, with no evidence of the beginning resolution of proteins within these peaks.

A marked improvement in separation of the nonhistones into fractions was achieved by the introduction of a complex gradient. Nearly complete separation of the two major envelopes of nonhistone proteins was obtained (Figure 7a). The first envelope was resolved into its components somewhat more effectively than with the simple linear gradient, while the second envelope also yielded evidence of beginning resolution of its components.

Individual fractions throughout the elution profile were analyzed by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate (Figure 7b). The first major peaks (50–75 ml) are seen to comprise primarily the higher molecular weight group of the nonhistone proteins. The second envelope (100–125 ml) contains the smaller molecular weight nonhistones. Complete separation of any particular protein in this highly heterogeneous mixture was not achieved by this single chromatographic procedure. However, fractionation of the major groups of nonhistones was achieved, and evidence of subfractionation within these major groups is provided by both the elution profile and the analytical gels.

Discussion

Study of the composition of chromatin has been fraught with difficulties arising from variability of the starting material, poor definition of the basis for classification of components, and lack of knowledge about the components. Thus, chromatin can be prepared by a variety of methods (*cf.* Zubay and Doty, 1959; Marushige and Bonner, 1966; Huang and Huang, 1969; Johns and Forrester, 1969), and the composition, particularly in terms of nonhistone proteins, can vary broadly, perhaps in relation to the methodology utilized for the preparation. Traditionally, in the absence of functional assays for these species, histones have been defined on the basis of their acid solubility and nonhistones by their acid insolubility. Such definitions are ambiguous at best, and have led to problems in both definition of the relative amounts of the two classes of chromatin proteins, and in attempts to fractionate chromatin by the use of acid extraction. Such problems as these had led to reports of acid-soluble nonhistones (Sadgopal and Bonner, 1970), acid insoluble histones (Sonnenbichler and Nobis, 1970) and of nonhistone proteins which appear to have electrophoretic mobilities identical with those of histone fractions (Elgin and Bonner, 1970; Marushige *et al.*, 1968).

In approaching the development of a method for fractionation of the components of chromatin, we have preferred to define chromatin operationally as the product of a given isolation procedure, rather than as a rigorously defined chemical entity. We have specifically studied liver and kidney chromatin which have reportedly large content and variety of nonhistone proteins (Bonner *et al.*, 1968b; Elgin and Bonner, 1970), feeling that fractionation methodology which succeeds with a large amount of these proteins (and potentially with contaminating nonchromatin proteins) present should be also applicable to chromatins that are less heterogeneous in their nonhistone protein content. In dissociating the chromatin complex, and separating histone from nonhistone, we have avoided the use of acid, alkaline, or phenol extraction, both because of variable solubilization of both histone and nonhistone, and because of the potentially irreversible effect on the native structure of some of the nonhistone proteins. Similarly, the use of ionic detergents has been avoided, due to their avid binding to proteins (Reynold and Tanford, 1970), and the consequent difficulty in the removal of the denaturants to allow separation by other than size criteria (Shirey and Huang, 1969). Furthermore, we have attempted to design a method which allows the preparation of all the components of chromatin from a single tissue sample. This was felt to be of particular import for further studies of the biological role of chromatin proteins in cellular development and differentiation, both ensuring that no significant fraction would be inadvertently discarded, and that precise comparisons could be made between the different components of the chromatin sample. The method developed meets the criteria set forth initially, and serves well for the analytical and preparative fractionation of chromatin from various tissue sources. The sedimentation of DNA is carried out under conditions that both lead to nearly complete dissociation of chromatin and are appropriate for the first chromatographic step, eliminating lengthy dialysis or concentration procedures. The initial chromatography on a cation exchanger leads to quantitative separation of histones from nonhistones, and allows the subfractionation of the histone classes.

After removal of any residual small nucleic acid components, the nonhistone proteins were subjected to chromatography on an anion exchanger. This chromatographic procedure does not lead to the purification of any single one of the nonhistone proteins, but it does spread these proteins throughout the elution profile of a single gradient. While the fractionation can be optimized with use of a complex gradient mixing device, a preliminary separation is obtainable with only a simple linear gradient. Since our goal is to apply these fractionation methods to the purification of proteins of biological significance in chromatin function, it was felt that a method which displayed all the proteins would be preferable to one that isolated one or more selectively. With this method and the use of pair-label experiments, it should be possible to localize a protein of interest in the elution pattern, and then either expand the gradient in that region, or proceed to another separation method, to isolate the protein of interest in pure form.

A number of reports have appeared which attempt to attribute functional significance to nonhistone proteins in cellular development or regulatory processes. For example, elevated nonhistone synthesis has been reported after hormonal or other stimulations (Shelton and Allfrey, 1970; Stein and Baserga, 1970; Barker, 1971; Rovera and Baserga, 1971). A particular protein has been localized on gel electrophoresis and shown to be differentially synthesized in livers of cortisol-stimulated, adrenalectomized rats (Shelton and Allfrey, 1970). Tissue-specific nonhistone proteins which may serve as hormonal acceptors have been described (Swaneck *et al.*, 1970; Spelsberg *et al.*, 1971). In the past, the methodology for fractionation and isolation of these proteins has been limited and has not allowed the preparative-scale isolation of particular nonhistone proteins in a form which allows the study of their biological function. It is expected that the currently presented methodology will overcome these difficulties.

Acknowledgment

We thank the late Mr. Jesse Hicks for his technical assistance in the preparation of chromatin.

Added in Proof

Several Bio-Rex 70 lots, obtained recently, although of advertised capacity for H^+, did not absorb histones completely when used with 0.4 M guanidine hydrochloride in buffer. Histones were quantitatively absorbed by these lots of absorbent by decreasing the guanidine hydrochloride concentration in the equilibrating and sample buffers to 0.35 M.

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A Circular Dichroism Study of Microtubule Protein†

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ABSTRACT: The conformation of tubulin, the 6S protein subunit of microtubules, has been studied by circular dichroism (CD) spectroscopy. The native protein at 4° and pH 6.5 contains approximately 22% α helix, 30% β structure, and 48% random coil. This protein is characterized by its ability to bind the antimitotic drug colchicine. At 37°, where colchicine binding is optimal the protein undergoes a slow conformational change resulting primarily in loss in α helix. This change increases the lability of the protein to irreversible denaturation accompanied by aggregation. The protein can be protected by the presence of various nucleotides such as GTP, GDP, GMP, GMPPCP, and the antimetabolites colchicine and vinblastine. Colchicine binding shows a sharp optimum at pH 6.5. Variations of pH away from this value lead to mild alternations in circular dichroism at 37°, much larger effects at 4°. All of the results obtained are consistent with the idea that colchicine-binding ability is a critical function of the protein conformation. A few preliminary measurements on the CD of intact sea urchin sperm tail microtubules are reported. GMPPCP and colchicine increase the rate of thermal dissociation of the tubule, as observed by changes in the CD spectra.

Microtubules, 240-Å diameter tubular structures, are widely distributed in plant and animal cells (Porter, 1966). These tubules make up the spindle fibers of the mitotic cell, the axonemal complex of cilia and flagella, and are prominent in the axons and dendrites of neurons. The precise function of the microtubule is unknown although they have been implicated in such diverse functions as axoplasmic transport, ciliary motion, morphogenesis, and form maintenance (Shelanski and Feit, 1971). While the microtubules in cilia and flagella appear to be stable over a wide range of temperature, pressure, and drug treatments, cytoplasmic microtubules are very labile. This lability is best demonstrated by the experiments of Tilney (Tilney *et al.*, 1966; Tilney and Porter, 1967) on the microtubule rich axopods of the heliozoan Actinosphaerium. In these thin processes the microtubules are depolymerized and the process retracted on exposure of the cell to low temperature (4°), high pressure (3000 psi), or the antimitotic drug colchicine. All these effects are reversible. The microtubules are stabilized by exposure to D_2O.

The subunit protein of the microtubule, tubulin or MTP, has been isolated from cilia (Renaud *et al.*, 1968), sea urchin sperm flagella (Shelanski and Taylor, 1967, 1968; Stephens, 1968), and numerous cytoplasmic sources, especially brain (Borisy and Taylor, 1967a,b; Weisenberg *et al.*, 1968). In all cases the subunit isolated was a 6S, 120,000 dimer which has approximately 2 moles of guanine nucleotide bound per dimer. The protein obtained from cytoplasmic sources and from the central pair of microtubules of the sperm flagellum

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