A New Method for Fractionating Histones for Physical and Chemical Studies†

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ABSTRACT: We have developed a method for the fractionation of histones using a single column (120 × 2.5 cm) packed with Bio-Gel P-100, 100 mesh resin and one eluent (0.01 N HCl + 0.02% sodium ozide) which is capable of separating and fractionating histone from vertebrates, invertebrates, and plants. Approximately 10 mg of whole histone was applied to the top of the column and the flow rate adjusted to 6.5 ml/hr under a head pressure of 60 cm. Fractions were collected (2 ml), histones assayed by absorption at 230 nm, and appropriate fractions pooled. Pure fractions were dialyzed *vs.* 0.4 N sulfuric acid overnight and precipitated with 95% ethanol. This method is capable of separating previously unfractionable histones such as fish gonadal tissue, *Arbacia punctulata* sperm, and pea. Recovery of total histone at 90% of the theoretical yield can be achieved with 98% purity of each fraction (pea F2a1 excepted). Using this method we have been able to purify histone F3 in sufficient quantities to allow for comparative tryptic peptide mapping of protein from calf thymus (two cysteines) and fish gonadal tissue (one cysteine) (K. Sommer (1973), Ph.D. Thesis, University of Iowa, Iowa City, Iowa) as well as sequencing of fish histone F3 (J. Hooper, E. Smith, K. Sommer, and R. Chalkley (1973), *J. Biol. Chem. 248*, 3245). In addition, we have used histone purified in this manner for studies including molecular weight determination and characterization of pea histone F2b and F2a2 and *Arbacia punctulata* sperm histone F3.

The widening scope of histone investigations has increased the demand for purified histone fractions for chemical and physical studies. Several lengthy procedures have been developed to produce both large amounts of a specific pure fraction of histone (Kinkade and Cole, 1966; Fambrough and Bonner, 1969; Iwai and Senshu, 1969; Mauritzen *et al.*, 1966; Sanders and McCarty, 1972) and individual separation of all histone fractions from a given creature or tissue (Johns, 1964; Oliver *et al.*, 1972). All methods are tedious and lengthy, requiring either multiple extractions with various aqueous–organic solvents or extensive column chromatography. With the exception of the method of Oliver *et al.* (1972), which is essentially a modification of the method developed by Johns (Kinkade and Cole, 1966), they require large amounts of starting material and frequently, there is cross-contamination of various fractions. Although the Oliver *et al.* method is capable of efficiently separating all five major fractions of histone from a number of vertebrates and an invertebrate, it was found that this method was not applicable for fractionation of histones isolated from at least one plant source, the pea (the high lysine content of pea F2b and F2a2 changes their characteristic solubility in 5% perchloric acid), from fish gonadal tissue, and from *Arbacia punctulata* sperm. Probably the most serious problem with the existing fractionation schemes is that they cannot be applied to less than 15 mg of total histone without serious loss of reliability and efficiency. This makes the physical–chemical studies of histones from tissues and organisms which yield only a small amount of these basic proteins almost impossible. This is likely to be the case for many plants and invertebrates, particularly those with few, small chromosomes and those with a high cytoplasmic/nuclear volume ratio.

We have recently developed a direct approach to the fractionation of vertebrate, invertebrate, and plant histones. It is applicable to as little as 5 mg of total histone. A high degree of purity (98%) is obtained with a minimal expenditure of time and effort. The major innovation and advantage of the method is that it utilizes a single column and a single eluent to achieve a separation of all five histone fractions. Data are presented showing that this method is capable of efficiently separating the five major fractions from vertebrates (calf thymus, human placenta, and fish gonadal tissue); an invertebrate, *Arbacia punctualata* sperm; and a plant, the pea.

We have used histone fractionated and purified by this method for several types of physical–chemical studies in our laboratory. We have examined specific fractions of pea histone more closely. The amino acid composition and molecular weight of pea histone fractions F2b and F2a2 have been determined. Histone F2b exists as an electrophoretically homogeneous band whereas histone F2a2 exists in two forms. The amino acid composition and heterogeneity of *Arbacia* histone F3 have been studied. Finally, calf thymus F3 and fish testis F3 have been used for comparative tryptic peptide mapping (K. Sommer and R. Chalkley, in preparation) and fish testis F3 for sequence work (Hooper *et al.*, 1973).

Materials and Methods

Histone Isolation. Histones were isolated from nucleohistone obtained from purified nuclei as described previously (Panyim *et al.*, 1971). Pea histones were kindly supplied from Dr. Steven Spiker, and *Arbacia punctulata* sperm histones by Dr. Douglas Easton.

Histone Fractionation. Forty grams of Bio-Gel P-100, 100–200 mesh (Bio-Rad Corp.), swollen overnight in 1 l. of 0.01 N hydrochloric acid + 0.02% sodium azide were packed into a column (2.5 × 120 cm) and equilibrated with 1 l. of 0.01 N HCl + 0.02% sodium azide (eluent). Whole histone (10 mg) was applied to the top of the column in 2 ml of eluent and the flow rate adjusted to 6.5 ml/hr under a head pressure of 60 cm. Fractions (2 ml) were collected and histone was assayed by absorption at 230 nm. Appropriate fractions

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were pooled (as determined by polyacrylamide gel electrophoresis of peak fractions). Pure fractions were collected into dialysis bags and concentrated against solid sucrose, dialyzed *vs.* 0.4 N sulfuric acid overnight, and precipitated with 95% ethanol. The pellets were washed three times with acetone and dried *in vacuo*. All manipulations were performed at 2° unless otherwise stated.

Although the above method utilizes volumes and quantities appropriate for processing 10–15 mg of total histone, it can easily be scaled down to accommodate as little as 5 mg or as much as 200 mg of total histone.

The yield of pure histone fractions may be increased by retreating cross-contaminated histone from peak overlap fractions. The most common cross-contaminations are those associated with the peak overlap regions of histone F2b, F3, and F2a1, although in pea, peak overlap occurs in the histone F2a2 and F2b region. Pooled column fractions containing histone F3 cross-contaminated with histone F2b or histone F2a1 were made 5% perchloric acid with concentrated perchloric acid and dialyzed *vs.* a solution of 80% ethanol + 0.25 N HCl (10 ml of concentrated HCl + 90 ml of water + 400 ml of 100% ethanol), two changes against the same solvent (Oliver *et al.*, 1972). Any precipitate (histone F2b) which appears should be removed by centrifugation. The solution was dialyzed *vs.* seven volumes of absolute ethanol overnight. Contents of the dialysis sac were removed and centrifuged at 12,000*g* for 5 min and the pellet (pure F3) was collected and dried. Clarified dialysate was made 0.4 N in H_2SO_4 and dialyzed *vs.* ethanol for 6 hr. The pellet (F2a1) was collected and dried. Essentially the same procedure may be used for removing F2b contamination from F2a2 in pea peak overlap regions. The pooled column fractions are treated with perchloric acid and ethanol–HCl, and F2b was precipitated. The clarified dialysate is made 0.4 N in H_2SO_4, and F2a2 is precipitated by dialysis *vs.* 95% alcohol. It should be noted that the initial concentration of histone F3 + F2b, F3 + F2a1, or F2b + F2a2 (pea) must be greater than 5 mg/ml to remove cross-contaminants by this method. Concentrations of this magnitude occur when total histone starting material is greater than 25 mg. If the amount of starting material is less than 25 mg, yield of pure histone fractions is maximized by judicious pooling of fractions from the column rather than by retreating cross-contaminated fractions or rechromatographing the fractions. Obviously, in chromatography of small amounts of whole histone (5 mg), it will be necessary to sacrifice yield for purity as the retreatment of specific fractions requires more histone than available under these conditions.

Electrophoresis. Polyacrylamide gel electrophoresis was performed as described in detail by Panyim and Chalkley (Panyim *et al.*, 1971). After completion of electrophoretic destaining, the gels were scanned at 600 nm with the Gilford Model 2000 spectrophotometer equipped with a gel scanner. The peaks obtained were quantitated with a Du Pont electronic curve analyaer.

Amino Acid Analysis. Protein samples were hydrolyzed *in vacuo* in 6 N HCl at 110° for 22 hr. Amino acid analysis was performed using a Jeol Model JLC-6AH analyzer equipped with Integrator JCC-SK. Corrections for loss of serine and tyrosine and incomplete liberation of isoleucine and valine were not made. Cysteine was determined by performic acid oxidation (Hirs, 1967).

Molecular Weight Determination. Molecular weights were determined using the modified sodium dodecyl sulfate electrophoretic method of Panyim and Chalkley (1971).

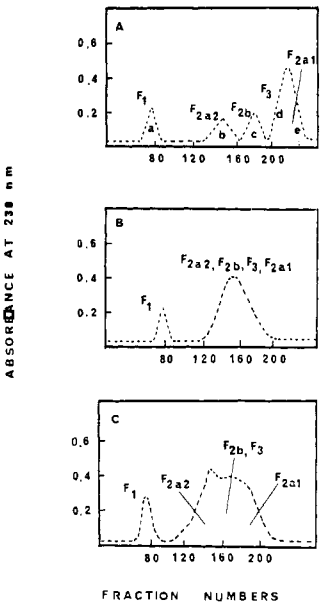

FIGURE 1: Elution profiles of total calf thymus histone applied to different column systems. (A) Histone applied to Bio-Gel column used for routine fractionation as described in Materials and Methods. In profile, a is histone F1, b histone F2a2, c histone F2b, d histone F3, and e histone F2a1. (B) Histone applied to a Sephadex G-100 (100–200 mesh) column. Column length and diameter, flow rate, head pressure and eluent were identical with those used for the Bio-Gel column in Figure 1A and described in the text. (C) Histone applied to same Bio-Gel column described in A. Column had been equilibrated with histone chromatographed with eluent of 0.01 N HCl + 0.02% sodium azide + 4 M urea.

Results and Discussion

The basic differences in molecular weight and the shape of the various histone fractions are important in terms of their resolution by exclusion chromatography. However, their separation is also critically a function of the molecular sieve resin used. The optimal condition for histone separation is on Bio-Gel P-100 as described in Materials and Methods and shown in Figure 1A. If a Sephadex resin is used under similar conditions, the retention times of calf thymus histones F2a2, F2b, F3, and F2a1 are decreased (Figure 1B), so that fractions are coeluted and, consequently, pure fractions cannot be obtained. Addition of 4 M urea to the eluent results in a decrease in the retention time of Bio-Gel of histones F2a2, F2b, F3, and F2a1 (Figure 1C) and in their coelution. Evidently there is a substantial amount of secondary and tertiary structure present in these molecules even at the low pH employed and this forms a critical aspect of the factors which contribute to the resolution of the various histone fractions.

Application of the technique described to total histones obtained from *Arbacia punctulata* sperm, calf thymus, fish gonadal tissue, and pea are shown in Figures 2 and 3. The fractions obtained from such chromatography are shown in Figure 2. They are electrophoretically pure and the extent of cross-contamination is less than 2% as judged by quantitative microdensitometer scans.

Column elution profiles (Figure 3) for each type of histone indicate that histone fractions are not necessarily eluted as a function of decreasing molecular weight as is the case in molecular sieve column chromatography of globular proteins. In calf thymus, histone F1 (molecular weight 20,500 and 21,500; Panyim and Chalkley (1971)) which is the largest of the histones is eluted first in the void volume. This is the result of its size and extended rod-like structure under acidic

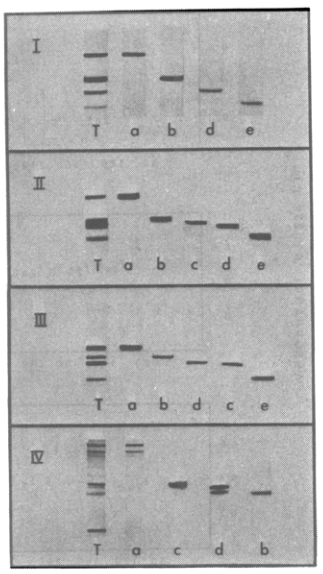

FIGURE 2: Polyacrylamide gel analysis of pooled purified histone fractions. Analysis of histone fractions were performed by electrophoresis on 10-cm acrylamide gels (15% acrylamide, 0.1% *N,N'*-bisacrylamide, 0.5% TEMED, 5.4% acetic acid, 2.5 M urea, and 0.025% $(NH_4)_2S_2O_8$ at 130 V for 3.5 hr, followed by staining for 5 hr with 0.1% Amido Black in 20% ethanol and 7% acetic acid (Panyim and Chalkley, 1969). (I) *Arbacia punctulata* sperm histones, (II) calf thymus histones, (III) fish gonadal tissue histones, (IV) pea histones. In all cases, T indicates total histone or starting material, a indicates histone F1, b indicates histone F3, c indicates histone F2b, d indicates histone F2a2, and e indicates histone F2a1.

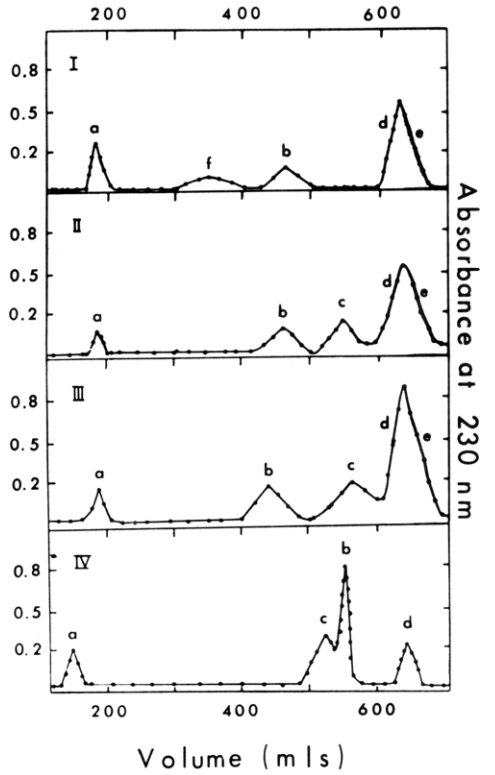

FIGURE 3: Column elution profiles. Procedure described in Materials and Methods. (I) *Arbacia punctulata* sperm histones, (II) calf thymus histones, (III) fish gonadal tissue histones, and (IV) pea histones. In all cases, a is histone F1, b is histone F2a2, c indicates histone F2b, d indicates histone F3, e indicates histone F2a1, and f indicates nucleic acid contaminant.

conditions. Histone F2a1 (molecular weight 11,282; DeLange *et al.* (1969)), the smallest of the histones, is eluted last from the column. However, the elution patterns of the three intermediate range histones are totally anomalous in terms of molecular weight. Histone F2a2 (molecular weight 13,100; Yeoman *et al.*, 1972) is eluted after F1, and followed in turn by histone F2b (molecular weight 13,700; Panyim and Chalkley (1971)) and in close proximity histone F3 (molecular weight 15,324; Hooper *et al.* (1973)).

Although it is not possible to provide a simple rationale for this order of elution, several explanations have been proposed. First it is possible that some residual conformational structure of these histones may play a role in the elution behavior of these proteins. Based upon the elution profiles and the known molecular weights we can predict that F3 with a molecular weight of 15,324 (Hooper *et al.*, 1973) may have a more compact conformation at this pH than histones F2a2 an F2b (molecular weight 13,100 and 13,700, respectively) since it is eluted after these two smaller proteins. These results are in good agreement with the studies on the conformation of isolated histones made by optical rotatory dispersion (Tuan and Bonner, 1969; Boublik *et al.*, 1970b) and several other methods (Jirgensons and Hnilica, 1965; Boublik *et al.*, 1970a), which indicate that arginine-rich histones in aqueous solution of ionic strength have a greater degree of secondary structure than lysine-rich histones. If elution of histones from this type of column is a function of both molecular weight and shape, histone elution could be related to histone mobility on polyacrylamide gels, where mobility is a function of molecular weight, shape, and charge density of the molecule. The anomalous behavior of the histone fractions upon exclusion chromatography provides an explanation for the electrophoretic mobility of fractions F3, F2b, and F2a2 in polyacrylamide gels at pH 3 in the absence of urea. In this system histones F2b and F3 have identical mobility, moving about 6% slower than histone F2a2. However, F3 had a lower charge density (6–7%) than either ot the other histone fractions and as a result of this factor, coupled with its higher molecular weight, we would have expected it to migrate significantly more slowly than either F2b or F2a2. However, if the F3 molecule is more compact than the other two fractions (as indicated by the chromatographic elution profile) its anomalously high rate of electrophoretic migration is more readily explained.

A second simpler explanation for the anomalous behavior of histones on Bio-Gel is plausible. It is possible that some interaction between column matrix, in the form of residual carboxyl groups, and histones may occur and that this in part may account for the anomalous elution of histones. Other workers (Sung and Dixon, 1970) have noted the anomalous behavior of histones on Bio-Gel resin. This would account for the trailing effect of histone F2a1, which is not completely eluted until 20 ml after the total volume of the column has been passed.

Whatever the cause for the anomalous behavior of histones on the Bio-Gel column, it does not alter the principle advantage of using this resin. That is that it allows for the fractionation of the five histones using a single column, and one eluent.

The data of Figure 3 indicate that the parameters governing the elution profiles of calf and fish histone F1, F3, and F2a1 have not changed during the course of vertebrate evolution. This observation is in good agreement with the remarkable conservation of primary structure shown by the arginine-rich histones (DeLange *et al.*, 1969; Fambrough and Bonner, 1968) and with earlier reports that the molecular weights of fish and calf lysine-rich histones are very similar (Panyim and Chalk-

TABLE I: Amino Acid Analysis of Purified Histone Fractions in Mole Per Cent.

	Total Amino Acid Content (mol %)							
	Histone F3			Histone F2a2			Histone F2b	
Amino Acids	Calf Thymus	Fish Testis	*Arbacia punctulata*	Calf Thymus	Pea	*Arbacia punctulata*	Calf Thymus	Pea
Lysine	8.0	9.0	9.4	11.4	15.5	15.9	14.0	17.7
Histidine	1.3	1.5	1.5	3.0	1.3	2.1	2.1	0.4
Arginine	13.0	13.1	12.3	10.3	6.9	10.6	8.3	3.9
Aspartic acid	4.1	3.9	4.2	5.6	5.4	7.5	5.1	2.4
Threonine	7.4	7.3	6.8	4.3	4.2	3.1	6.0	4.8
Serine	4.2	4.7	6.3	3.5	5.5	5.3	9.0	9.1
Glutamic acid	11.3	11.5	10.6	9.6	6.7	8.4	8.5	7.4
Proline	3.4	3.4	2.6	3.9	7.8	3.1	7.7	9.5
Glycine	5.6	5.5	7.5	10.8	9.2	13.2	6.1	7.6
Alanine	14.4	14.6	13.3	12.0	15.1	16.2	10.0	21.9
Cysteine	1.5	1.0	0.6	0.0	0.0	0.0	0.0	0.0
Valine	4.6	4.6	6.0	6.6	8.0	6.5	6.6	6.1
Methionine	1.3	1.2	0.9	0.4	0.6	0.02	1.6	0.5
Isoleucine	5.3	5.2	5.1	4.1	2.9	4.1	4.5	2.0
Leucine	9.6	9.9	8.8	11.0	8.9	12.4	5.5	5.9
Tyrosine	1.9	2.0	2.3	2.6	2.0	0.9	3.2	0.5
Phenylalanine	2.8	3.0	2.5	0.9	1.4	1.5	1.6	0.5

ley, 1971). However, small but reproducible changes in the elution profiles are noted for histone fractions F2a2 and F2b (Figure 3). Fish F2a2 is eluted slightly ahead of calf F2a2 and fish F2b is eluted somewhat later than calf F2b. This later observation is consistent with the previous reports based on sodium dodecyl sulfate electrophoresis, namely that fish F2b has a lower molecular weight than mammalian F2b. However, all vertebrate F2a2 histones appear to have the same molecular weight and we interpret the change in the elution profile of fish F2a2 as an indication that it has a slightly more extended overall structure than mammalian F2a2. This provides an attractive explanation for the slightly lower electrophoretic mobility of fish F2a2 relative to mammalian F2a2 in gel electrophoretic systems since other reports have indicated that the reduced mobility could not be explained on the basis of differences in charge density or molecular weight. The change in the elution profile of fish F2a2 and F2b as compared with that of calf thymus is predictable then, considering their corresponding change in electrophoretic mobility on gel analysis.

The elution profiles of calf and the invertebrate *Arbacia punctulata* are similar except for the striking absence of histone F2b in *Arbacia* sperm whole histone. In this particular preparation of total sperm histone, some nucleic acid contamination was co-isolated with the histone and consequently eluted from the column (Figure 3-If). That this fraction (f) is not a histone F2b has been deduced from amino acid analysis and inability of the fraction to migrate on our polyacrylamide gel system. The fact that there appears to be no histone F2b in *Arbacia* sperm is all the more surprising as clear evidence had been provided for the existence of histone F2b in somatic tissues of the sea urchin (Easton and Chalkley, 1972). Easton and Chalkley (1972) had reported the presence of a minor band tentatively identified as histone F2b in *Arbacia punctulata* sperm histone, and its absence was attributed initially to the reported low levels of this particular fraction in the total histone. However, we have found that if column purified *Arbacia* sperm histone F3 is allowed to remain in a solution of 0.01 N HCl or 0.9 N acetic acid for 2 days at 2°, a degradation product is formed whose electrophoretic mobility on polyacrylamide gel is identical with that previously described as *Arbacia* histone F2b (Figure 4). We therefore suspect that the *Arbacia* "histone F2b" is in fact a degradation product of histone F3.

Amino acid analysis of *Arbacia punctulata* sperm histone F3 shows that it has a characteristic arginine-rich composition very similar to that of histone F3 isolated from calf thymus and fish gonadal tissue (Table I). There are small differences in the amino acid composition of the F3 of sea urchin and calf as seen in slightly larger amounts of glycine, valine, and serine in *Arbacia*. But, in general, the amino acid analysis agrees with the evidence of Panyim and Chalkley (1971) that histone F3 from a variety of different sources is similar.

The elution profile of pea histone shows significant differences from that seen for the animal histones. The lysine-rich histone is eluted slightly ahead of its animal counterpart indicating an increased molecular weight in agreement with other workers (Spiker, 1971). The arginine-rich histone F3 is eluted at the same point in both pea and calf histone analysis and this is consistent with the increasing evidence in favor of an extreme conservation of both amino acid sequence and molecular weight in this histone fraction. Surprisingly F2a1

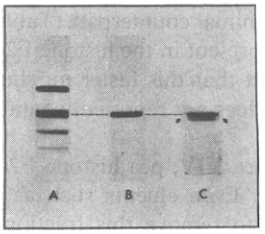

FIGURE 4: *Arbacia punctulata* sperm histone F3 and degradation product. Electrophoretic analysis conditions as described for Figure 2. (A) Total *Arbacia* histone or starting material; (B) column purified histone F3; (C) column purified histone F3 after 2 days n acid solution described in the text.

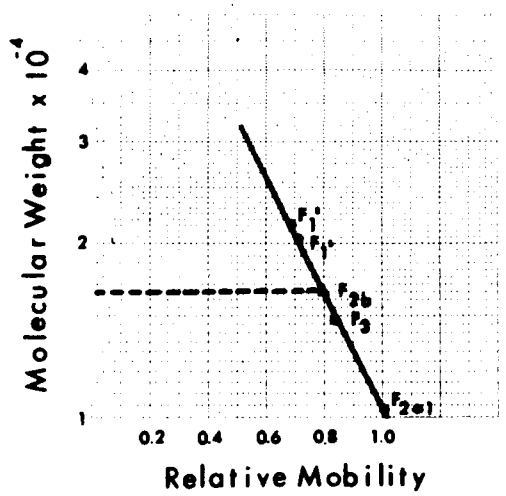

FIGURE 5: Determination of the molecular weight of pea histone F2b. Semilog plot of molecular weight against relative mobility (mobility of histone F2a1) used to determine the molecular weight of pea histone F2b according to the method of Panyim and Chalkley (1971).

was not eluted from the column and this is discussed in more detail below. The major differences between the pea and animal histone elution profile is seen for histones F2b and F2a2. So great is the difference that the order of elution is reversed. Pea F2b is eluted in advance of its position in all the animal systems we have studied, and F2a2 is retained on the column for a significantly longer period. The decreased retention time of F2b is most likely due to an increase in molecular weight which is approximately 10% higher in peas than in vertebrates. We calculate the molecular weight of pea F2b to be 16,500 (Figure 5) as opposed to the molecular weight of calf thymus F2b of 13,700. This is a rather surprisingly large difference of molecular weight especially for a histone fraction which has in general been thought of as conservative in its primary structure. The change in chemistry of this fraction is mirrored in its amino acid composition as seen in Table I. The lysine content of pea histone F2b is much higher than that of calf thymus F2b, which probably accounts for its increased solubility in 5% perchloric acid, which is of course one of the main reasons why the previous methods for fractionation proved unsatisfactory.

Pea histone F2a2 exists in two forms. It is interesting tht the smaller F2a2 species has an electrophoretic mobility similar to that of histone F3 (Figure 3-IV) and therefore was not previously seen in electrophoretic analyses. Quantitatively this band comprises 40% of the total histone F2a2. The slowest electrophoretic moving species has a molecular weight of 15,000 while the faster moving species has a molecular weight of 14,200. Amino acid analysis of the histone F2a2 fractions indicates that this fraction contains somewhat less arginine than its animal counterpart (Table I) (Phillips, 1971). Cysteine was not present in the histone F2a2 material. We are therefore confident that this faster moving band is a distinct F2a2 species and does not represent contamination by histone F3.

As seen in Figure 3-IV, pea histone F2a1 was never eluted from the column. Even eluents such as 4 M urea or 0.4 N sulfuric acid fail to remove this fraction from the column. This behavior was seen in repeated chromatographic analyses of whole pea histone which contained *bona fide* F2a1 as determined by electrophoretic studies. We suspect that this histone fraction may have aggregated with starch granules frequently co-isolated from pea chromatin preparations with the histone (S. Spiker, personal communications). Thus we have been able to purify four pea histone fractions, which was previously difficult, but we have run into technical difficulties in obtaining the fifth pea fraction.

Summary

We have developed a method using a single column and one eluent capable of separating vertebrate, invertebrate, and plant histones into their five component fractions. We have found our method to be applicable to previously unfractionable histones such as pea, *Arbacia punctulata* sperm, and fish gonadal tissue. Recovery of total histone at 90% of the theoretical yield can be achieved with 98% purity of each fraction (pea F2a1 excepted). We have used histone purified by this method for several types of studies in our laboratory including molecular weight determination and characterization of pea, histone F2b and F2a2, and *Arbacia punctulata* sperm histone F3 as well as comparative peptide mapping and sequencing (K. Sommer and R. Chalkley, in preparation; Hooper *et al.*, 1973).

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Immunospecificity of Nonhistone Proteins in Chromatin†

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ABSTRACT: A fraction of nonhistone proteins of chromatin (NP fraction) was found to contain protein species which can form immunochemically tissue-specific complexes with the DNA isolated from the same animal species (homologous DNA). When alone, complexed with heterologous DNA, or other polyanionic molecules, the NP fraction lost its tissue specificity detectable by the complement fixation method. The NP fraction from rat liver was found to contain proteins which were firmly retained on polyacrylamide–agarose columns containing rat-spleen DNA; there was no measurable retention of these proteins by calf-thymus DNA. The affinity of rat-liver NP proteins for homologous DNA was very strong. While all the rat-liver histones and other nonhistone proteins could be eluted from rat-spleen DNA columns with 0.2 M KCl, a concentration of 0.4 M KCl was necessary for the elution of NP proteins. When analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, the DNA-binding NP proteins eluted from rat-spleen DNA column with 0.4 M KCl consisted mainly of two major and one minor polypeptide bands with mobilities corresponding approximately to 12,000–15,000 daltons.

The biochemistry and morphology of a cellular phenotype are determined by the process of cytodifferentiation and generally do not change during the lifetime of differentiated cells. Parental phenotype is passed onto the new cells produced by each division and a major change in the differentiated cellular state occurs only rarely. Perhaps the best known example of self-perpetuating change in cellular differentiation is the neoplastic growth.

Experiments performed with isolated chromatin indicate that a definite restrictional pattern is imposed on the DNA genome during differentiation and that certain nonhistone proteins together with the histones determine the tissue specificity of DNA transcription in chromatin (Paul and Gilmour, 1968; Gilmour and Paul, 1969, 1970; Spelsberg and Hnilica, 1970; Spelsberg *et al.*, 1971; Bekhor *et al.*, 1969; Smith *et al.*, 1969; Kamiyama and Wang, 1971). It was shown by Henning *et al.* (1962) and more recently by Chytil and Spelsberg (1971) that a fraction of chromatin nonhistone proteins can elicit the formation of tissue-specific antibodies when injected together with DNA into the rabbits. A preliminary report from this laboratory (Wakabayashi and Hnilica, 1973) showed that the tissue-specific antibodies are formed against the complexes of chromosomal nonhistone proteins with homologous DNA. Here we report that a nonhistone protein fraction of a limited heterogeneity interacts with the DNA in chromatin in a highly specific manner.

Materials and Methods

Unless specific, all preparative work was performed at 2–4°.

Preparation of Chromatin and Nonhistone Protein–DNA Complexes. Male albino rats (Sprague-Dawley) fed *ad libitum* were sacrificed by cervical dislocation and their livers were excised and placed immediately into an ice-cold solution of 0.25 M sucrose. The rat-liver nuclei were solated according to the method of Blobel and Potter (1966).

Thymus glands of young calves were obtained from a local slaughter house. The excised glands were placed into crushed ice, transported to the laboratory, and processed as was described for the livers.

Transplantable rat neoplasms (Walker carcinosarcoma, Novikoff hepatoma, rat 30D hepatoma) were maintained by weekly transplantations. The cells of both hepatomas (ascites) were collected by centrifugation and washed 2–3 times with several volumes of isotonic sucrose solution and centrifuged. This treatment removed most of the contaminating erythrocytes. The method described by Wilhelm *et al.* (1972) was used to prepare hepatoma nuclei; Walker tumor nuclei were obtained by the procedure of Busch *et al.* (1959). All nuclear preparations received a final wash with 0.25 M sucrose–5.0 mM $MgCl_2$ in 10 mM Tris-HCl buffer (pH 7.5). Chromatin was prepared from the isolated nuclei by the method of Spelsberg and Hnilica (1971a) as modified by Wilhelm *et al.* (1972). After washing with 0.3 M NaCl, chromatin preparations were dissociated in buffered 2.5 M NaCl–5.0 M urea solutions.

Bekhor *et al.* (1969) as well as Gilmour and Paul (1969) have shown that essentially all the chromatin proteins can be separated from DNA by dissociation in 2.0 M NaCl–5.0 M urea–10 mM Tris-HCl buffer (pH 8.3) and prolonged ultracentrifugation. If this procedure is performed at pH 6.0, all the histones and about 20–30% of the chromatin nonhistone proteins remain in the supernatant while the DNA and associated nonhistone proteins form a gelatinous pellet (Spelsberg *et al.*, 1971).

In our experiments, the viscous solution containing about 0.2–0.3 mg/ml of chromatin DNA in 2.5 M NaCl–5.0 M urea–

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