STUDIES ON PLANT AND ANIMAL HISTONES

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to

Mom and Boo

Acknowledgments

To the many people who have been counsellors and generous companions in our quest for knowledge and in the living of our lives,
And to the many others who appear in the jumble of exciting and happy incidents which crowd my memory,
And to the often unrecognized who keep quiet order in our microworld
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Abstract

The histones of the pea plant, Pisum sativum L., and of calf thymus have been fractionated and further characterized in order to determine the extent of heterogeneity and the main chemical features of these basic nuclear proteins. Histones were fractionated by chromatography on Amberlite CG-50 and by preparative disc electrophoresis. The resulting highly purified histone fractions were futher characterized by analytical disc electrophoresis, amino acid analysis, N-terminal and C-terminal analyses, and the preparation of tryptic peptide maps. Calf thymus histones Ia, Ib, IIb1, III and IV (f1, f1, f2a2, f3, and f2a1 in the nomenclature of Johns, Phillips and Butler) and pea bud histones Ib, IIb, III and IV were obtained as electrophoretically pure components and each appears to be a single molecular species on the basis of N-terminal and C-terminal analysis and the number of tryptic peptides. The total number of major histones in calf thymus appears to be six, in pea bud, eight. The apparent heterogeneity of calf thymus histones demonstrated by disc electrophoresis is largely due to the formation of histone III complexes by disulfide bridges between histone III monomers. While calf thymus histone III contains two cysteines per molecule pea bud histone III contains but one and thus can form only dimers.

For each calf thymus histone there appears to be an homologous pea bud histone. It is proposed that the homologous pea and calf histones are related by evolution and perform identical biological functions. This hypothesis is based upon remarkable similarities in chromatographic

and electrophoretic behavior, amino acid compositions, terminal amino acids, and in some cases even peptide maps of corresponding pea and calf histones. Peptide maps of the arginine-rich histone III contain 29 soluble peptides of which 26 are common to calf and pea; maps of histone IV contain 32 peptides of which 27 are common to calf and pea.

By chromatography and electrophoresis the histones of various pea tissues are qualitatively identical to those of pea bud. There are, however, quantitative differences and these have been accurately measured by a method of quantitative analytical disc electrophoresis. Young pea cotyledons contain only about a third as much lysine-rich histone as do mature cotyledons. Exploratory experiments on the synthesis of histone in pea cotyledons as a function of development and in relation to other macromolecular parameters are described in an appendix.

The dissociation of histones from pea bud nucleohistone by NaCl was studied, employing quantitative disc electrophoresis. Histone I (lysine-rich) is selectively dissociated by 0.5 M NaCl and the remaining histones are non-selectively dissociated primarily over the range 0.5 - 1.5 M NaCl. These data are compared with data for the dissociation of calf thymus histones from nucleohistone by NaCl and the general similarities are noted.

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General Introduction

Histones are basic proteins which are natively complexed with DNA in the chromosomes of higher organisms. This definition is intentionally vague, for it is not known whether histones are natively complexed with the DNA of cytoplasmic organelles such as mitochondria or only with nuclear DNA, and although it is clear that histones are not found in bacteria (Zubay and Watson, 1959; Butler, 1964; Raaf, 1966), it is not known whether the protozoa and all the metazoa contain histones or only those organisms which are quite phylogenetically advanced. The definition does, however, embody a very important criterion: histones are native components of the genetic apparatus. Direct evidence for the native association of histones and DNA comes from cytological investigations (Alfert and Geschwind, 1953; Alfert, 1957; Horn and Ward, 1957; Bloch and Hew, 1960; Swift, 1964; Holtzman, 1965).

In practice it is often difficult to establish beyond reasonable doubt that a protein isolated as protein-DNA complex is actually associated with DNA in the living cell. This difficulty has led to the use of such terms as "major histones" and "minor histones" to distinguish between those basic proteins present in rather large quantities even in highly purified deoxyribonucleoprotein (DNP) and those basic proteins present in only trace amounts in DNP. The former are certainly native components of DNP; the latter are more likely artifacts of preparation. While these minor proteins will receive mention from time to time, this thesis is primarily concerned with the major basic proteins, henceforth referred to simply as histones.

Although the histones were discovered around 1880, little was known about their characteristics before Stedman and Stedman (1950, 1951) proposed on the basis of very tenuous evidence that histones might function as the regulators of genes. Their theory of the chemical nature of genes was incorrect, but this proposal together with exciting new insights into the nature of genes and the mechanisms of gene duplication and gene usage (DNA synthesis, RNA synthesis and protein synthesis) provided impetus to the study of the histones.

The biological functions of the histones are still not well understood. A large body of data has been amassed to support the hypothesis that histones are directly involved in repression of genetic information (Huang and Bonner, 1962; Bonner and Huang, 1963; Bonner et al., 1963; Allfrey et al., 1963; Barr and Butler, 1963; Hindley, 1963; Hnilica and Billen, 1964; Johns and Butler, 1964; Huang et al., 1964; Littau et al., 1965; Marushige and Bonner, 1966; Holoubek, 1966). It is also apparent that at least some components of histone confer special structural properties on native nucleohistone (Zubay and Doty, 1959; Wilkins et al., 1959; Peacocke and Preston, 1962; Giannoni and Peacocke, 1963; Ohba, 1966; Tuan, 1967; also MHF Wilkins, S. Pardon and B. Richards, unpublished observations). The compromise view has also been proposed: that control of gene expression is effected via regulation of nucleohistone structure by histones. However, there is strong evidence that histones are not directly involved in determining the structural differences between template active euchromatin and template inactive heterochromatin (Frenster, 1965; Grogan et al., 1966; Maio and Schildkraut, 1967; Comings, 1967).

It remains quite possible that some histone components may be gene regulators while others are purely structural elements of chromatin.

The most central problem of histone chemistry concerns histone heterogeneity. For many years after their discovery (Kossel, 1884) the histones were treated as a single substance (see Greenstein, 1944). Stedman and Stedman reported a crude fractionation of histones in 1950. Four years later the first clear-cut fractionation of histones into lysine-rich and arginine-rich was accomplished (Daly and Mirsky, 1954). Subsequently a wide variety of techniques were developed for the analytical and preparative fractionation of histones. These included methods of fractional precipitation, velocity sedimentation, moving boundary electrophoresis, zone electrophoresis, selective extraction from DNA, ion exchange chromatography, and exclusion chromatography (see Phillips, 1961, for a complete review). The great spectrum of amino acid compositions, terminal amino acids and molecular weights reported for the fractions produced by these methods forms a body of data interpreted by many as being indicative of tremendous molecular heterogeneity (Murray, 1964). The histones appeared to constitute a nearly continuous spectrum of molecular species, having lysine/arginine ratios ranging from infinity for the α1 fraction of Cruft et al.(1957) to zero for most of the protamines (Felix, 1960).

Improved methods of preparation, fractionation and analysis have during the past few years shown that much of the supposed heterogeneity of histones can be attributed to artifacts such as proteolysis (Johns, 1964), aggregation (Busch et al., 1962; Hnilica and Bess, 1965), and

cross-contamination of fractions (Power and Butler, 1965). Comparative studies of the histones of various organs of a given species and comparative studies of the histones from different species (Crampton et al., 1957; Hnilica et al., 1962; Neidle and Waelsch, 1964; Laurence et al., 1963, 1966; Palau and Butler, 1966; Hnilica, 1966; Fambrough and Bonner, 1966) demonstrate a lack of tissue or even species specificity of histones, findings more compatible with limited than with extensive histone heterogeneity.

These comparative studies deal more directly with a second major problem in histone chemistry -- the problem of determining which chemical and physical characteristics of histones are essential to histone function. At the molecular level the whole multiplicity of modern-day organisms is believed to be the result of countless small and seemingly random alterations in the information content of genes (via processes such as crossing-over, base changes, and the addition and deletion of bases) coupled with natural selection for the resultant phenotypes best suited to various ecological niches. Those alterations which adversely affect information vital to the survival of the organism express themselves generally as "lethal mutations" and are weeded out of the gene pool by death of the organism. By this mechanism, characteristics essential to survival are preserved relatively unchanged through great time-spans of evolution (see Handler, 1964). Through chemical studies of the histones of organisms of various phylogenetic groups identification of the characteristics common to the histones of phylogenetically remote organisms can be made. The data in this thesis

demonstrate a high degree of similarity in chemical characteristics between the histones of calf thymus and pea bud. These common characteristics probably include the most essential properties of histones. Such chemical studies also provide a description of histones from which a more refined definition of histones can be drawn.

The research reported in this thesis began as an attempt to characterize the histones of the pea plant Pisum sativum L. var Alaska. The availibility of methods for the preparation of highly purified pea plant deoxyribonucleoprotein (Huang and Bonner, 1962), the very remote phylogenetic relationship of the pea plant to all other organisms whose histones have been studied (all of the latter are vertebrates), and the use of the pea plant in studies of the functions of histones (reviewed in Bonner et al., 1967) all have made the characterization of pea histones a feasible and timely enterprise. During the course of this research a number of methods were applied which had not previously been employed in histone research. In order to make meaningful comparison of the pea histones with those of vertebrates, it was necessary to simultaneously study the histones of some vertebrate. Calf thymus histone was the material of choice, since the histones of calf thymus have been more thoroughly studied than histones from any other source.

This parallel study of pea and calf histones has been directed toward solutions to the two principal problems discussed above: how heterogeneous are the histones? and what chemical and physical characteristics are common to the histones of pea and calf? Partial answers to these two questions are provided in this thesis and form a basis for a much clearer conception of the nature of histones in general.

References

Alfert, M. (1957) in The Chemical Basis of Heredity (W.D. McElroy and B. Glass, eds.) Johns Hopkins Press, Baltimore, p. 186.

Alfert, M. and Geschwind, I.I. (1953), Proc. Nat. Acad. Sci. (Wash.) 39, 991.

Allfrey, V.G., Littau, V.C., and Mirsky, A.E. (1963), Proc. Nat. Acad. Sci. (Wash.) 50, 1026.

Barr, G.C. and Butler, J.A.V. (1963), Nature 199, 1170.

Bloch, D.P. and Hew, H.Y.C. (1960), J.Biophys. Biochem. Cytol. 8, 69.

Bonner, J., Dahmus, M., Fambrough, D., Huang, R.C., Marushige, K., and Tuan, D. (1967), Science,in press.

Bonner, J. and Huang, R.C. (1963), J. Mol. Biol. 6, 169.

Bonner, J., Huang, R.C., and Gilden, R.V. (1963). Proc. Nat. Acad. Sci. (Wash.) 50, 893.

Busch, H., Hnilica, L.S., Chien, S., Davis, J.R., and Taylor, C.W. (1962), Cancer Res. 22, 637.

Butler, J.A.V. (1964), in The Nucleohistones (J. Bonner and P.O.P. Ts'o eds.) Holden-Day, San Francisco, p 36.

Comings, D. (1967) J. Cell Biol., in press.

Crampton, C.F., Stein, W.H., and Moore, S. (1957), J. Biol. Chem. 225, 363.

Cruft, H.J., Hindley, J., Mauritzen, C.M. and Stedman, E. (1957), Nature 180, 1107.

Daly, M.M. and Mirsky, A.E. (1954-1955), J. Gen. Physiol. 38, 405.

Fambrough, D., and Bonner, J. (1966), Biochemistry 5, 2563.

Felix, K. (1960), Advan. Protein Chem. 15, 1.

Frenster, J.H. (1965), Nature 206, 680.

Giannoni, G. and Peacocke, A.R. (1963), Biochim. Biophys. Acta 68, 157.

Greenstein, J.P. (1944), Advan. Protein Chem. 1, 209.

Grogan, D.E., Desjardins, R., and Busch, H. (1966), Cancer Res. 26, 775.

Handler, P. (1964), Fed. Proc. 23, 1229.

Hindley, J. (1963), Biochem. Biophys. Res. Comm. 12, 175.

Hnilica, L.S. (1966), Biochim. Biophys. Acta 117, 163.

Hnilica, L.S. and Bess, L.G. (1965), Anal. Biochem. 12, 421.

Hnilica, L.S. and Billen, D. (1964), Biochim. Biophys. Acta 91, 271.

Hnilica, L.S., Johns, E.W., and Butler, J.A.V. (1962), Biochem. J. 82, 123.

Holoubek, V. (1966), J. Cell. Biol. 31, 49A.

Holtzman, E. (1965), J. Histochem. Cytochem. 13, 318.

Horn, E.C. and Ward, C.L. (1957), Proc. Nat. Acad. Sci. (Wash) 43, 776.

Huang, R.C. and Bonner, J. (1962), Proc. Nat. Acad. Sci. (Wash.) 48, 1216.

Huang, R.C., and Bonner, J. and Murray, K (1964), J. Mol. Biol. 8, 54.

Johns, E.W. (1964), Biochem. J. 93, 161.

Johns, E.W. and Butler, J.A.V. (1964), Nature 204, 853.

Kossel, A. (1884), Z. Physiol. Chem. 8, 511.

Laurence, D.J.R., Phillips, D.M.P. and Butler, J.A.V. (1966), Arch. Biophys. Biochem. 113, 338.

Laurence, D.J.R., Simson, P., and Butler, J.A.V. (1963) Biochem. J. 87, 200.

Littau, V.C., Burdick, C.J., Allfrey, V.G., and Mirsky, A.E. (1965). Proc. Nat. Acad. Sci. (Wash.) 54, 1204.

Maio, J.J. and Schildkraut, C.L. (1967), J. Mol. Biol. 24, 29.

Marushige, K. and Bonner, J. (1966), J. Mol. Biol. 15, 160.

Murray, K. (1964), in The Nucleohistones (J. Bonner, and P.O.P. Ts'o, eds.) Holden-Day, San Francisco. p. 15.

Neidle, A., and Waelsch, H. (1964), Science 145, 1059.

Ohba, Y. (1966), Biochim. Biophys. Acta 123, 76.

Palau, J., and Butler, J.A.V. (1966), Biochem. J. 100, 779.

Peacocke, A.R. and Preston, B.N. (1961), Nature 192, 228.

Phillips, D.M.P. (1961), Progr. Biophys. Biophys. Chem. 12, 211.

Power, D.F. and Butler, J.A.V. (1965) Biochem. J. 97, 32p.

Raaf, J. (1966), D. Phil., Oxford University, Oxford.

Stedman, E. and Stedman, E. (1950) Nature 166, 780.

Stedman, E. and Stedman, E. (1951), Phil. Trans. Roy. Soc (London) Ser. B., 235, 565.

Swift, H. (1964), in The Nucleohistones (J. Bonner and P.O.P. Ts'o, eds.) Holden Day, San Francisco. p 169.

Tuan, D. (1967), Ph.D. Thesis, California Institute of Technology.

Wilkins, M.H.F., Zubay, G., and Wilson, H.R. (1959), J. Mol. Biol. 1, 179.

Zubay, G. and Doty, P. (1959), J. Mol. Biol. 1, 1.

Zubay, G. and Watson, M.R. (1959), J. Biophys. Biochem. Cytol. 5, 51.

CHAPTER I

Introduction

At the present time there is great interest in the role of histones in the structure of chromatin and in the control of gene expression in higher organisms. Knowledge of the nature of histones is essential to a clear understanding of the mechanisms of histone function. Toward providing such knowledge, many investigators are involved in the characterization of vertebrate histones (see Phillips, 1961; Murray, 1965). Surprisingly, the histones of invertebrates and of plants have been virtually neglected. The present study, previously reported in part (Fambrough and Bonner, 1966), provides sufficient comparative data on the histones of the phylogenetically remote organisms, calf and pea plant, to allow some conclusions to be drawn concerning the origin, evolution and general nature of histones.

Methods

Preparation of chromatin

For the preparation of histones minimally contaminated by non-chromosomal protein it is necessary to use purified chromatin as the starting material for histone extraction. For the preparation of pea bud chromatin (Bonner, et al., 1967) approximately 5 kg of pea seeds were soaked overnight in water, planted in vermiculite, and germinated in the dark for 4 days at 25°C. The apical buds (approximately one cm of stem plus bud) were then harvested to yield about 600 g fresh weight of buds. The buds were homogenized with approximately one liter of grinding medium (0.25 M sucrose, 0.05 M tris buffer pH 8.0, 0.001 M $MgCl_2$) for 1.5 minutes at 100 volts in a Waring blendor. This and all subsequent steps were performed at 0-5°C. The homogenate was filtered through four layers of cheesecloth and then through two layers of Miracloth (Chicopee Mfg. Co., Miltown, N.J.). The filtrate was next centrifuged at 4000 x g for 30 minutes. The soft pellets were scraped from the underlying layers of starch, suspended in 300 ml of grinding medium, and centrifuged at 10,000 x g for 20 minutes. The pellets were again separated from the starch, suspended in 300 ml. of 0.05 M (or 0.01M) tris buffer (pH 8.0), and centrifuged at 10,000 x g for 20 minutes. This step was twice repeated. The resulting pellets of crude

chromatin were suspended in a total of 30 ml of 0.01 M tris buffer (pH 8.0), homogenized with a Potter-Elvehjem homogenizer (about 20 strokes) and layered in 5 ml portions on 25 ml aliquots of 1.7 M sucrose in cellulose nitrate tubes. The upper two-thirds of the contents of each tube were stirred to form a rough gradient. The tubes were then centrifuged at 22,000 rpm for 3 hours in the SW-25 Spinco rotor. The resulting gelatinous pellets (purified chromatin) were suspended in 0.01 M tris buffer and dialyzed against 100 volumes of the same buffer overnight. Recovery of DNA from the tissue homogenate was 70 to 80%.

For the preparation of calf thymus chromatin the method of Marushige and Bonner (1966) was used without modification. Except for the use of saline EDTA (0.075 M NaCl, 0.024 M sodium ethylene-diamine-tetraacetate, pH 8.0) as grinding medium, this method is very similar to that described above.

Preparation of histone

For extraction of histones the dialyzed chromatin was diluted to a concentration of less than 400 μg DNA/ml with cold 0.01 M tris buffer. This suspension was stirred on ice and one-fourth volume of cold 1 N sulfuric acid slowly added. After 30 minutes of stirring, the suspension was centrifuged at 17,000 x g for 20 minutes. The sediment was broken up and extracted with 0.4 N sulfuric acid (half the final volume of the first extract). To the combined supernatants four volumes of cold absolute ethanol were added and the histone sulfate precipitated at -20°C for 36 hours. The histone sulfate was recovered by centrifugation, washed

three times with ethanol, and dried in a vacuum desiccator.

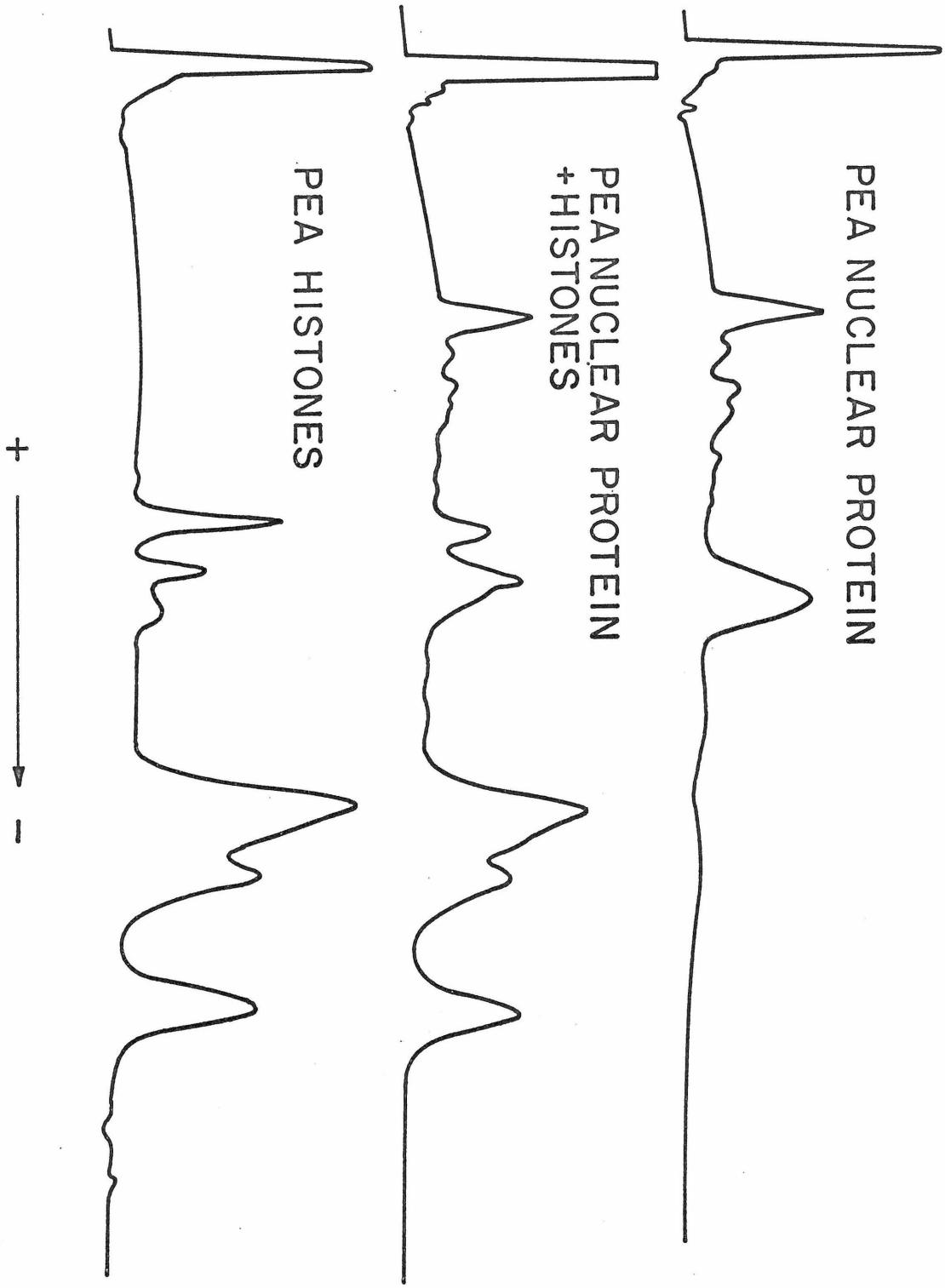
It is necessary to use very pure nucleoprotein to obtain suitable pea histone sulfate preparations. When crude nucleoprotein or whole nuclei are extracted with acid, histone may constitute less than 10% of the acid-soluble protein. Contamination, apparently largely by ribosomal proteins, is extensive. Crude histone preparations made from such crude chromatin are wholly soluble only in concentrated urea, and as little as 20% may be again soluble in acid. Densitometric tracings of the electrophoretic fractionation of such a crude nuclear extract, of pure histone, and of a mixture of the two are presented in Figure 1.

Extraction of histones with HCl is less satisfactory for preparative purposes. Histone chlorides are not totally precipitable from acidic ethanol solution, are more hygroscopic, and are more difficult to redissolve than are histone sulfates.

Preparation of histones by the method of Johns

Alternative to the extraction of whole histone from chromatin, a crude fractionation of histones can be effected by the selective extraction of histones based upon dissociation of histone from DNA by acids and acidified ethanol and upon the different solubilities of the several histone salts in ethanol and acetone (Johns, 1964). This method of preparation was used for the large scale preparation of histone fractions from calf thymus nucleoprotein and with partial success for the preparation of crude pea bud histone fractions.

Figure 1. Densitometric tracings of the electrophoretic patterns of the acid soluble proteins from a crude preparation of pea cotyledon nuclei and from pea nucleohistone and from a mixture of the two. The crude nuclear preparation was a 500 x g pellet from a strained homogenate of pea cotyledons in grinding medium. The acid soluble protein extracted from this preparation amounted to more than 1 mg protein per gram pea seeds or about 100 x the content of histones. Note that most of the non-histone proteins are of lower mobility than characteristic of histones. Minute amounts of such electrophoretically slow components can often be seen in the electrophoretic pattern of pea histones but usually in quantities too slight to be detected by densitometry (but see Figure 7 of Chapter II).


PEA NUCLEAR PROTEIN
PEA NUCLEAR PROTEIN
+HISTONES
PEA HISTONES
+
—

Purified chromatin at a concentration of less than 5 mg DNA/ml in 5% perchloric acid (PCA) was homogenized at full speed in a Waring Blendor for 2 minutes (4°C). The homogenate was centrifuged for 20 minutes at 17,000 x g and the pellets extracted twice more in the same way, but each time with half the initial volume of 5% PCA. The combined supernatants were clarified by filtration through a sintered glass funnel or through Miracloth, and trichloroacetic acid (TCA) was added to a final concentration of 1.1M. The precipitate of lysine rich histone (fraction f1) was allowed to form overnight at 4°C and then was collected by centrifugation, washed with acidified acetone (200 ml acetone: 0.1 ml conc. HCl) and three times with acetone, and then dried in vacuo.

The pellet from the 5% PCA extraction of the lysine-rich histone was suspended in ethanol (less than 2.5 mg DNA/ml) and allowed to stand overnight at 4°C. The suspension was then centrifuged and the resulting pellet extracted twice with 80% ethanol (less than 2.5 mg DNA/ml). The combined ethanolic supernatants were clarified by centrifugation and the histone quantitatively precipitated by the addition of five volumes of acetone and .005 volumes concentrated HCl. After standing in the cold overnight this precipitate was collected by centrifugation and washed once with acetone. The histone was then dissolved in distilled water, and ethanol and conc. HCl were added to make a final solution 80% ethanol, 0.25N HCl at less than 1mg/ml histone. This solution was dialyzed twice against an equal volume of absolute ethanol, and the resulting protein precipitate (fraction f3) was removed by centrifugation,

washed twice with acetone, and dried *in vacuo*. The soluble histone was precipitated from the fraction f3 supernatant by the addition of three volumes of acetone, collected by centrifugation, washed twice with acetone and dried *in vacuo* (fraction f2a).

The insoluble material which remained after extraction into ethanol of histone fractions f3 and f2a was next extracted with 0.25 N HCl to remove the remaining histone (fraction f2b) by homogenization of the material (5 mg DNA/ml solution) in a blendor. The homogenate was centrifuged and the pellet extracted with 0.25 N HCl twice more. The combined supernatants were clarified by filtration, and the histone fraction f2b was precipitated overnight by the addition of five volumes of acetone, washed three times with acetone, and then dried *in vacuo*.

Column Chromatography

For the separation of the major classes of histones, they were applied to and eluted from Amberlite CG-50, using a gradient of Guanidinium chloride buffered at pH 6.8 (Luck *et al.*, 1958; Satake *et al.*, 1960; Rasmussen et al, 1962). Chromatographic procedures are described below.

Preparation of the Resin and of Guanidinium Chloride

A thin slurry of Amberlite CG-50 (200-400 mesh, chromatographic grade, Mallinckrodt Chemical Works) was made in water. The heavier material was allowed to settle for several minutes and the suspension of finer particles then decanted. The defined Amberlite was next successively suspended with stirring in each of the following solutions,

followed in each case by filtration (Buchner funnel on suction line, using two sheets filter paper): 2 N HCl; distilled water; 2 N NaOH (the filtrate is very yellow and turbid if new Amberlite is used); distilled water; 2 N HCl; distilled water; 2 N NaCl; 2 N NaCl (the resin was then titrated to pH 7 with NaOH); 8% guanidinium chloride (GuCl) buffered with 0.1 M sodium phosphate at pH 6.8 (GuCl-PO_4). Finally the resin was suspended in 8% GuCl-PO_4 and the slurry used to pack the columns. The resin may be stored in this form.

Practical grade GuCl (Eastman Organic Chemicals) was purified by filtration of two liters of 60-80% solution through an 8 x 30 cm activated charcoal column (Celite (#545 Johns-Minville) and activated charcoal (#655 Matheson Chem. Co.) 2:1 w/w). The concentration of GuCl in the purified solution was determined from its refractive index, using the relation:

$$\frac{n^{25^\circ}\ \text{GuCl} - n^{25^\circ}\ H_2O}{\text{weight \% of GuCl in } H_2O} = .00166$$

which holds for solutions up to more than 60%.

Operation of a 2.5 x 60 cm column

Approximately 50 mg of histone were powdered with a glass rod and dissolved in 2.0 ml of 8% GuCl-PO_4 at room temperature. Pure histone samples were totally soluble. If a histone solution was turbid, the insoluble impurities were removed by centrifugation at 16,000 x g

for 20 minutes. The clear supernatant was allowed to flow into the column and then washed in with three 1 ml portions of 8% GuCl-PO_4. Ten ml of 8% GuCl-PO_4 were then layered on the resin and continuous flow initiated.

A linear gradient from 8-13% GuCl-PO_4 was used to elute the lysine-rich and slightly-lysine-rich histone fractions in a total 700 ml of solution. The column was then flushed with 100 ml of 40% GuCl-PO_4 followed by 150 ml 8% GuCl-PO_4. Flow of solution through the column was then discontinued, and the column was ready for reuse.

A flow rate of 30-40 ml per hour was found to be most satisfactory. The first 75 ml of effluent were collected in a graduated cylinder. During this time adjustment of the flow rate was completed. Four ml fractions were then collected until termination of the run (~200 fractions).

For the determination of chromatographic profiles on an analytical scale, a scaled-down fractionation procedure was developed (column 0.6 x 55 cm). A micro-column 0.2 x 20 cm, taking a load of about 0.5 mg and using a 20 ml salt gradient was also developed. In these cases the entire fractions were used for protein assay, but after such assay the precipitated protein can be recovered by centrifugation and used, for example, for electrophoretic analysis.

Protein assay

Guanidinium chloride interferes with colorimetric protein assays and with OD_{230} measurements. Because histones have a low and variable content of aromatic amino acids, OD_{280} is also a poor measure for them. Measurement of turbidity after TCA precipitation is, however, suitable.

Two-tenths ml aliquots from every second or every third fraction were transferred by syringe into small test tubes and diluted with 0.6 ml distilled water. Four-tenths ml of 3.3 M TCA were then added by syringe to groups of 12 tubes and the tubes shaken vigorously for a few seconds. After thirteen minutes, measurement of OD at 400 mμ was begun. Development of turbidity is virtually complete after 13 minutes and OD_{400} remains constant within 2% for the next 5 minutes.

The measured turbidity at the plateau level is a linear function of protein concentration over practically the entire range of experimental determinations (up to OD 400 mμ 0.5 or perhaps greater). Turbidity measurements are independent of the fraction of histone being assayed. Ten ug/ml of any histone in the final TCA containing solution yields an OD 400 mμ of 0.083.

Dilution of the samples with water is necessary since GuCl is only sparingly soluble in 1.1 M TCA. Even with dilution, the GuCl concentration in the final fractions is so high that voluminous precipitation of GuCl crystals occurs when the samples are vigorously shaken. Since the crystals are large, solution free of them can be pipetted off.

Recovery of Histones

When the histone sample introduced into the column is completely dissolved, all of the protein is recovered in the effluent. After analysis of column fractions, which for a 2.5 x 60 cm column consumes about 3% of the histone, the remaining material from each histone peak

was pooled in dialysis tubing (previously boiled in EDTA, (2.5×10^{-2}M) and washed exhaustively with water and ethanol) and dialyzed against 20 volumes of distilled water or 0.1 M acetic acid, with a change of dialysis medium every 4 hours for six changes. The volume of each dialysate was then reduced to about 6-7 ml by flash evaporation at room temperature. These concentrated solutions were dialyzed against 200 volumes of 0.1 M acetic acid for several changes, lyophilized, and stored in airtight vials. Recovery for the entire procedure is about 70%. The 0.1 M acetic acid is used to prevent precipitation of the arginine-rich histones during dialysis. It does not interfere with lyophilization and does not affect the protein in any detected way.

Electrophoresis

Disc electrophoresis was performed, using a modification (Bonner et al., 1967) of the method of Reisfeld et al. (1962). A pH 4.3 gel which was 15% in acrylamide and 6 M in urea was prepared by mixing 1 volume Temed solution (48 ml N KOH, 17.2 ml glacial acetic acid, 4 ml (NNN'N')-tetramethylethylenediamine, deionized water to 100 ml), two volumes of acrylamide solution (60 g acrylamide, 0.4 g NN'methylene bis acrylamide, deionized water to 100 ml), and five volumes of 0.2% (w/v) ammonium persulfate in freshly deionized 10 M aqueous urea solution. For 7.5% gels the acrylamide solution contains 30 g acrylamide, 0.8 g NN'methylene bis acrylamide, deionized water to 100 ml.

Aliquots (0.9 ml) were pipetted into 6.5 cm lengths of 5 mm ID glass tubing and overlaid with 0.1 ml of 3 M urea for anaerobic poly-

merization. After polymerization, this layer was removed. Each histone sample was dissolved at a concentration of 1 mg/ml in 10 M urea and 1-20 µl applied to a gel. The sample solutions were overlaid with tray buffer (31.2 g β-alanine, 8 ml acetic acid, water to 1 liter), and electrophoresis was performed in a standard disc electrophoresis apparatus at constant current of 4 milliamperes per tube for 1.5 hours. Currents of greater than 5 milliamperes per gel produce excessive ohmic heating within the gel, which in turn causes curved bands. Lower currents require longer runs to give acceptable band resolution. Diffusion limits resolution if electrophoresis times are greater than 2-3 hours.

Gels were stained for at least 4 hours in 1% amidoschwarz 10b, 50% ethanol, 7% acetic acid aqueous solution. The gels were then destained by electrophoresis and stored in 40% ethanol containing 7% acetic acid. The ethanol in the staining and storage solutions prevents swelling of the gels without affecting staining and fixation of the proteins. The electrophoretic destaining was performed at less than 2 ma per gel and a trace of stain was added to the destaining solution, both procedures to prevent discoloration of the protein bands.

Any background color remaining in the gels after destaining was removed by dialysis against destaining solution. The completely destained gels were scanned using a Canalco Model E microdensitometer (Canal Industrial Corp., Rockville, Md.) and stored in destaining solution in screw-top glass vials. No appreciable alteration in the optical density of the stained bands was noticed during two months of storage.

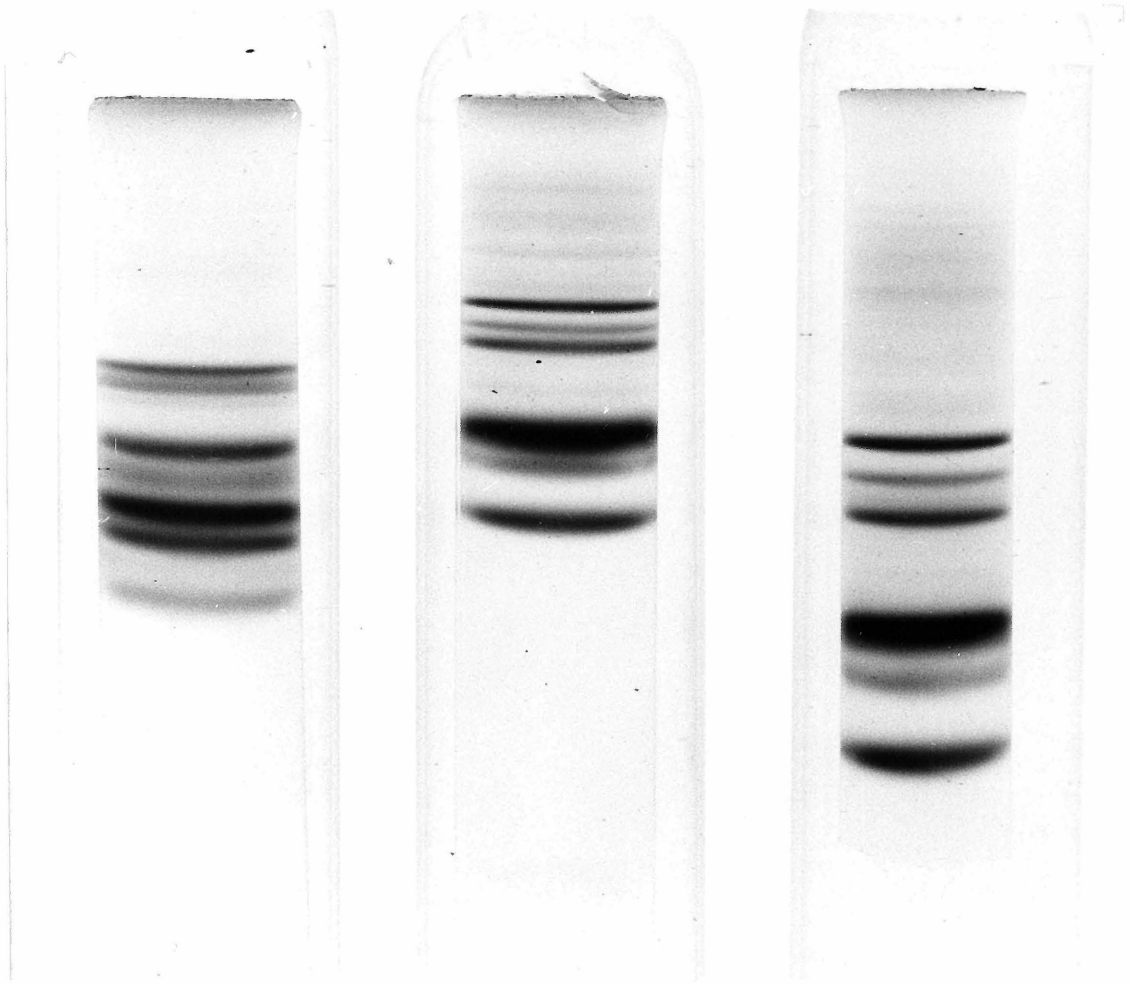
Fifteen % acrylamide gels containing 6 M urea are most useful for the disc electrophoresis of histones. The procedure for non-urea gels is identical to that for urea gels except that the persulfate solution is made in aqueous solution and gelpolymerization is carried out for 2 hours under a layer of water rather than under 3M urea. Histone mobilities are similar in urea and urea-free gels. Urea gels, however, have the advantages of shorter polymerization time and sharper band resolution. There is no difference in histone electrophoretic pattern in 15% and 7.5% acrylamide gels (see Fig. 2). Seven and one-half % gels yield greater band separation, but also more band diffusion. Furthermore, the lower concentration of acrylamide produces more fragile gels which undergo noticeable shrinkage during storage.

There is reasonable latitude in the solvent used for the histone sample . Solvents of high ionic strength such as 8% GuCl are unsuitable because they greatly decrease histone mobilities and interfere with stacking. Solvents containing 0.2 N HCl, 0.2 N H_2SO_4, or 10 M urea have been used successfully.

The ideal amount of whole pea histone for fractionation by disc electrophoresis is 15-20 μg. Overloading occurs with over 50 μg of whole histone and non-linear staining with over 20 μg (see Chapter II). The volume of sample solution placed on the gel is not critical when urea solution is used. As little as 1 μl and as much as 0.1 ml were used without affect on band resolution.

All electrophoretic data are presented in the form of densitometric tracings of the stained polyacrylamide gels. As discussed in Chapter II,

Figure 2. Fractionation of pea bud histones by disc electrophoresis in polyacrylamide gels. Electrophoretic migration was from top to bottom; gels were stained with amidoschwarz. Three different gel conditions are illustrated. From left to right they are: 15% gel, 15% gel containing 6 M urea, and 7.5% gel containing 6 M urea.



the areas under such tracings are directly proportional to protein concentration. These tracings show, however, somewhat less resolution of histone components than the original gels. An appreciation of the difference in resolution can be obtained by studying Figure 3.

In some experiments radioactively labelled histones were fractionated by disc electrophoresis. To measure radioactivity in the fractions, the stained bands were cut from the gels and each band was dissolved by incubation at 70°C in 0.5 ml of 30% hydrogen peroxide. Then 10 ml of scintillation counting fluid[1] were added to each solution and the solutions were counted in a Beckman scintillation counter. The counting efficiency in such solution was found to be about 45% (^{14}C).

Preparative Disc Electrophoresis

For the preparation of electrophoretically pure histone fractions a Canalco Prep-Disc Apparatus was used. By systematic variation of parameters, conditions were established for the maximal resolution of histone components in minimal time. It is not feasible to prepare pure histone fractions from whole histone, so histone fractions prepared by column chromatography were used as starting materials for prepar-

[1]The composition of this scintillation counting fluid is:

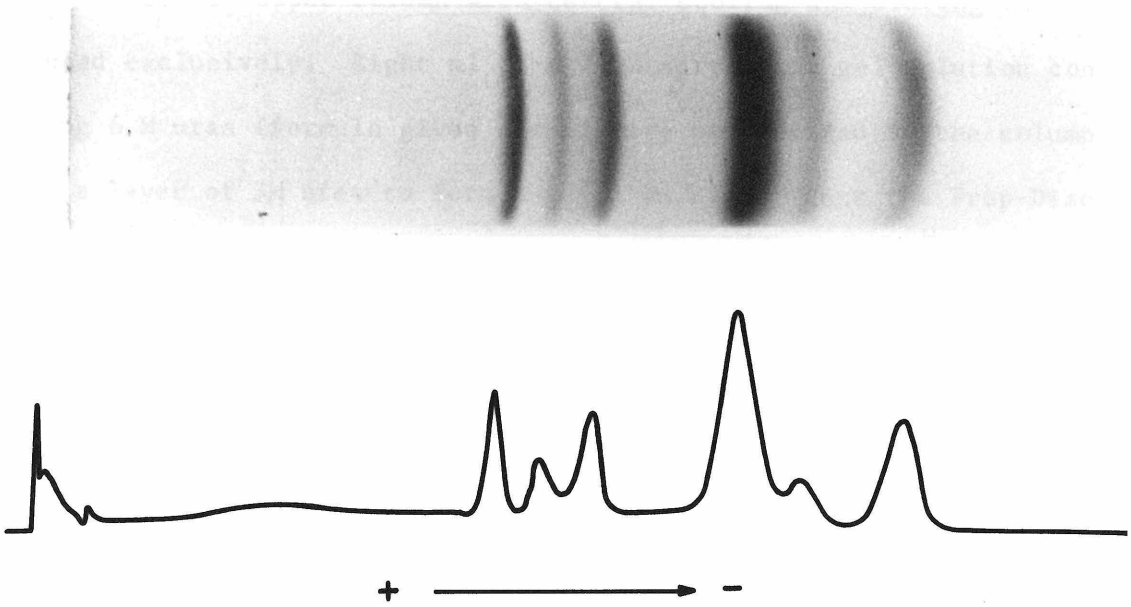
4 g PPO (Packard Instrument Co.)

0.05 g POPOP (Packard Instrument Co.)

985 ml Dioxane (Eastman Organic Chemicals)

120 g Naphthalene (Eastman Organic Chemicals)

Figure 3. Photograph of the electrophoretic pattern of pea bud histones (above) and densitometric tracing of the same pattern (below). Compare resolution of histone components in the gel with the densitometric tracing thereof.



ative electrophoresis, with the single exception that the fastest electrophoretic component (histone IV) can be prepared from whole histone.

The PD2/320 upper column with central cooling and eluting tube was used exclusively. Eight ml of a 7.5% acrylamide gel solution containing 6 M urea (formula given above) were polymerized in the column under a layer of 3M urea to form a gel 2 cm high. Next the Prep-Disc apparatus was assembled, the urea solution was removed from the gel, and the upper column filled with β -alanine buffer. A solution of histone in approximately 1 ml of 8M urea was then applied to the gel surface, using a syringe and long needle, and electrophoresis was performed at constant current of 35-40 milliamperes. Tap water was circulated through the cooling jacket and the central column. A flow rate of eluting buffer (β -alanine buffer) of about 30 ml/hour was generally used, and a slit width of about 1 mm between the gel surface and the lower electrode slit disc was found satisfactory. Fraction collection was set at 1 ml/tube; a drop counter was used to insure the collection of equal volume samples. Most preparative electrophoretic runs were complete in two to four hours.

The maximum sample load is about 1 mg of any single histone component and about 3 mg of total histone. Greater sample loads result in marked tailing of the bands so that all fractions are contaminated by fractions of higher electrophoretic mobility, and little improvement in yield of the fastest component is achieved by overloading. The progress of the electrophoresis can be monitored by the use of 1 lambda of 0.1%

methylene blue tracking dye, which migrates with the salt front; the histone bands can be directly visualized, due to their indices of refraction, until they reach the last half centimeter of the gel. As in analytical disc electrophoresis, no sample gel or stacking gel is required.

The fractions were assayed for protein by the direct addition of ½ volume of 3.3 M TCA and subsequent measure of turbidity (OD 400 mμ) after fifteen minutes. The fractions from each peak were then combined and the precipitated histone collected by centrifugation at 10,000 x g for 20 minutes, washed once with acidified acetone (0.1 ml conc. HCl per 200 ml acetone) and twice with acetone and then dissolved in 1 ml of 0.1 M acetic acid and lyophilized. Recovery of histones sometimes approached 100%. Lower recovery in other experiments is probably due to the slow elution rate. There is no protein remaining in the gel after completion of electrophoresis.

Amino acid analyses

Histone samples (0.3 - 3 mg) were hydrolyzed in 1-2 ml constant boiling HCl in evacuated, sealed tubes for 22 hours at 105°C. Oxygen was expelled from the hydrolysis tubes by repeatedly flushing with nitrogen followed by evacuation. Amino acid analyses were performed using a Beckman/Spinco Automatic Amino Acid Analyzer.

For the analysis of the amino acid composition of simple peptides or the identification of single amino acids and simple mixtures, the method of Dreyer and Bynum (1967) was used. The amino acids were

separated by high voltage paper electrophoresis in 6.7% formic acid at 40-45°C and the dried electrophoreograms dipped in cadmium ninhydrin reagent (10 ml H_2O, 2 ml acetic acid, 100 mg cadmium acetate, 100 ml acetone, 2g ninhydrin) and dried overnight in a dry oven at 37°C to develop the stain. Quantification of the stained amino acids was accomplished by elution of the spots from the paper with anhydrous methanol and determination of absorbance at 500 mμ. Duplicate standards were simultaneously measured to obtain staining constants. Staining is virtually linear with amino acid concentration over the range 1 to 30 nanomoles.

This method of analysis does not adequately detect proline. Proline was determined by paper chromatography of amino acid mixtures in 70% n-propanol, 30% ammonium hydroxide and staining with isatin reagent (100 mg isatin, 50 ml n-butanol, 5 ml acetic acid). The chromatograms were dipped in the stain, air dried, and then heated over a bunsen flame or hot plate to bring out the bright blue proline spots. Accurate quantitative determination of proline is not possible by this method but an estimate can be made by comparison of the staining of the unknown spot with the color produced by known amounts of proline.

N-terminal analysis

N-terminal amino acids of histone fractions were determined by a modified three-cycle Edman procedure (Edman, 1960; L. Hood and W.R. Gray, personal communication). Each histone fraction was dried *in vacuo* over phosphorus pentoxide and weighed immediately. Two to five

mg of histone were suspended in 1 ml of coupling buffer (15 ml pyridine, 10 ml water, 1.18 ml dimethylallylamine - this mixture titrated to pH 9.0 with approximately 2 ml of 20% trifluoroacetic acid). To the suspension 50 µl of redistilled phenylisothiocyanate were added, the suspension was flushed with nitrogen, and the coupling reaction allowed to proceed at 40°C for 1 hour. The suspension was next washed with 5 ml of benzene and then four times with 4 ml portions of butyl acetate. One-half ml of water was added to each tube and the mixture lyophilized. The dried material was washed three times with 1 ml portions of ethyl acetate.

The phenylthiocarbamyl derivatives resulting from the above procedures were next cleaved by the addition of 0.2 ml of trifluoroacetic acid and incubation for 15 minutes at 40°C under nitrogen. This cleavage and the subsequent cyclization produces 5-thiazolinone derivatives which were then extracted with successive 2, 2, and 1 ml portions of dichloroethane. The dichloroethane was removed by flushing with nitrogen. To the dried thiazolinones 0.3 ml of conversion buffer (30% ethanol titrated to pH 1.0 with 0.15 M HCl) were added, and the conversion reaction was carried out at 80° for 1 hour under nitrogen. The resulting 3-phenyl-2-thiohydantoin (PTH) amino acids were extracted with three 1 ml portions of ethyl acetate, the ethyl acetate was removed by flushing with nitrogen, and the PTH amino acids were dissolved in 50 ul of dichloroethane. For determination of yield 5 µl of the PTH amino acid solution were mixed with 1 ml ethanol, its UV spectrum was determined, and the yield calculated, using 16,000 and

14,300 for the molar extinction coefficients at 269 mμ of the alanine and proline derivatives (Edman and Sjöquist, 1956).

PTH amino aicds were identified by thin layer chromatography on Eastman TSC fluorescent sheets, using two solvent systems: m-xylene and 1:2:2 heptane: 75% formic acid: dichloroethane (Systems D and F of Sjöquist (Sjöquist, 1960)). For system D the thin layer sheets were first treated with 1:4 formamide: acetone and dried briefly.

Identifications were confirmed and the existence of minor components investigated by conversion of the PTH amino acids or thiazolinones to dansyl amino acids followed by electrophoresis in two buffer systems (Gray, 1967). To prepare dansyl amino acids, 10 μl of the PTH amino acid-dichloroethane solution or a comparable amount of thiazolinones were combined with 30 μl of 0.1 N NaOH and hydrolyzed for 12 hours at 105° in a sealed tube. The pH of the hydrolysate was adjusted to about 8 by exposure to a CO_2 atmosphere. Sixty μl of dansyl chloride (1 dimethylaminonaphthalene-5-sulfonyl chloride - 3 mg/ml in acetone) were then added and the solution was incubated at 40° for 2 hours. The acetone was evaporated and the remaining solution extracted twice with 60 μl portions of ethyl acetate (water saturated) and twice more at pH 4 (citrate buffer). The low pH extracts were then dried and dissolved in 10 μl of 1 M ammonium hydroxide. Aliquots were spotted on paper and electrophoresed on a flat plate at pH 4.4 (pyridine/acetic acid/water - 10/20/2500 by volume). This electrophoresis does not separate dansyl-proline and -alanine. Therefore the section of the electrophoreograms containing those dansyl derivatives

was cut out and sewn onto fresh paper and a second electrophoresis was performed at pH 1.7 (6.7% formic acid) or at pH 12.6.(0.1 M trisodium phosphate, 0.1 M sodium hydroxide). Electrophoreograms were inspected in UV light, the dansyl amino acids appearing as strongly fluorescing spots at characteristic positions (some dansyl amino acids such as dansyl proline also have characteristic hues).

C-terminal Determinations:

For the determination of the carboxy-terminal amino acids of the histone fractions two methods were used: cleavage of the c-terminal amino acids from the proteins with carboxypeptidases and hydrazinolysis of the fractions by the method of Nui and Fraenkel-Conrat (1955) as modified by Bennett and Dreyer (1967). In the former method, 1 mg samples of histone fractions were dissolved as well as possible in 100 µl of 0.2 M ammonium carbonate solution containing 5 M urea. These solutions were heated to 100°C for 60 seconds, cooled, and after the addition of 5 µl of carboxypeptidase A or B (Worthington 2x recrystalized) solution (10 mg/ml in ammonium carbonate buffer, DFP treated) the samples were incubated at 37°C for one to sixty minutes. Each incubation mixture was then chromatographed on a calibrated Sephadex G-25 column (10 ml bed volume) to separate the liberated amino acids from the remains of the protein. The amino acids were subsequently identified as described under "Amino Acid Analyses" above.

For the second method, pure, anhydrous hydrazine was prepared by vacuum distillation of hydrazine pretreated with barium oxide; the

constant boiling fractions were collected under nitrogen and stored under nitrogen in sealed ampules at -20°C. Histone samples were dried under vacuum with P_2O_5. Three hundred µl aliquots of hydrazine were added to 0.2 to 1.0 mg histone samples in thick walled tubes, the solutions were frozen, and the tubes were evacuated and flushed with nitrogen several times and then sealed under vacuum. Hydrazinolysis was carried out at 70°C for forty-eight hours. The unreacted hydrazine was removed under vacuum. The remaining free amino acids and hydrazide derivatives were dissolved in 200 µl of distilled water and the pH adjusted to neutral with acetic acid. The solutions were applied to 1 x 10 cm XE-64 columns equilibrated with 0.5 M ammonium acetate buffer, pH 5.6, and chromatographed with the same eluting buffer. Fifteen 1 ml fractions were collected, and these were dried under vacuum separately. The fractions were then redissolved in 10 µl of distilled water and each fraction analyzed for amino acids by the high voltage electrophoresis technique described above.

When even trace amounts of water are present in the protein sample, measureable amounts of the amino acids serine, glycine, and alanine are always observed. Their contribution to the total yield of amino acids is on occasion quite significant - as much as 20% or more. When such hydrolysis was significant, the data were discarded. A yield of more than 90% of a single amino acid and the presence of no free amino acids besides the major ones and traces of the three hydrolysis products serine, glycine and alanine were taken as criteria of C-terminal purity in hydrazinolysis experiments.

The principal losses of C-terminal amino acids probably occur during the transfer of sample to the column for chromatography and to the paper for electrophoresis. These losses are probably minor, since a yield of one mole of C-terminal amino acid per 20,000 to 35,000 grams of protein was obtained in nearly every case. The two amides, glutamine and asparagine, are not detected in this method of C-terminal analysis. In only one case, however, was the yield sufficiently low to suggest that some C-terminal group might have been missed in the analysis. Since no amino acid amides are found in the carboxypeptidase digestions of any of the histone fractions, it is unlikely that these amino acids occur as C-terminal in any histone.

Reduction and Alkylation

For the reductive cleavage of disulfide linkages, histone was dissolved at a concentration of 1 mg/ml in 10 M urea and β -mercapto ethanol was added to give a final concentration of 0.1 M. The solution was incubated for one hour at 37°C to effect complete reduction. An aliquot was removed for analytical disc electrophoresis to confirm the completeness of the reduction.

To the reduced histone solution was next added one fifth volume of 1 M iodoacetate in 1 M tris buffer, pH 8, containing an additional measured amount of ^{14}C labelled iodoacetate of known specific activity. After incubation at 37°C for one hour, the alkylated histone was precipitated from solution by the addition of one half volume 3.3M TCA, collected by centrifugation, washed with 1.1M TCA and then with

absolute ethanol. The washed protein pellet was dissolved in 8M urea and the protein concentration determined by the method of Lowry et al. (1951). Known quantities of histone were then subjected to disc electrophoresis and the amount of ^{14}C label in each histone band was determined as described in the section on disc electrophoresis above. To obtain control values for the non-specific reaction of iodoacetate with histone, histone in the oxidized state and histone fractions lacking cysteine were subjected to identical alkylation conditions, and the value of ^{14}C labelling used as the level of non-specific reaction.

Preparation of N-terminal peptides

Advantage was taken of the fact that in the enzymatic hydrolysates of proteins with blocked N-terminal amino acids the N-terminal peptides are unique in having no positive charge (unless they contain basic amino acids). The N-terminal peptides which have no positive charge can be easily separated from the other peptides by chromatography on a cation exchange resin which will retain positively charged molecules. Histone fractions with blocked end groups were digested with pronase (CalBiochem Co.) or subtilysin (Nutritional Biochemical Corp.) in 0.1 M ammonium carbonate buffer at 37°C for several hours. The resulting hydrolysates were passed through 1 x 10 cm Dowex-50 columns in the hydrogen form at neutral pH, using distilled water as solvent for elution. The unretarded peptides were collected in several ml of effluent.

Analysis for N-terminal Acetyl groups

Several histone fractions contain no demonstrable N-terminal amino acids and are presumed to be blocked by acetylation of the terminal amino group. N-terminal peptides obtained by the method described immediately above were subjected to hydrazinolysis. The hydrazide products were chromatographed on Schleicher & Schuell #589 paper in two solvent systems: pyridine: acetic acid: water 10:1:4 and pyridine: analine: water 9:1:4. Acetyl hydrazide was synthesized by refluxing ethyl acetate and hydrazine in ethanol solution for several hours and subsequently removing the unreacted materials by vacuum desiccation (Narita, 1958). The resulting crystalline acetyl hydrazide was used as standard in the paper chromatography. Chromatograms were sprayed with Ehrlich's reagent. Acetyl hydrazide appeared first as a fluorescent spot which later became a bright visible orange.

Determination of peptide sequence

The amino acid sequence of one peptide was determined by partial acid hydrolysis of the peptide in 2 N HCl for 10 minutes, separation of the resulting peptide fragments by paper electrophoresis, and determination of the fragment peptide sequences by the subtractive Edman-dansyl technique of Gray (1967). This method differs from that described for determination of N-terminal amino acids in that portions of the peptides were subjected to multiple cycles of the Edman reaction to remove amino acids from the N-terminal end of the peptide prior to dansylation of the remaining peptide pieces, acid hydrolysis of the

dansylated pieces (6 N HCl, 105°C, 12 hours) and identification of the dansyl derivatives.

Preparation of peptide maps

Histones were dissolved at a concentration of 10 mg/ml in 0.2 M ammonium bicarbonate, trypsin (free of chymotryptic activity (Kostka and Carpenter, 1964)) was added to give a final concentration of 20 ug/ml, and the tryptic hydrolysis was carried out at 37°C for 3 or 4 hours. Each incubation mixture was then applied in small aliquots to a sheet of Whatman #3 paper, and descending chromatography along the short direction was performed, using 70% n-propanol, 30% ammonium hydroxide. About twenty hours were required to move the most mobile peptides near to the bottom of the paper. The chromatograms were then air dried at room temperature, rewet with 0.55 M pyridine acetate buffer, pH 3.5, and electrophoresis in the second dimension was performed at 20°C, 3300 volts, for 60 minutes. The peptide maps were dried in a ventilated oven at 65°C for 30 minutes, dipped in collidine-ninhydrin stain (600 ml ethanol, 200 ml acetic acid, 80 ml 2-4-6 trimethylpyridine, 1 g ninhydrin) and dried 10 minutes at 65°C.

To selectively stain peptides containing tyrosine and histidine residues, some maps were sprayed with Pauly reagent (0.025 M sulfanilic acid, 0.05 M $NaNO_2$ in 0.5 M HCl, followed by a spray of 10% Na_2CO_3), which stains tyrosine containing peptides orange and histidine containing peptides pink. To selectively stain peptides containing arginine, a modified Sakaguchi stain was used (C. Bennett, unpublished method).

Peptide maps were dipped in 0.0125% alpha-naphthol in absolute ethanol and dried at room temperature. The maps were then sprayed with a fresh solution of 6 parts 1.5 N sodium hypochlorite, 94 parts 10% NaOH. After one minute papers were lightly sprayed with a solution of 12 parts iodine - potassium iodide (30g KI, 22.4 g I_2, 400 ml H_2O) and 100 parts 10% NaOH. Arginine containing peptides appear as bright pink spots.

Results

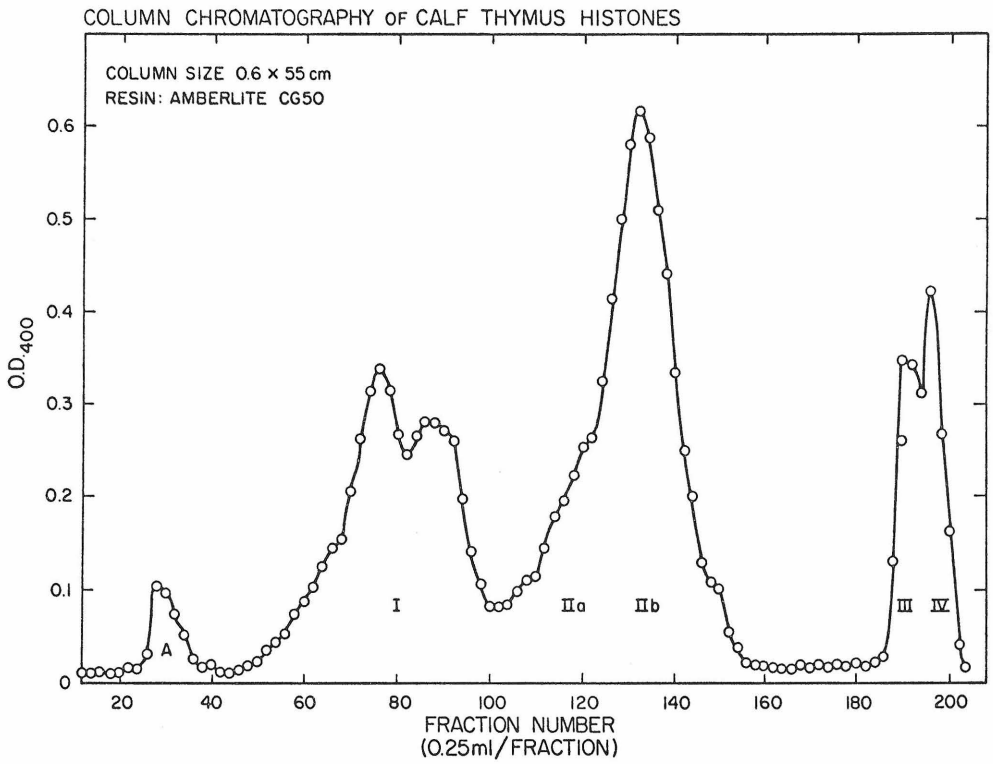
Column chromatography

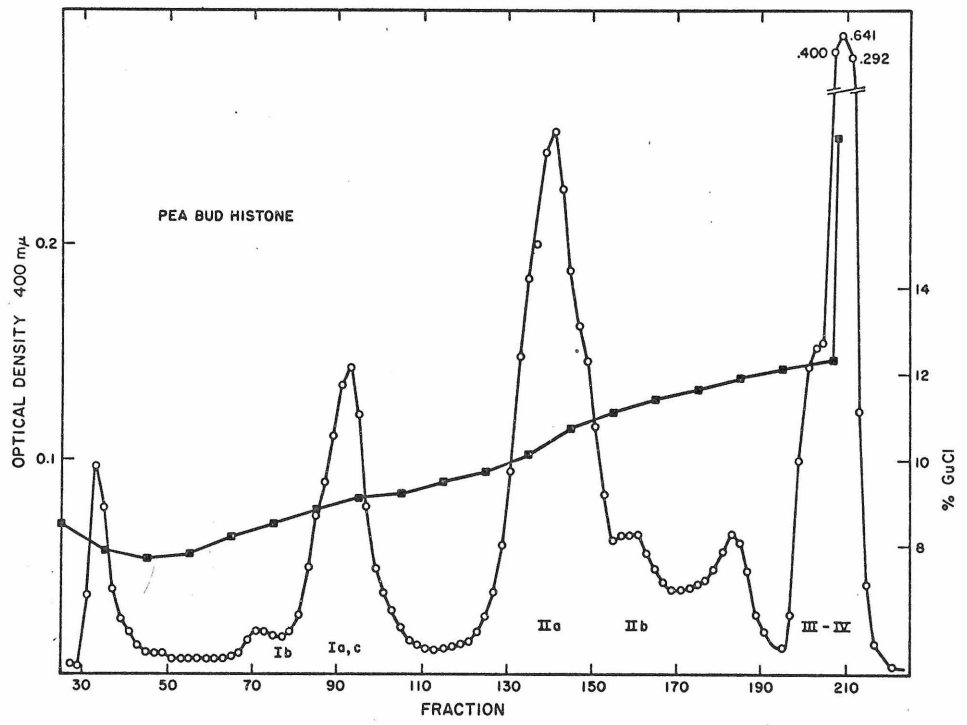
Pea bud and calf thymus histones chromatographed on Amberlite CG-50 with a guanidinium chloride gradient yielded chromatographic patterns presented in Figure 4ab. Protein in the first peak (A) is not retained by the resin and appears immediately after one hold-up volume of effluent. Three major histone fractions are separated by column chromatography. These fractions are labeled I, II, and III-IV to correspond to existing nomenclature for calf thymus histone fractions similarly separated (Rasmussen et al., 1962). Amino acid analyses confirm that in both cases the three major fractions are, in order of elution, lysine-rich, slightly lysine-rich, and arginine-rich.

The finer detail of column fractionation became understood only after analysis of the fractions by several other methods, as will be discussed below. However, a more detailed description of the column fractionation at this time will serve to clarify the nomenclature used to denote the different histones.

The major fractions of calf thymus histone all appear to be heterogeneous, each fraction containing at least two components. Some of this apparent heterogeneity is a manifestation of true heterogeneity and some is artifact, due possibly to aggregation or other histone interactions and to the nature of the salt gradient. Histone I chromatographs as a double peak, with the components well separated in the example elution pattern shown in Figure 4a. The two components are

Figure 4. Fractionation of histones by column chromatography on Amberlite CG-50. a) Calf thymus histones. b) Pea bud histones. Protein was measured by precipitation in 1.1 M TCA and subsequent measurement of OD 400 mμ (--o--o--). Concentration of guanidinium chloride in the effluent is indicated by --■--■--.


COLUMN CHROMATOGRAPHY OF CALF THYMUS HISTONES
COLUMN SIZE 0.6 × 55 cm
RESIN: AMBERLITE CG50
O.D.400
0.6
0.5
0.4
0.3
0.2
0.1
0
A
I
IIa
IIb
III
IV
20
40
60
80
100
120
140
160
180
200
FRACTION NUMBER
(0.25ml/FRACTION)


PEA BUD HISTONE
.641
.400
.292
OPTICAL DENSITY 400 mμ
0.2
0.1
% GuCl
14
12
10
8
Ib
Ia, c
IIa
IIb
III - IV
30
50
70
90
110
130
150
170
190
210
FRACTION

named, in order of elution, Ia and Ib. These two subfractions have been shown to have very similar amino acid compositions (Rasmussen et al., 1962), and to differ primarily in that histone Ia contains slightly more arginine than does histone Ib. Further, these fractions have been shown to possess very similar primary structures (Kinkade, 1966; and see below).

Calf thymus histone IIa is apparently identical with histone III-IV: both are arginine-rich histone, containing the rare amino acid e-methylysine. Calf thymus fraction IIa is also apparently responsible for the N-terminal alanine found to contaminate the otherwise proline N-terminal group of the slightly lysine-rich histone (Luck et al., 1958). Histone IIb, which superficially appears to be rather chromatographically homogeneous, is actually composed of two electrophoretic components, termed IIb1 and IIb2 by Rasmussen et al., (1962). Both are slightly lysine-rich.

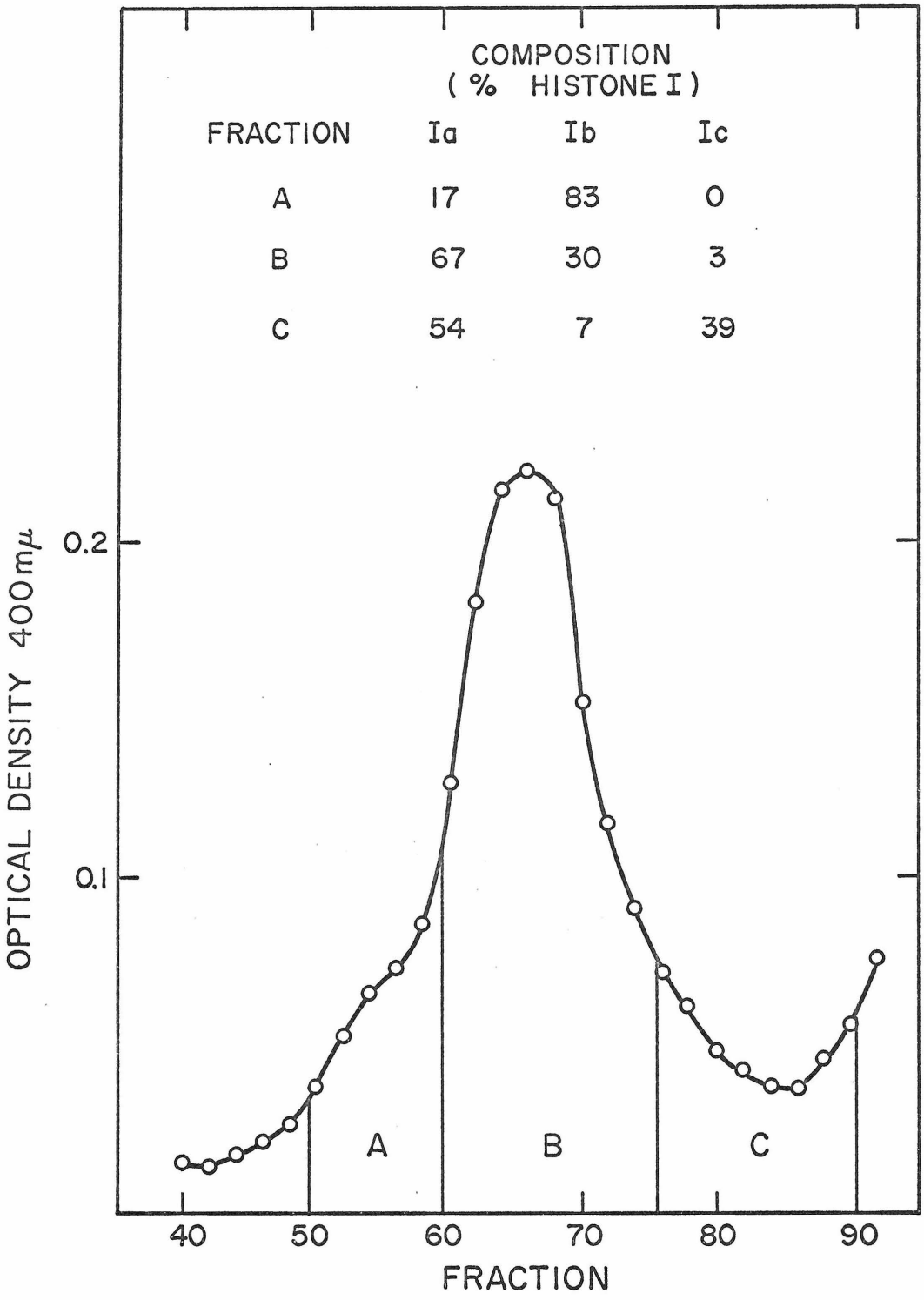
The separation of the arginine-rich histones into two fractions is apparently an artifact of the stepwise elution of this material. Fractions III and IV are identical by every criterion of analysis. However, each fraction contains two different kinds of molecules which can be distinguished in many ways. Thus, slightly modifying the meaning of the names III and IV, the author has reassigned them to the two identifiable components. The electrophoretically slower one, containing N-terminal alanine, is designated "III" while the electrophoretically faster one, containing blocked N-terminal, is designated "IV". This choice of nomenclature is not an arbitrary one. The other major nomenclature of the

histones, based principally on the chemical fractionation of Johns (see Methods) has assigned to the arginine-rich histones the names f2a1 and f3. The present choice of nomenclature equates III with f3. For a complete summary of the relation between the two systems of nomenclature see "Fractionation of Histones by the Method of Johns" (page 62) and table V.

A degree of complexity similar to that of the calf thymus profile is found in the chromatographic fractionation profile of pea bud histones. The lysine-rich histone I peak is rarely symmetrical and is composed of three components termed Ia, Ib and Ic in order of increasing electrophoretic mobility. Chromatographically, the order of elution is Ib, Ia and Ic, the first two comprising the bulk of the histone I peak, the Ic component eluting principally in the trough between histone I and II (see Figure 5). These three components appear to bear the same structural relation to each other as do calf thymus histones Ia and Ib.

Pea bud histone includes three slightly lysine-rich components. The first two are practically superimposed in elution profile, but can be distinguished by different C-terminal amino acids and by their electrophoretic properties. They bear the names IIa1 and IIa2 in order of increasing electrophoretic mobility. Component IIb appears as a shoulder on the descending edge of the histone IIa peak. Histone IIb is closely followed in elution by an unnamed material which contains all of the histone components, but which is usually richest in the arginine-rich histones III and IV. This peak is quite variable in size and poses a major obstacle to the use of column chromatography for quantitative analysis of histones.

Figure 5. Chromatographic fractionation of pea bud lysine-rich histones on Amberlite CG-50. The histone I peak was divided into three cuts (A, B and C) and the composition of each cut in terms of histone I components was determined by quantitative disc electrophoresis (see Chapter II). Compositions of cuts are presented in the figure. The histone II contamination in these cuts was ignored in the quantification.


COMPOSITION
(% HISTONE I)
FRACTION Ia Ib Ic
A 17 83 0
B 67 30 3
C 54 7 39
OPTICAL DENSITY 400mμ
0.2
0.1
A
B
C
40
50
60
70
80
90
FRACTION

The arginine-rich histones constitute the last peak of the elution profile. Again, there may be a partial separation of two components in this peak, but these subfractions appear to be alike. I have therefore again designated the two molecular species of this material, by homology with the calf thymus arginine-rich histones, "III" and "IV".

Amino acid analyses of the column fractions

The amino acid compositions of pea bud histone fractions are presented in table I. These compositions are expressed as moles of each amino acid per 100 moles of total amino acids recovered. Although serine and threonine are partially degraded during hydrolysis, the losses are relatively small (approximately 10% and 5% respectively (Rees, 1946)), and no correction has been made for them. For comparison with the pea bud histone fractions, amino acid compositions of calf thymus histone fractions (taken from Rasmussen et al., 1962) are also given in table I. The calf thymus histone amino acid compositions are corrected for serine and threonine decomposition. The amino acid analyses were performed on histone fractions comparable to those, the electrophoretic patterns of which are given in Figures 7 and 8. Thus there is slight contamination of each fraction by material of other fractions, and amino acid compositions are composites of the amino acid compositions of the different molecular species present in each fraction.

Although caution should be used in evaluation of the amino acid data of heterogeneous proteins, comparison of the amino acid compositions does reveal striking similarities between the corresponding

Table I.

The Amino Acid Compositions of Chromatographic Histone Fractions

Amino Acid	Pea A	Pea I	Calf Thymus[a] Ib	Pea IIa	Pea IIb	Calf Thymus[a] IIb	Pea III-IV	Calf Thymus[a] III-IV
Lys	8.2	22.9	26.2	16.8	14.1	13.5	9.7	9.7
His	1.5	0.9	0.2	1.6	1.1	2.8	1.9	1.9
Arg	3.2	2.7	2.6	7.2	7.6	7.9	10.8	11.9
Asp	7.0	3.0	2.5	6.7	6.2	5.6	6.1	5.0
Thr	5.2	4.6	5.4	4.7	4.8	5.2	6.1	6.7
Ser	7.8	5.6	6.5	7.3	6.2	7.0	4.4	4.6
Glu	9.7	7.8	4.3	8.6	7.7	8.7	8.8	10.4
Pro	6.8	10.0	9.1	6.9	5.3	4.7	3.9	4.2
Gly	11.8	3.7	7.3	10.5	10.0	8.2	9.8	8.6
Ala	12.9	22.9	24.2	7.9	11.8	11.5	9.7	11.6
Val	7.3	6.2	4.1	4.5	6.9	6.7	6.7	5.9
Met	1.0	Trace	0.1	0.7	0.6	0.8	0.4	1.3
Ile	4.8	2.9	1.2	3.8	5.0	4.5	5.9	5.3
Leu	8.5	4.6	5.0	7.9	8.4	8.6	10.5	8.9
Tyr	2.3	0.9	0.7	2.4	1.8	3.0	2.4	2.2
Phe	2.2	1.3	0.6	2.7	2.4	1.3	3.1	2.5

[a]Taken from Rasmussen *et al.* (1962).

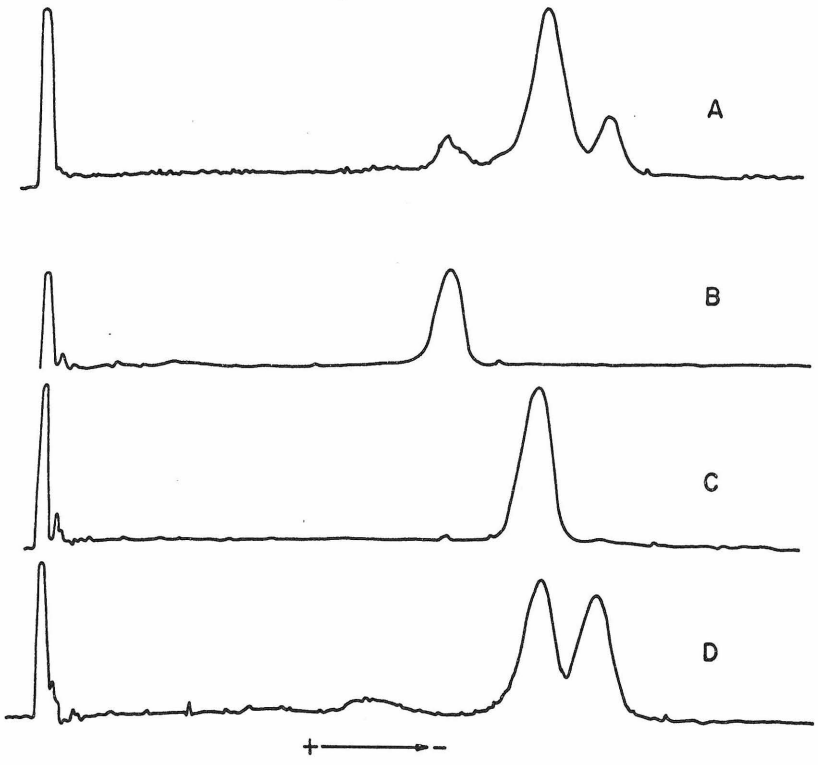
pea bud and calf thymus histone fractions. The lysine-rich histones of pea bud and calf thymus show a particularly striking resemblance due to their unusual compositions. Their arginine/lysine ratios are approximately 0.1. In addition to their high lysine content, these histones are almost equally rich in alanine, the two amino acids accounting for about half of the total compositions. Proline content is also remarkably high -- about 9-10 mole per cent. Aromatic amino acids are present in very small amounts and methionine is practically lacking. Of the two pea bud slightly lysine-rich histone components, IIb particularly resembles calf thymus histone IIb, both possessing arginine/lysine ratios of about 0.5. The III-IV fractions are also extremely similar, differing in content by more than one mole per cent in only six amino acids.

Disc electrophoresis

Electrophoresis of histones in polyacrylamide gels was first used in the course of this work as a means of analysis and identification of the histone fractions produced by column chromatography. Although disc electrophoresis has proved to be a far superior tool for the resolution of histone components, especially for the separation of pea histones, little additional heterogeneity of histone fractions is revealed by it.

Figure 6 shows densitometric tracings of the electrophoretic patterns of whole calf thymus histone and calf thymus histone fractions Iab, IIb, and III-IV. In this figure a partial separation of histones

Figure 6. Densitometric tracings of the electrophoretic patterns of whole calf thymus histone (A) and chromatographic fractions I, IIb and III-IV (B, C and D respectively). Peaks at the far left represent origins of gels, not protein.


A
B
C
D
+
-

Ia and Ib is evident. These two are, however, not reproducibly resolved by disc electrophoresis. The patterns reveal no indication of separation of components IIb1, IIb2 and III. After longer electrophoresis times III does, however, often emerge as a band of greater mobility than that of IIb.* The electrophoretic pattern of calf thymus histone IIa is identical with that of III-IV. Histones III and IV are quite different in electrophoretic properties, histone IV having the greatest mobility of all the histones. Note the presence of some material of lesser mobility in the III-IV fraction. The nature of this material is discussed under "Arginine-rich Histones" below.

Densitometric tracings of the electrophoretic patterns of whole pea bud histone and of pea bud histone fractions Iab, IIa, IIb and III-IV are presented in Figures 7 and 8. Here the identification of column chromatography fractions with electrophoretic components is quite apparent. The tracings also give some indication of the degree of cross-contamination between fractions. Histone Iab contains about 20% histone IIa, histone IIa contains a trace of histone I, and histone III-IV contains some histone II, especially IIb. The degree of purity of the chromatographic fractions here illustrated is typical with the exception that histone IIb is rarely obtained in such pure form. More often the best cut of IIb contains some IIa as well as appreciable III-IV. That the bands identified as contaminant histones in the column fractions are indeed such can be demonstrated by purification of compo-

*Histones IIb1 and IIb2 can be separated (for example) by electrophoresis in starch gel.

Figure 7. Densitometric tracings of the electrophoretic patterns of whole pea bud histone (A) and chromatographic fractions Iab, IIa and III-IV (B, C and D respectively).

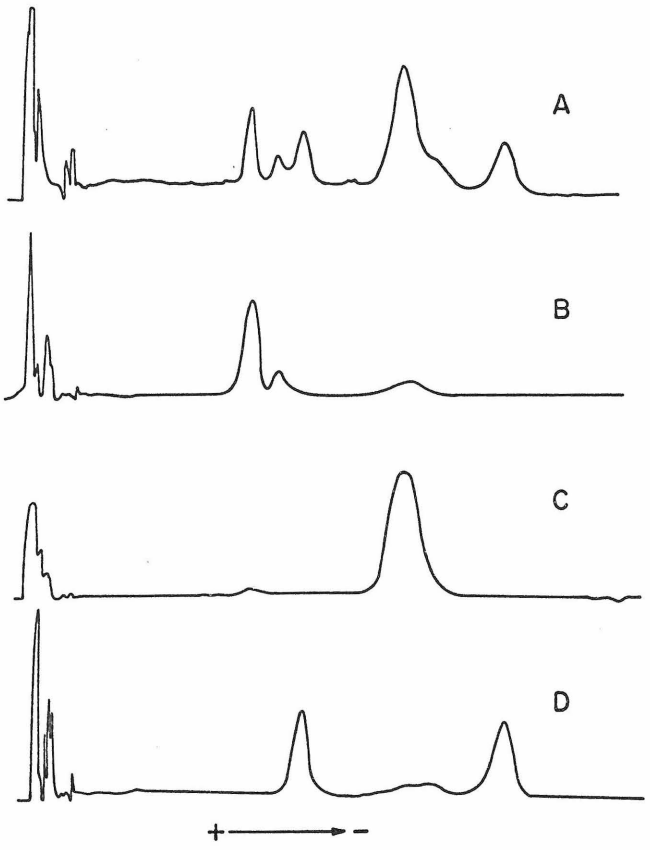
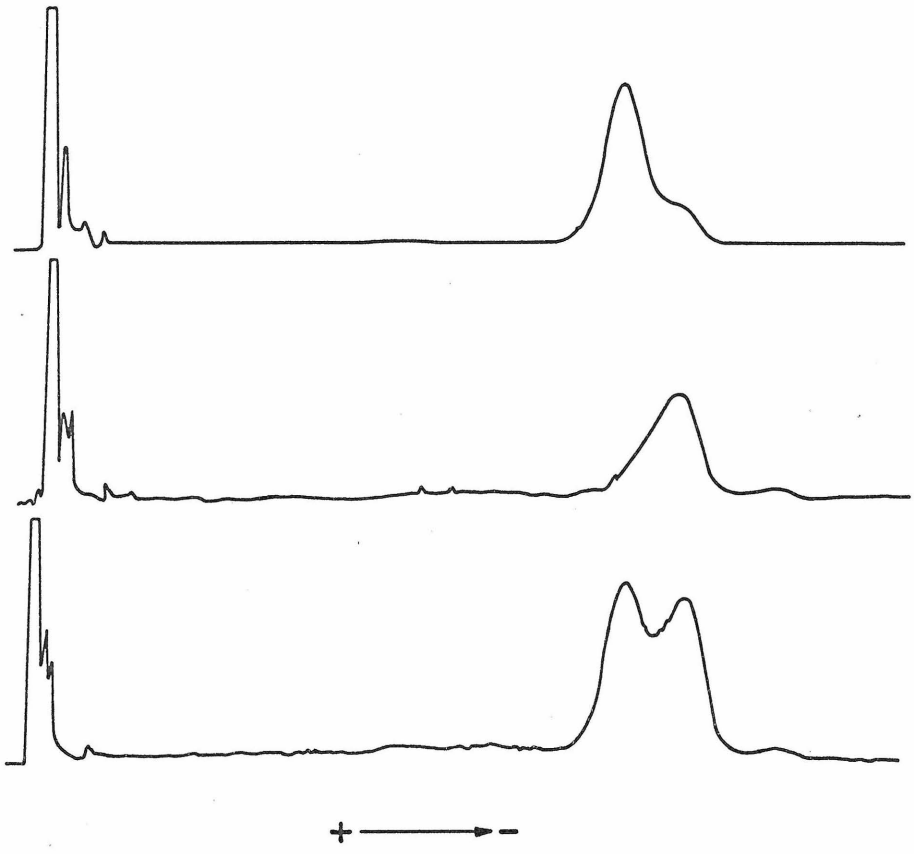

A
B
C
D
+
-

Figure 8. Densitometric tracings of the electrophoretic patterns of pea bud histones IIab (top), IIb (center), and IIab plus IIb (bottom).



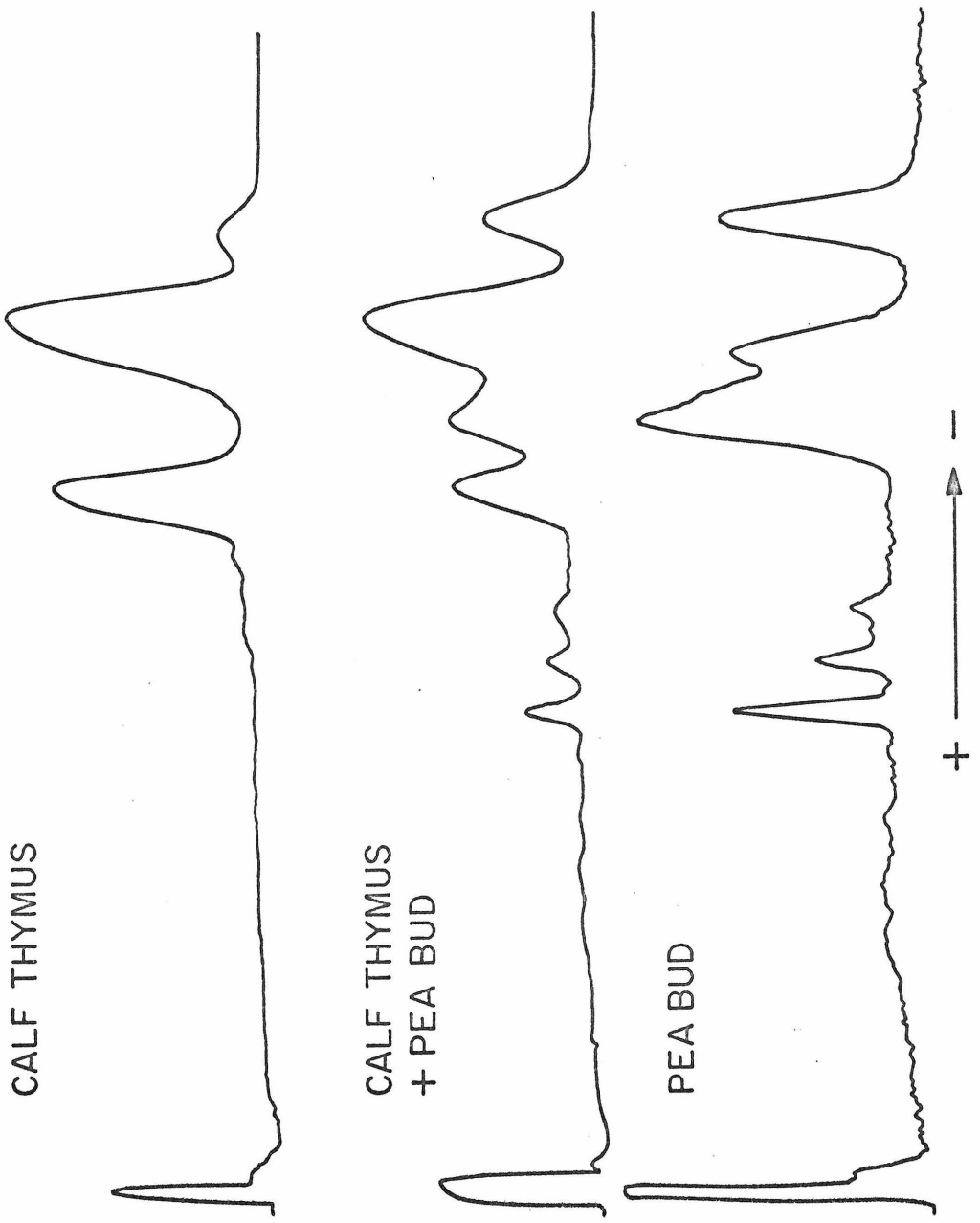
nents by repeated column chromatography. Such twice-purified fractions contain little or no cross contamination.

Figure 5 presents a more detailed analysis of the histone I components, showing the relation between order of chromatographic elution and composition. Identification of histone Ic as a bona fide component of histone I and not as simply a trace of histone III is based upon several lines of evidence gathered as peripheral information in the course of other investigations. This information may be summarized as follows: histone Ic is extracted with the lysine-rich histones by treatment of nucleohistone with 5% perchloric acid. Histone Ic is removed from nucleohistone together with histones Ia and Ib by 0.5 M NaCl. Histone Ic is not removed from histones Ia and Ib by repeated column chromatography. Peptide maps of histone I samples rich in histone Ic resemble maps of histones Ia and Ib. Finally, histone Ic is not affected by reduction of whole histone with β-mercaptoethanol, a treatment which changes the electrophoretic mobility of histone III.

The resolution of histone IIa1 and IIa2 is not apparent in the tracings of figures 8 and 9, but the two components can be distinguished by the different colors of their amido schwarz stained bands. The slower IIa1 stains a more brownish blue than does the faster IIa2. The separation in later experiments was, for no apparent reason, much better, and this superior resolution is demonstrated, for example, in Figure 3 of Chapter II.

Disc electrophoresis has also been used to compare the electrophoretic mobilities of calf thymus and pea bud histones. Figure 9 shows the electrophoretic patterns of pea bud histones, calf thymus histones, and a mixture of the two. The electrophoretic mobilities of the pea histone fractions I and IIa are somewhat less than those of the corresponding calf thymus histone fractions, particularly in the case of the histone I fractions. The differences may be due to the higher content of free carboxyl groups in the pea histone fractions, to the lower content of basic amino acids in the pea histone fractions, to a larger size of the pea histones, or to differences in content of phosphoserine and phosphothreonine (Kleinsmith et al., 1966). Amino acid composition data and peptide maps presented below suggest that the first three factors may be involved; there is no information upon which to evaluate the importance of the last factor. The pea bud and calf thymus histone IV fractions have identical mobilities, and pea histone IIb has a mobility identical to that of calf histone IIb1. The electrophoretic properties of pea bud and calf thymus histone III fractions are more complex than indicated above. These properties are discussed under "Arginine-rich Histones" below.

Figure 9. Densitometric tracings of the electrophoretic patterns of calf thymus histones (top), pea bud histones (bottom), and a mixture of the two (center).


CALF THYMUS
CALF THYMUS
+ PEA BUD
PEA BUD
+
−

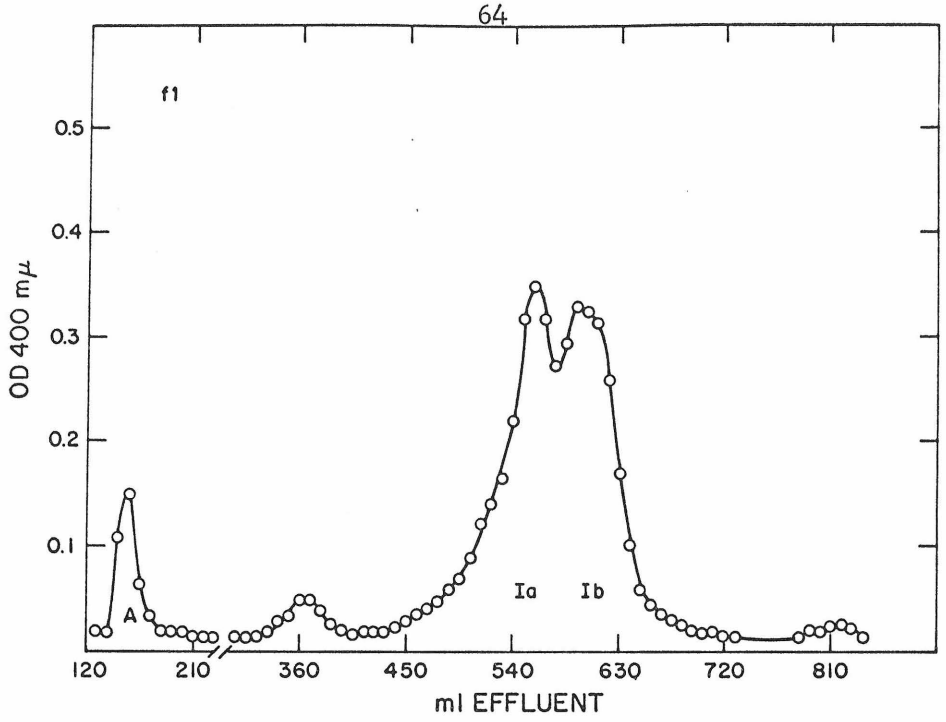
Fractionation of histones by the method of Johns

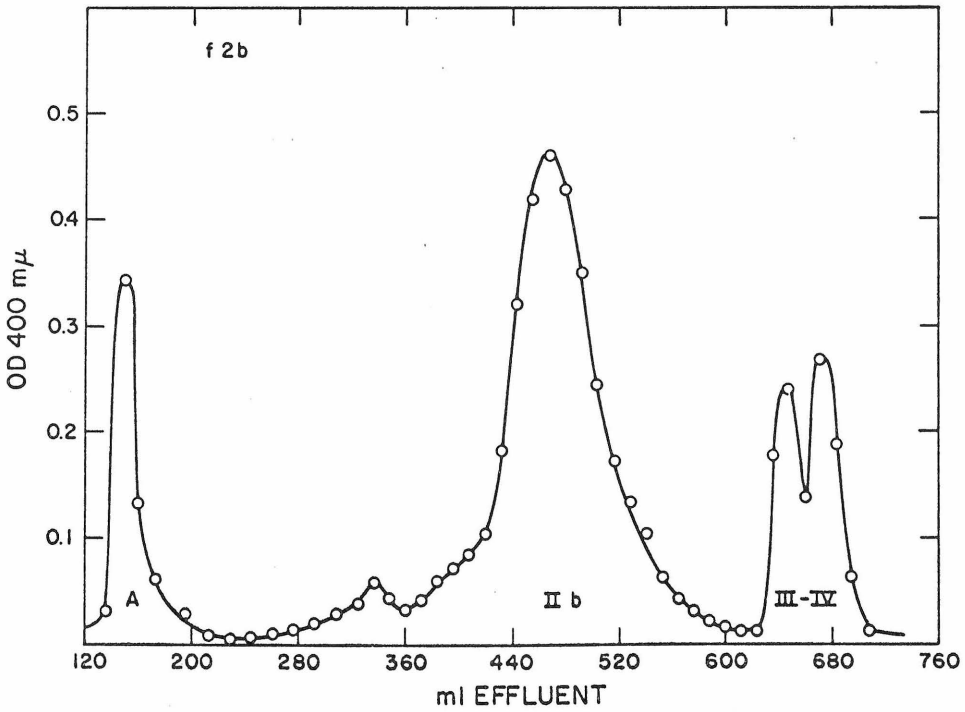
Nomenclature of calf thymus histones

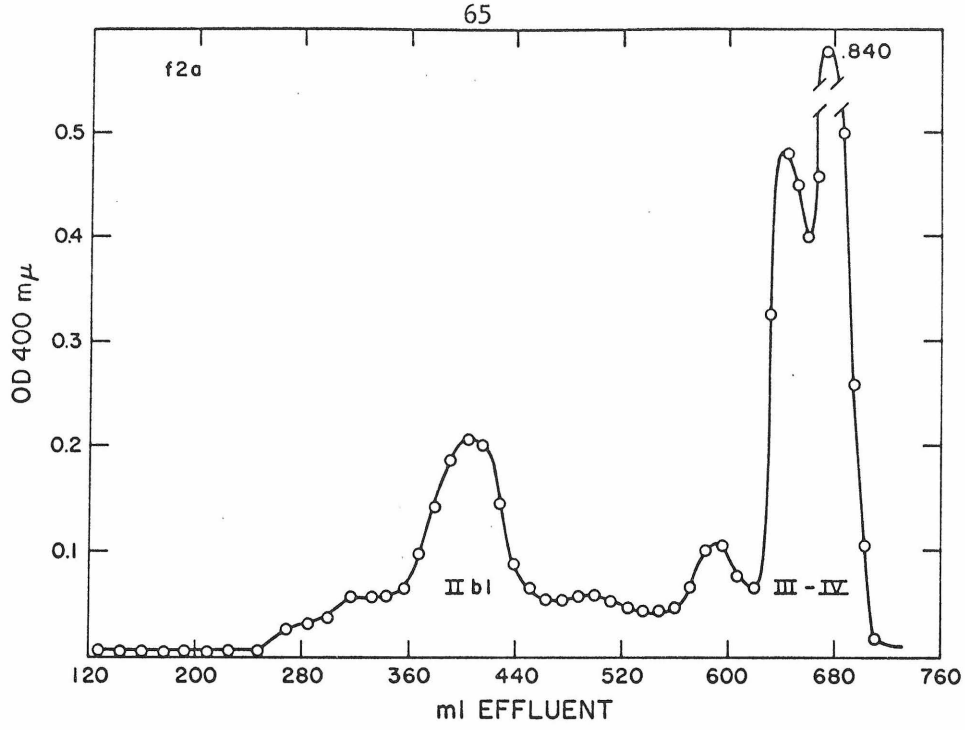
The problem of histone nomenclature has been a serious one. There has been a great deal of confusion due to the simultaneous development of many different methods of histone fractionation in many different laboratories and to the misimpression that the histones were very likely extremely heterogeneous. The problem is well illustrated by Murray's attempt to standardize the nomenclature in 1964 (Murray, 1964). Since that time the situation has simplified due to the adoption of the nomenclature of Johns, Phillips and Butler for calf thymus histones by most laboratories. This has been due, in turn, to the simplicity of the methods developed by those workers for the fractionation of calf thymus histones (Johns and Butler, 1962; Johns, 1964; Phillips and Johns, 1965). Unfortunately, these methods are not entirely satisfactory for complete purification of histone components and do not work well with sources other than calf thymus (Lawrence et al., 1966; Palau and Butler, 1966; MacGillivray, 1966).

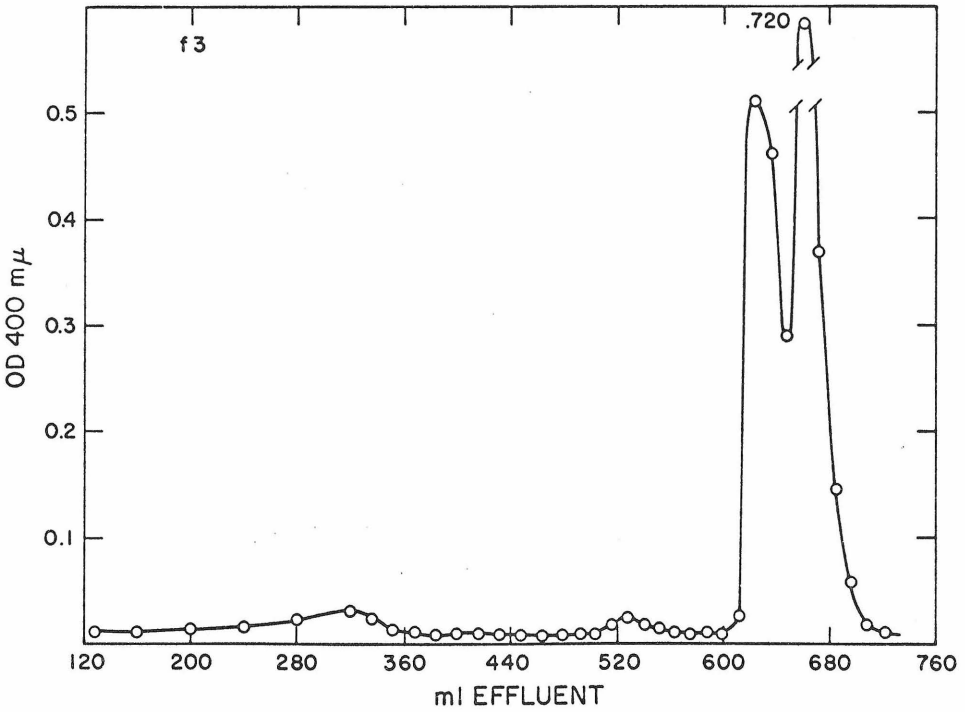
In order to relate the nomenclature used in this laboratory to that of Phillips, Johns and Butler, calf thymus histone fractions were prepared by the method of Johns (1964) and these fractions then fractionated by column chromatography on Amberlite CG-50. The chromatographic patterns of the Johns' fractions are presented in Figure 10 a-d. These chromatographic analyses are in full agreement with amino acid composition data of Rasmussen et al., (1962) and of Johns (1964) and of Phillips and Johns (1965) and indicate the following identities: the

Figure 10. Chromatographic fractionation of histone fractions prepared by the method of Johns (1964). a) Column chromatography of histone fraction f1 (40 mg) on Amberlite CG-50. For this chromatography a 1.5 x 120 cm column was used with a flow rate of about 15 ml/hr. Two hundred ml of 8% GuCl-PO_4 were passed through the column after application of the sample. Subsequently a 600 ml linear gradient of 8% to 13% GuCl-PO_4 was used to elute histones Ia and Ib, and the column was finally flushed with 40% GuCl-PO_4. These chromatographic conditions were employed in an attempt to reproduce the elution patterns obtained by Kinkade (1966) in which more than two lysine-rich chromatographic peaks were observed. Several such attempts were made, using different preparations of calf thymus lysine-rich histone, but an elution profile like those reported by Kinkade was never obtained. b, c, and d) Column chromatography of histone fractions f2b, (50 mg), f2a (50 mg) and f3 (46 mg) respectively on a 2.5 x 60 cm column of Amberlite CG-50. A 600 ml linear gradient of 8% to 14% GuCl-PO_4 followed by 40% GuCl-PO_4 was used in each case.


f1
0.5
0.4
0.3
0.2
0.1
OD 400 mμ
A
Ia
Ib
120
210
360
450
540
630
720
810
ml EFFLUENT


f 2b
0.5
0.4
0.3
0.2
0.1
OD 400 mμ
A
II b
III-IV
120
200
280
360
440
520
600
680
760
ml EFFLUENT


f2a
.840
0.5
0.4
0.3
0.2
0.1
OD 400 mμ
II b1
III - IV
120
200
280
360
440
520
600
680
760
ml EFFLUENT


f3
.720
0.5
0.4
0.3
0.2
0.1
OD 400 mμ
120
200
280
360
440
520
600
680
760
ml EFFLUENT

lysine-rich histone fractions f1 is equivalent to histones Ia and Ib; fractions f2b and a part of f2a are equivalent to histone IIb; and the remaining histone f2a and f3 are equivalent to histone III-IV.

By analytical disc electrophoresis and terminal analyses (see below) it was determined that the portion of f2a chromatographing in the IIb region is histone f2a2 (IIb1). By the same methods it was found that the material in fraction f2b chromatographing as histone III-IV is actually some histones f2a and f3 which contaminate the fraction. Further, amino acid analyses reveal that the f2b fraction also contains much f2a2. Separation of fractions f2a and f3 by the fractional precipitation method of Johns resulted in only partial separation of these two fractions. Fraction f3 was obtained in low yield and fraction f2a still contains a component identified by end group analysis and disc electrophoresis as fraction f3 histone. These observations are summarized in Figure 10 a-d.

The fractions prepared by the method of Johns do not represent clean separations of individual histone components but can serve as crude fractions for further preparative purification. Somewhat cleaner separations are reported by Johns and by Hnilica (1966) and by Hnilica and Bess (1965), but usually the method of Johns has been used only as a first step in purification (see, for example, Hnilica and Bess (1965), Mauritzen et al., (1967)).

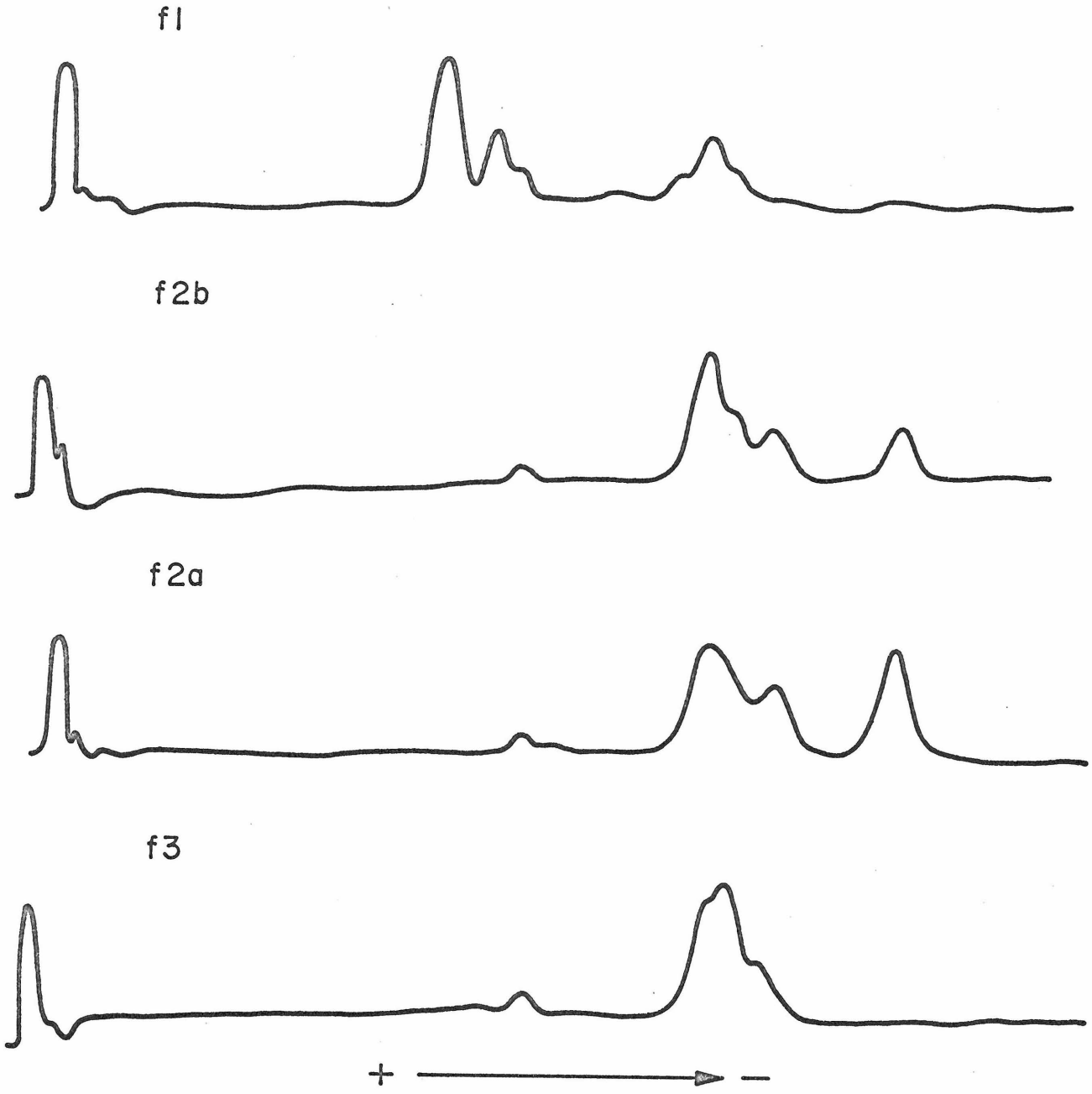
In the course of the present experiments an attempt was made to use the method of Johns for the preparation of pea bud histone fractions. The fractions so obtained were analyzed by disc electrophoresis, as

illustrated in Figure 11. The extraction of the lysine rich histone with 5% perchloric acid is quite effective in removing all of this histone, although it is grossly contaminated by others. The remaining fractionations are also highly cross-contaminated. Fraction f2a contains not only histone IV and IIb, but also much of histone IIa and a trace of histone III. Fraction f2b contains not only histone IIa but also substantial amounts of the arginine-rich histones. And fraction f3 is composed predominantly of histones IIa and IIb. Thus, while this method of fractionation is useful for the selective extraction of histone I, it cannot be used effectively as a purification step for the pea bud histones. Based upon amino acid analyses of fractions of wheat germ histones prepared in this manner, Butler and Johns (1962) concluded that plant histones lack arginine-rich fractions. The data presented above may explain the failure of these workers to find arginine-rich fractions.

Chemistry of histone fractions

A major hypothesis derived from study of calf thymus and pea bud histone fractions is that in these organisms and, by extension, in all higher organisms there are homologous histone fractions which are related by evolution and which remain similar in structure and therefore in function. The further discussion of the characterization of the histone fractions is therefore organized according to histone type to emphasize these structural similarities.

Figure 11. Densitometric tracings of the electrophoretic patterns of pea bud histone fractions prepared by the method of Johns (1964). Fractions are identified by the nomenclature used for calf thymus histone fractions prepared by the same recipe.


f1
f2b
f2a
f3
+
−

1. The Lysine-rich histones

a. Amino acid compositions

The first clue to the structural relationships between calf thymus and pea bud histone fractions came from the similarity in amino acid compositions of chromatographically prepared calf thymus and pea bud histone I. By repeated column chromatography and preparative disc electrophoresis these histones were further purified, and their amino acid analyses are presented in Table II .

The further purification resulted in increased similarity between pea bud and calf thymus histone I amino acid compositions. Again the main features in the amino acid compositions of these histones are the high content of lysine, alanine and proline and the very low content of tyrosine and phenylalanine and the absence of methionine, cysteine and tryptophan. Calf thymus and pea bud histone I differ primarily in that pea bud histone I contains somewhat less lysine and more arginine, contains histidine, and contains substantially less glycine and more glutamic acid. The difference in glutamic acid content may contribute to the lower electrophoretic mobility of pea histone I in polyacrylamide gel at pH 4.3. It is not known for either pea bud or calf thymus how much of the glutamic acid is present in native histone I as the amide.

Amino acid compositions of the pea histone I subfractions have not been obtained, but a relation similar to that found between calf thymus histones Ia and Ib is to be expected on the basis of the further characterization presented below.

Table II.

Amino Acid Compositions and Terminal Amino Acids of Lysine-rich Histones

	Pea Bud Histone I	Calf Thymus Histone Ia
Lys	25.5	27.7
His	1.1	----
Arg	2.8	1.8
Asp	2.3	2.1
Thr	4.0	5.7
Ser	4.9	6.6
Glu	7.3	3.6
Pro	9.9	9.2
Gly	2.3	6.8
Ala	22.8	25.6
Val	5.3	4.9
Met	----	----
Ile	1.9	1.0
Leu	4.1	4.1
Tyr	0.4	0.5
Phe	0.4	0.6
N-terminal	blocked	blocked
C-terminal	lysine	lysine

b. Terminal amino acids

The amino termini of both calf thymus and pea bud lysine-rich histones are blocked; no N-terminal amino acids can be detected by the Edman procedure or using the dansyl reagent.

Advantage was taken of the fact that upon enzymatic hydrolysis of a protein with blocked N-terminal amino group, the N-terminal peptide is often unique in bearing no positive charge at neutral pH, so that it can be purified on the basis of this characteristic. Digestion with pronase liberates such an N-terminal peptide from both calf thymus and pea bud lysine-rich histones. N-terminal analyses reveal that these peptides are indeed blocked. Enough of the calf thymus peptide was obtained to allow analysis of its amino acid composition (amino acid analyzer). Its composition is:

Thr	1.0
Ser	1.3
Glu	1.3
Pro	0.9
Ala	1.0

The N-terminal peptide was also prepared from a subtilisin digest of calf thymus histone I and has a composition Glu, Thr, Ser, Pro, Ala_{2-3}, Val as determined by high voltage electrophoresis and paper chromatography. Both the pronase and the subtilisin peptide are resistant to carboxypeptidase digestion and the smaller, pronase peptide has carboxyterminal proline, determined by hydrazinolysis.

From a partial acid hydrolysis of the pronase peptide three ninhydrin positive peptides were isolated by paper electrophoresis and sequenced by the dansyl-Edman procedure. Their sequences are Ser-Glu, Ser-Glu-Thr-Ala-Pro, and Ser-Glu-Thr-Ala-Pro. The high electrophoretic mobility of one of the pentapeptides suggests that it possesses two positive charges and is thus the result of an acid catalyzed acyl shift so that glutamic acid and threonine are linked in ester linkage.

The products of hydrazinolysis of the calf thymus pronase peptide were examined by paper chromatography. Among the hydrazides is a compound which has the same rf as synthetic acetylhydrazide and possesses identical fluorescence properties after reaction with Ehrlich's reagent (Hinman, 1956). Phillips (1963) has previously determined that calf thymus lysine-rich histone possesses one mole of acetyl group per 15,000 g protein. The present data support his conclusion that the N-terminal blocking group is indeed acetyl.

The pea histone I N-terminal peptide liberated by pronase digestion was similarly prepared. It also exhibits the composition Thr, Ser, Glu, Pro, Ala as determined by paper electrophoresis and probably also possesses a terminal acetyl group. Sufficient pea peptide for sequence determination has not yet been available. It was, however, subjected to partial acid hydrolysis and the resulting ninhydrin positive fragments examined by paper electrophoresis. The major resulting ninhydrin positive peptide is of mobility identical to that of the slower moving calf thymus pentapeptide and contains all of the same amino acids. No peptide of mobility equal to that of Ser-Glu could, however, be identified.

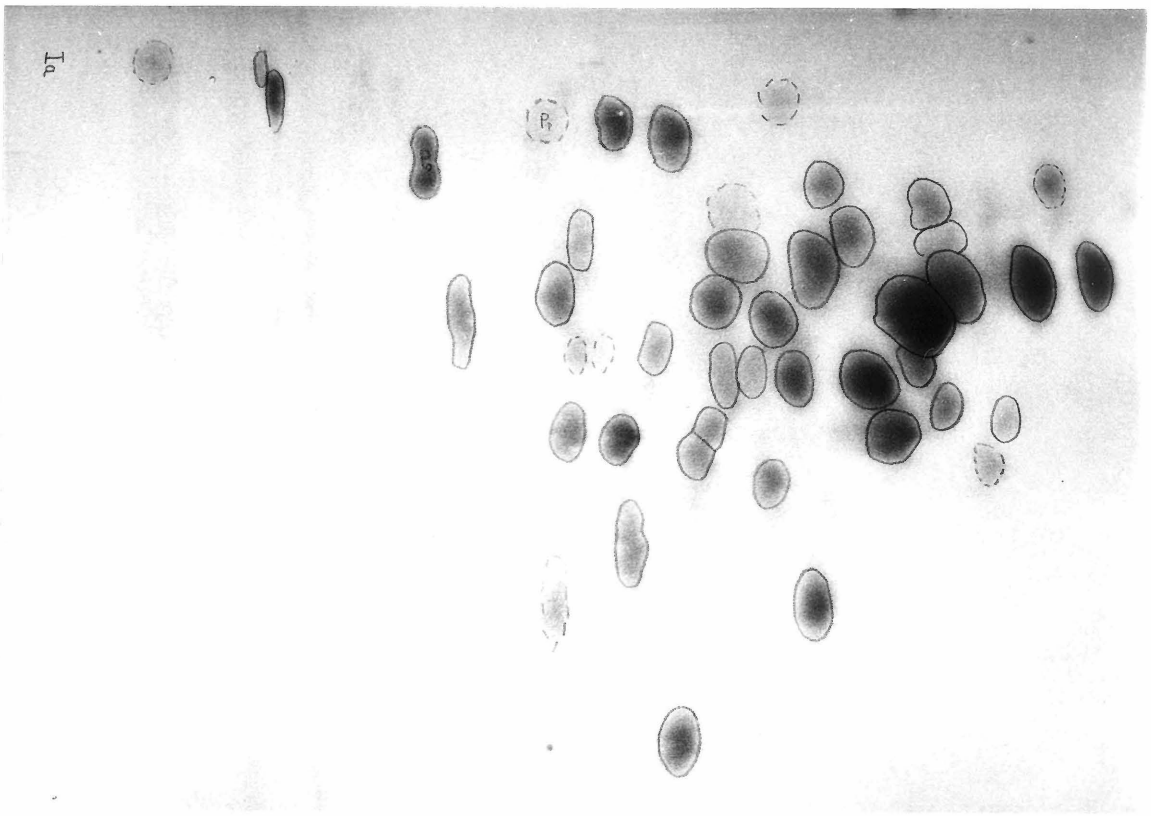
Important to the understanding of heterogeneity among the lysine-rich histone components of pea bud and calf thymus is the fact that only a single N-terminal peptide has been discovered in each case. The calf thymus N-terminal peptide can be prepared equally well from histone Ia or Ib.

C-terminal analysis by hydrazinolysis indicates that both calf thymus and pea bud histone I have lysine as the C-terminal amino acid of all components. Quantitative amino acid analysis indicates a yield of about 90% lysine as one mole per 62,000 grams of pea bud histone I and about 95% lysine as one mole per 67,000 grams protein for calf thymus histone I. These values are uncorrected for losses during the separation of the terminal amino acids. Since all other histone fractions yield on the order of one mole of C-terminal amino acids per 25,000-35,000 grams protein, the low yield of C-terminal amino acids from the lysine-rich histone is difficult to understand. One possible explanation is that a second C-terminal amino acid exists -- a C-terminal glutamine or asparagine which would be converted to a mono-hydrazide and lost during amino acid purification. This possibility is diminished by the demonstration that only lysine and a trace of alanine are released by carboxypeptidase AB digestion (37^{o}C, 30 min) of whole calf thymus histone I and of calf thymus histones Ia and Ib.

c. Peptide maps

"Tryptic fingerprints" or "peptide maps" of calf thymus histones Ia and Ib are presented in Figures 12 and 13. The first figure is a

Figure 12. Peptide maps of tryptic peptides of calf thymus histones Ia and Ib. Chromatography: n-propanol, ammonium hydroxide 7:3. Electrophoresis: pyridine acetate buffer, pH 3.5.


Ia

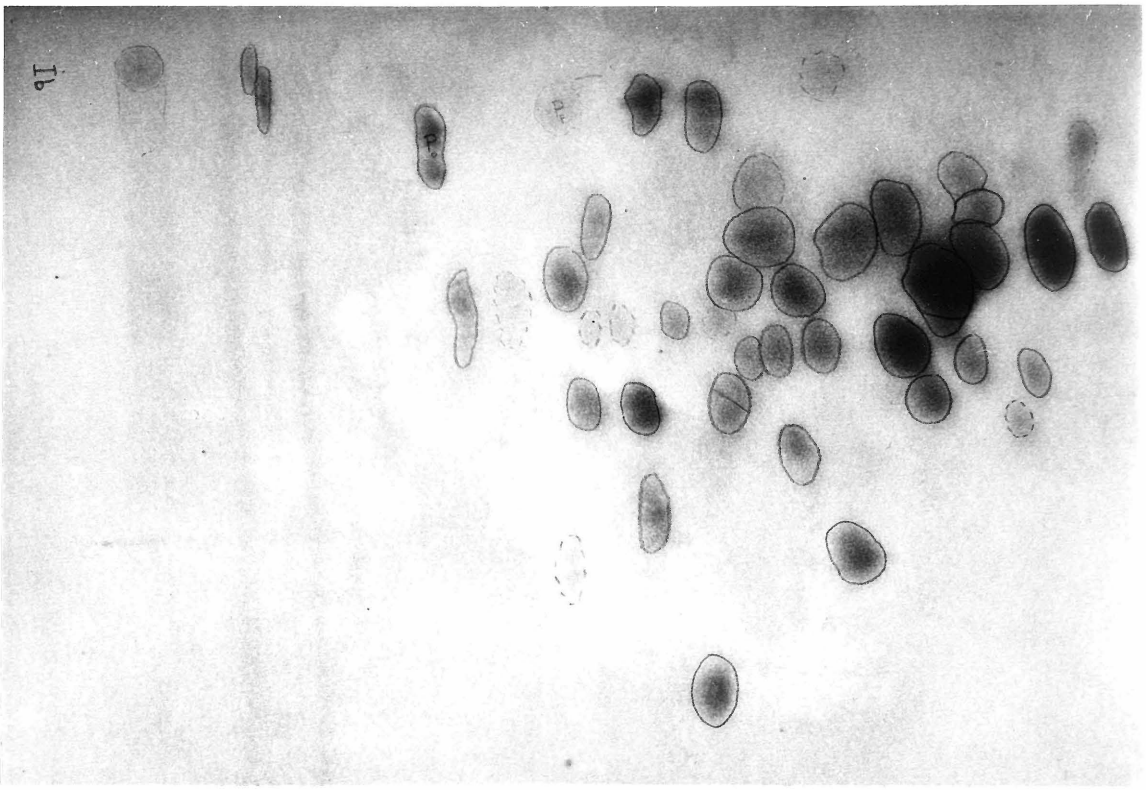
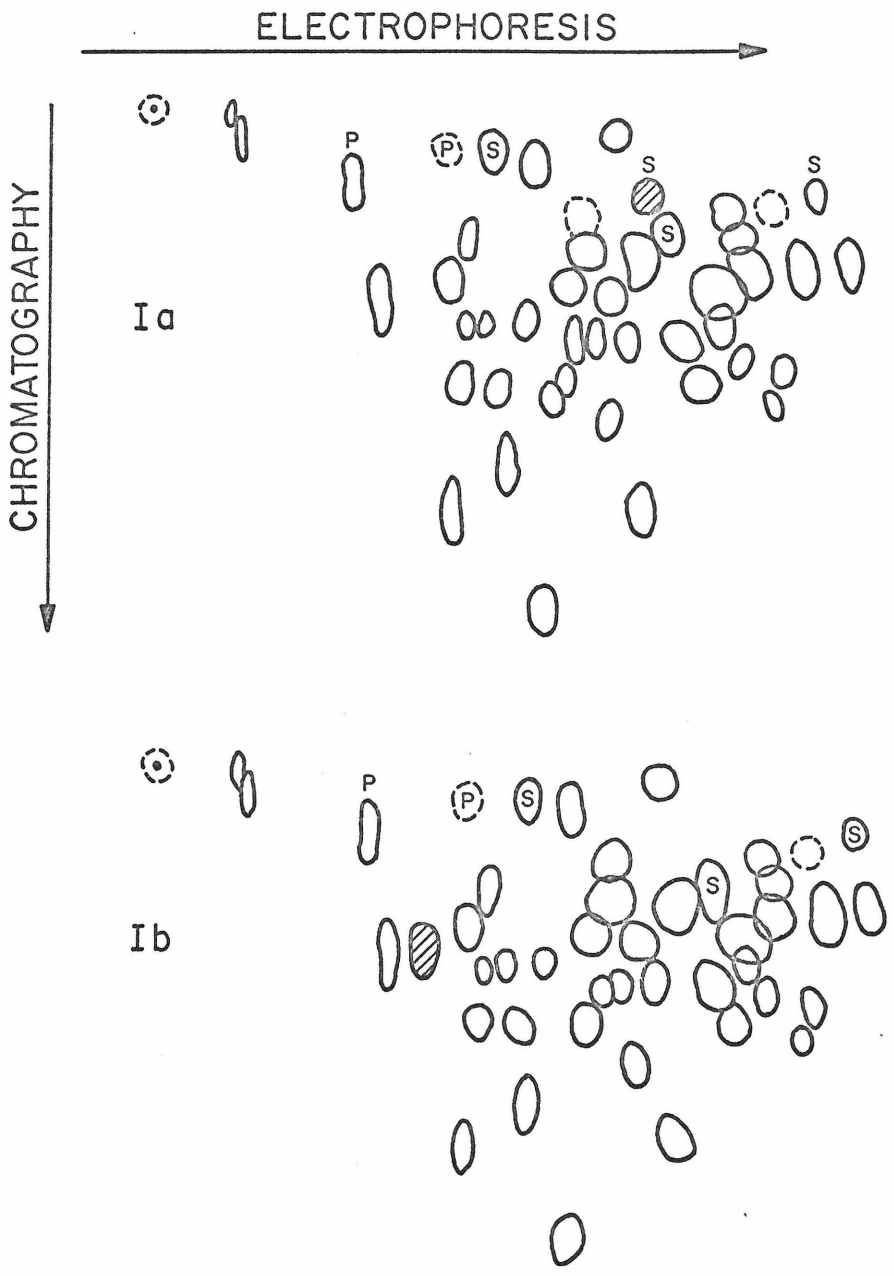

Ib

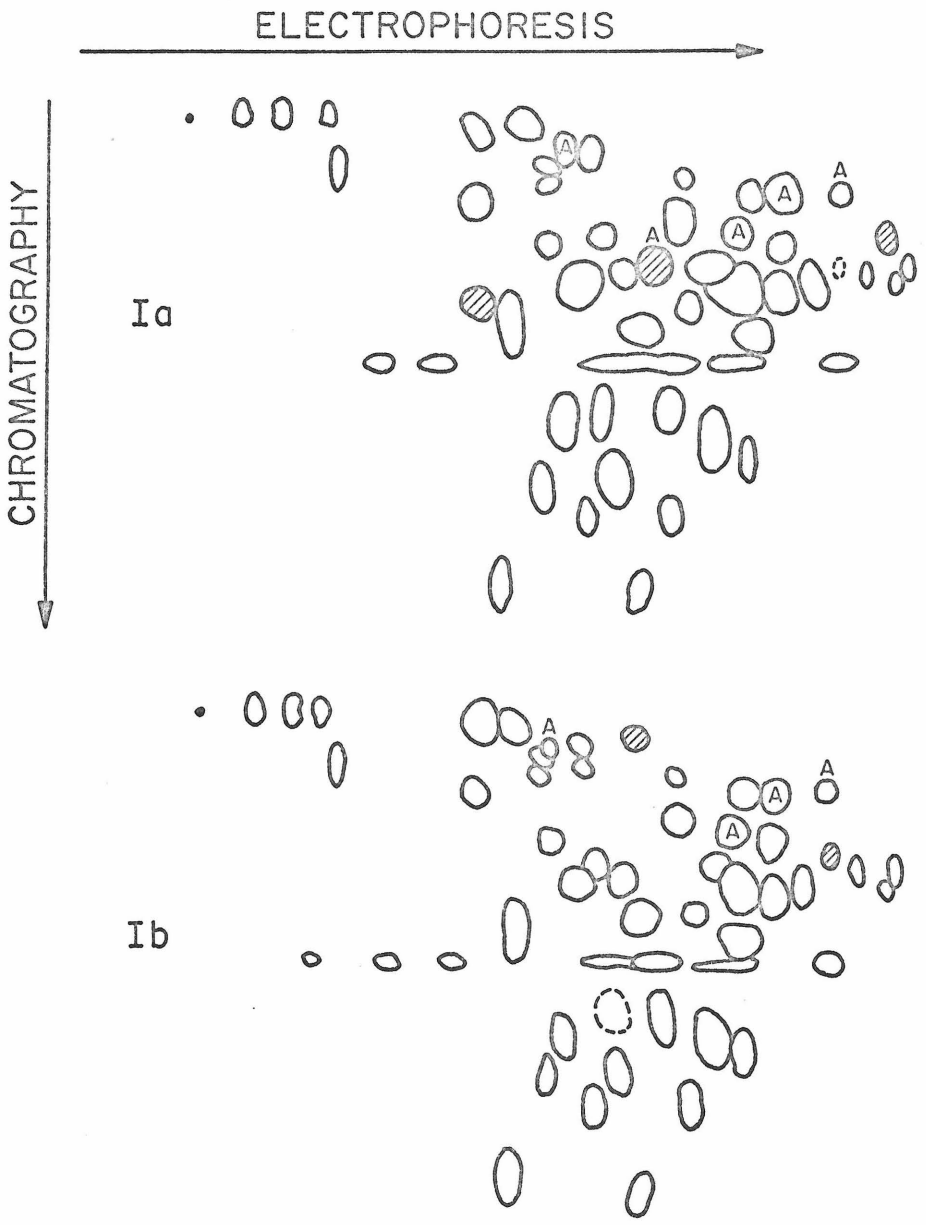
Figure 13. Distribution of arginine (S) and tyrosine (P) among the tryptic peptides of calf thymus histones Ia and Ib. ⌀ indicates peptide specific to Ia or Ib; ◌ peptide staining only faintly with ninhydrin. Note the extra arginine-containing peptide in histone Ia.


ELECTROPHORESIS
CHROMATOGRAPHY
Ia
Ib
P
S

pair of photographs of the collidine-ninhydrin stained maps, the second identifies the Pauly and Sakaguchi positive peptides. There is a single strong ninhydrin positive and Pauly reagent positive peptide common to Ia and Ib and a second weakly Pauly positive peptide. These same peptides in a ratio of about 10:1 have also been identified by Kinkade (1966) in chromatographic separation of histone I peptides. Three arginine containing peptides are also common to Ia and Ib, but the fourth arginine peptide is found exclusively in Ia. This is in agreement with the amino acid composition data, which show a slightly greater content of arginine in histone Ia than in Ib (Rasmussen et al., 1962; Kinkade, 1966).

Completely pure subfractions of pea bud histone I components have not been attainable even by use of preparative disc electrophoresis. However, subfractions rich in individual components were prepared and used for the preparation of peptide maps. These subfractions are described in the legend to Figure 14. Figure 14 shows the distribution of peptides from histones Ia and Ib. Peptides appearing on only one map or which show extremely different intensity of staining on the two maps are shaded. There are 4 arginine containing peptides common to pea histones Ia and Ib and a fifth found exclusively in Ia. This is quite analogous to the situation in calf thymus. That the pea histone I components contain 4 and 5 arginines while the calf thymus histone I components contain 3 and 4 arginines is in good agreement with the amino acid compositions reported above. These values of arginines per molecule are also in quite reasonable accord with the hypothesis that all lysine-

Figure 14. Distribution of tryptic peptides in peptide maps of pea bud histones Ia and Ib. Peptide maps were prepared from histone samples containing a) 96% histone Ib, b) 67% histone Ia and 30% histone Ib, c) 73% Ia, 7% Ib and 21% Ic. All of these peptide maps were quite similar. Peptides appearing with approximately equal intensity of staining in maps in which histone Ia or histone Ib peptides predominate are indicated by O; peptides judged to be specific to either Ia or Ib by Ø. Peptides containing arginine are marked "A". It is not possible to accurately judge which peptides are specific to histone Ic from the available data. However, peptide maps of the tryptic hydrolysates of histone I samples containing appreciable histone Ic do not contain many peptides in addition to those found in maps of histone I samples lacking Ic. Thus it is concluded that the primary structure of histone Ic is largely identical to that of histones Ia and Ib.


ELECTROPHORESIS
CHROMATOGRAPHY
Ia
Ib
A

rich histones contain one tyrosine and one phenylalanine per molecule. Assuming these values of arginine, tyrosine and phenylalanine per molecule the molecular weights of the lysine-rich histones must be somewhat greater than 20,000. Such a size is also in good agreement with the number of peptides seen in the peptide maps (45 for calf thymus histones Ia and Ib and 52 for pea bud histones Ia and Ib), assuming that some of the very intensely staining spots in the peptide maps represent several peptides and that there are probably some sequences such as Lys-Lys and Lys-Pro which are not cleaved during the tryptic hydrolysis.

Despite their marked compositional similarity and the similarity in end groups there is no obvious similarity in the peptide maps of calf thymus and pea bud histone I. However, large differences in chromatographic and electrophoretic properties may accompany small differences in peptide composition, so that even close structural homology may not be revealed in peptide maps.

On the other hand, the individual components of calf thymus and of pea bud histone I must have exceedingly similar primary structures. It would appear quite plausible that these components are closely related via common ancestor genes. The sort of heterogeneity exemplified by the calf thymus histone I components will henceforth be referred to as "microheterogeneity".

II. The slightly lysine-rich histones

a. Amino acid compositions

The amino acid compositions of pea bud and calf thymus slightly-lysine-rich histones are presented in Table III. The analyses of the calf thymus fractions are taken from recent publications by Mauritzen et al. (1967) and Hnilica et al. (1966). These are representative analyses, differing in only minor respects from those reported earlier by Rasmussen et al. (1962). These two calf thymus fractions can be separated cleanly only with great difficulty and the separation is best accomplished using methods different from thœ employed in our laboratory for the other fractionations. For this reason calf thymus fraction f2b (IIb2) was not prepared in pure form, but fraction f2a2 (IIb1) was prepared by Amberlite CG-50 chromatography of Johns method fraction f2a. It was found to chromatograph in the same region as histone f2b, as expected. A single analysis of this material is in agreement with the amino acid analyses of Mauritzen et al. (1967) and others, and indicates that this fraction IIb1 is rich in leucine and contains only slightly more lysine than arginine.

Both pea bud and calf thymus contain two very different fractions of slightly lysine-rich histone: one fraction of lysine/arginine ratio greater than 2 and a second of lysine/arginine ratio of approximately 1.2. Pea bud histone IIa and calf thymus histone IIb2 differ especially in content of serine and tyrosine. However, pea histone IIa is definitely composed of two molecular species (as shown by disc electrophoresis, C-terminal analysis and peptide mapping -- see below), one

Table III.

Amino Acid Compositions and Terminal Amino Acids of Slightly Lysine-rich Histones

	Pea Bud Histone IIa	Calf Thymus[a] Histone IIb2	Pea Bud Histone IIb	Calf Thymus[b] Histone IIb1
Lys	16.1	16.4	10.6	11.9
His	1.1	2.5	1.6	2.8
Arg	6.5	6.6	9.0	9.8
Asp	6.0	4.9	6.1	5.8
Thr	4.8	6.2	4.1	4.9
Ser	6.7	11.0	5.6	5.0
Glu	8.0	8.0	6.6	9.3
Pro	6.7	4.6	7.1	4.5
Gly	8.8	5.7	11.4	9.6
Ala	12.3	10.5	12.8	12.0
Val	6.7	7.0	7.9	5.9
Met	0.5	1.4	---	0.7
Ile	4.5	4.8	3.1	4.6
Leu	7.9	5.0	10.6	10.5
Tyr	1.7	3.8	1.9	1.7
Phe	1.9	1.5	1.6	1.2
N-terminal	proline	proline	blocked	blocked
C-terminal	serine alanine	lysine	alanine	lysine
grams histone per mole C-terminal*	30,500	30,200	34,700	29,000

[a]Taken from Hnilica *et al.* (1966).

[b]Taken from Mauritzen *et al.* (1967).

*Values uncorrected for losses.

of which may be more like calf thymus IIb2 than the mixture. There is some evidence that calf thymus histone IIb2 is also composed of two components (Phillips, 1967; Hnilica, 1965), although this may again represent microheterogeneity, since peptide maps of this histone are quite simple. Pea bud histone IIb and calf thymus histone IIb1 appear more nearly alike in general composition, both containing large amounts of leucine, glycine and alanine (more than 10 mole % each).

b. Terminal amino acids

The terminal amino acids of calf thymus and pea bud slightly lysine-rich histone fractions are presented in Table III. Proline is found as N-terminal amino acid in the pea bud and calf thymus components of higher lysine content. Pea bud histone IIb and calf thymus histone IIb1 are blocked at the amino terminal end and are presumably acetylated (Phillips, 1963). This has definitely been shown to be the case for calf thymus IIb1 (Phillips, 1967).

Pea histone IIa contains two C-terminal amino acids, alanine and serine, both obtained in high yield and in roughly equal amounts. In one experiment the histone IIa peak obtained by preparative disc electrophoresis was divided into early and late eluting protein and the C-terminals of these two subfractions were identified. The earlier eluting material (richer in histone IIa2) contained 62% alanine and 38% serine while the later eluting material (richer in histone IIa1) contained 55% alanine and 45% serine. Another sample of pea histone IIa, containing more IIa1 than IIa2 by quantitative analytical disc electrophoresis (see Chapter 2, "Methods") contained 64% serine and 36% alanine. Thus

it seems likely that the electrophoretically slower histone IIa1 contains C-terminal serine while IIa2 contains C-terminal alanine. Calf thymus histones IIb1 and IIb2 both have C-terminal lysine. It is interesting, but perhaps of doubtful significance, that calf thymus histones IIb1 and IIb2 share a common C-terminal amino acid while this is also true of one component of pea histone IIa and pea histone IIb.

c. Peptide maps

Pea fraction IIa yields 70 peptides on tryptic hydrolysis, compared with about 25 for calf thymus histone IIb2 (Hnilica, 1966), 43 for calf thymus histone IIb1, and 39 for pea bud histone IIb. This is quite compatible with the fact that pea IIa consists of two different components while pea histone IIb and calf thymus histones IIb1 and IIb2 consists of one molecular species each. There is little obvious similarity in the peptide maps of pea histone IIb and calf thymus histone IIb1.

III. The arginine-rich histones

a. Amino acid compositions

Amino acid compositions of pea bud and calf thymus arginine-rich histones are presented in Table IV. These analyses indicate that histones III and IV are quite distinct in composition. The analyses of corresponding pea bud and calf thymus histones show close compositional similarities indicative of homology. Histones III of both pea bud and calf thymus are particularly rich in glutamic acid and alanine

Table IV.

Amino Acid Compositions and Terminal Amino Acids of Arginine-rich Histones

	Pea Bud Histone III	Calf Thymus Histone III	Pea Bud Histone IV	Calf Thymus Histone IV
Lys	8.6(1.6)*	8.8(1.4)*	8.5	9.7(1.1)*
His	2.1	1.4	2.4	1.7
Arg	13.1	13.4	15.6	14.1
Asp	4.5	4.5	5.6	5.9
Thr	6.6	7.4	7.3	7.2
Ser	4.1	4.1	2.2	3.2
Glu	10.8	11.6	6.2	6.6
Pro	4.5	4.1	1.4	1.2
Gly	6.6	6.0	17.2	15.3
Ala	12.9	14.0	7.5	7.7
Val	5.2	4.4	6.6	7.4
Met	trace	trace	trace	trace
Ile	5.1	4.7	6.3	5.3
Leu	9.4	8.9	7.6	8.1
Tyr	1.0	1.7	3.0	3.5
Phe	3.9	2.8	2.7	1.9
N-terminal	alanine	alanine	blocked	blocked
C-terminal	alanine	alanine	glycine	glycine
grams histone per mole C-terminal**	24,400	36,300	31,700	22,200

*Values in parentheses are for e-methyl lysine in addition to lysine content.

**Values uncorrected for losses

and leucine, while pea bud and calf thymus histones IV are particularly rich in glycine and contain , for histones, an unusually small amount of proline. The unusual amino acid e-methyl lysine (Murray, 1964) is found in both pea bud and calf thymus histone III and in calf thymus histone IV. This amino acid is eluted as a shoulder on the descending slope of the lysine peak in the amino acid analyses. The constant for this amino acid in our analyzer is unknown and was therefore estimated to be the average of the constants for lysine, histidine and arginine. Since the constants for all amino acids except proline are rather similar, this estimate probably yields a reasonably accurate value for e-methyl lysine -- the error in its estimation is certainly no more than 10%.

b. Terminal amino acids

Homologous pea bud and calf thymus arginine-rich histones contain identical terminal amino acids (Table IV). Pea bud and calf thymus histones III contain N-terminal alanine and C-terminal alanine, while their histones IV contain blocked (probably acetylated; Phillips (1963)) N-terminal and contain C-terminal glycine. All terminal amino acids were recovered in greater than 90% purity and yields uncorrected for losses are reasonable for molecules with molecular weights of about 20,000. The C-terminal glycine of pea bud and calf thymus histone IV is in both cases resistant to liberation by carboxypeptidase, suggesting the presence of proline as penultimate amino acid at the carboxyl end of both pea bud and calf thymus histone IV.

c. Properties of histone III related to the presence of cysteine

When whole histone from pea bud or calf thymus is freshly prepared and fractionated by disc electrophoresis, histone III travels with a mobility 0.85 that of histone IV. If whole histone is allowed to remain in 8 M urea solution for one or two days, the mobility of histone III is drastically altered. The same phenomenon occurs when purified histone III is maintained in solution at neutral pH. Pea bud histone III is thereby converted to a form with mobility 0.55 that of histone IV, while calf thymus histone III is converted to a complex set of components with mobilities ranging from zero to 0.55 that of histone IV. During preparation of arginine-rich histones by either the method of Johns (1964), by that of Mauritzen et al., (1967) or by column chromatography on Amberlite CG-50 some of these components of lower mobility are produced. These are in all cases stable in 10 M urea, 0.2 N HCl or H_2SO_4, and 4 M guanidinium chloride and are unchanged by precipitation with trichloroacetic acid. The complex electrophoretic pattern of the calf thymus arginine-rich histone has long been taken as indicative of great molecular heterogeneity.

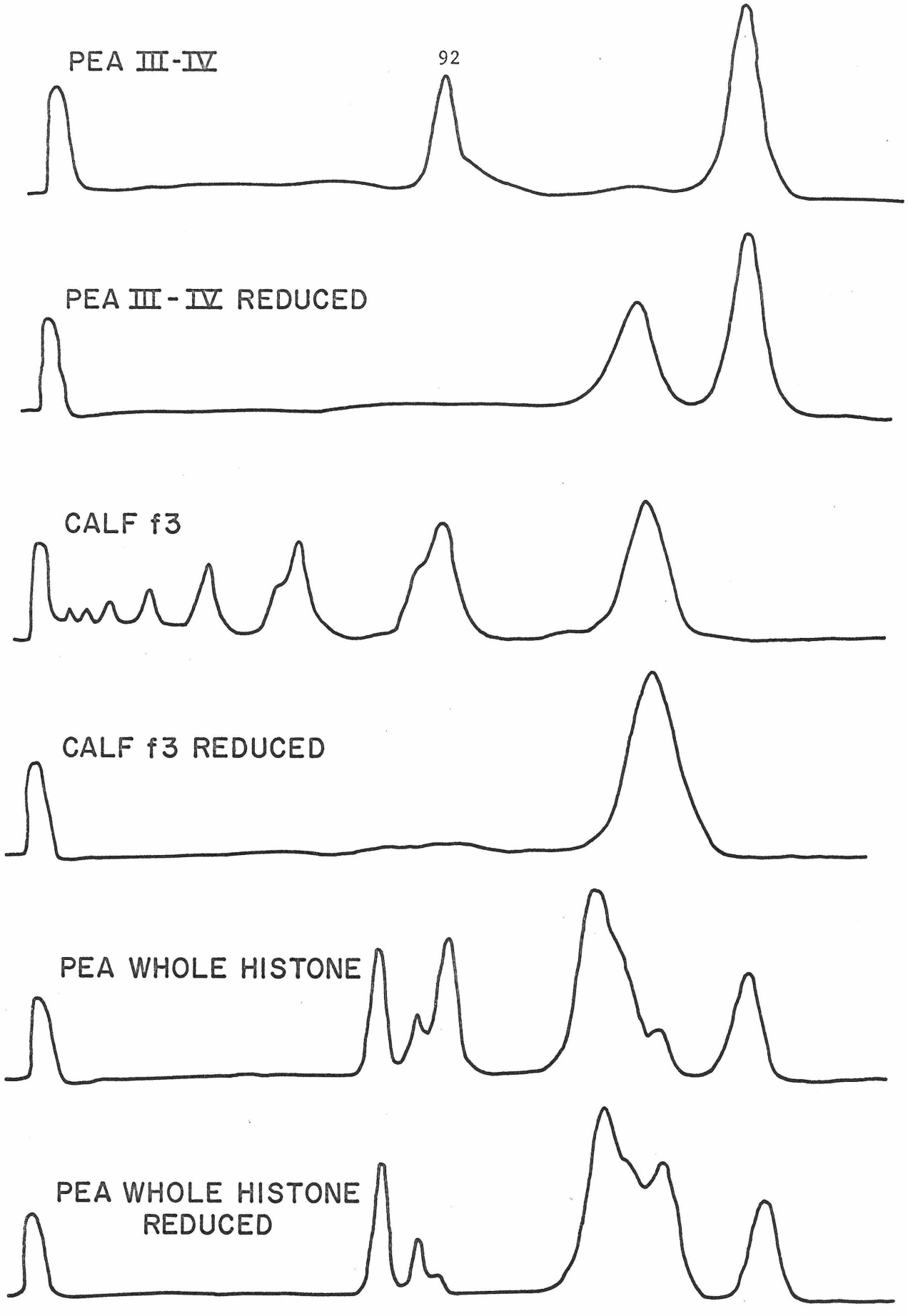
When pea histone III of low electrophoretic mobility is treated with β-mercaptoethanol, it is quantitatively converted to the more highly mobile form. When the electrophoretically complex mixture of calf thymus histone III is similarly treated, it is all converted to the single component of high electrophoretic mobility. This change in mobility is interpreted as due to change in size of the histone III molecules by reduction of inter-polypeptide chain disulfide bridges.

Thus the slow pea histone III component is interpreted as representing a dimer of the faster component, while the complex pattern of calf thymus histone III is interpreted as due to the presence of a mixture of dimer, trimer, tetramer, etc. The phenomena described above are illustrated in Figure 15. That the stated interpretation is correct will be shown below.

The difference in behavior between the pea bud and calf thymus oxidized (disulfide bridge containing) forms is most readily understood if one assumes that the pea bud histone III contains a single cysteine so that only dimers can form upon oxidation and that calf thymus histone III contains at least two cysteines so that various degrees of polymerization are possible, and so that structural isomers of the multimers can exist. The double bands present in what are thought to be the dimer and trimer components of the electrophoretic patterns may represent such alternative structural forms.

To test the hypothesis that pea bud histone III contains a single cysteine per molecule and calf thymus histone III contains at least two cysteines, pea bud and calf thymus histone III were each reduced with β -mercaptoethanol and then reacted with 14-C labelled iodoacetate under identical conditions. Correction for background reaction was made by using oxidized histone III and histone IV as controls. The controls indicate that about one third of the label found in the protein after the reaction is due to side reactions of iodoacetate with non-cysteine residues (probably largely histidine). One mole of acyl group was complexed specifically with reduced histone III for every 9,500 $\pm$ 1,000

Figure 15. Densitometric tracings of the electrophoretic patterns of pea bud and calf thymus histone fractions containing histone III in reduced and oxidized forms. From top to bottom they are: a) Pea bud histone III-IV as isolated by column chromatography (histone III in oxidized form). b) Pea bud histone III-IV prepared by column chromotography and subsequently treated with 0.1 M β-mercaptoethanol (8 M urea, 37°C, 30 minutes) (histone III in reduced form). c) Calf thymus histone f3 prepared by the method of Johns and further purified by column chromatography (histone III in both reduced and oxidized forms). d) Same material as in "c" except reduced with β-mercaptoethanol (histone III all in reduced form). e) Whole pea bud histone after prolonged storage in 8 M urea solution (histone III in oxidized form). f) Same material as in "e" except reduced with β-mercaptoethanol (histone III in reduced form).


PEA III-IV
PEA III-IV REDUCED
CALF f3
CALF f3 REDUCED
PEA WHOLE HISTONE
PEA WHOLE HISTONE
REDUCED

grams of calf thymus histone III and for every 23,000 ± 4,000 grams of pea histone III. Assuming a monomeric molecular weight of about 20,000, pea bud histone III thus contains one cysteine residue and calf thymus histone III two residues of cysteine per molecule.

It has been shown by Phillips (1965) that cysteine is present in the histone f3 fraction of calf thymus in the amount of one mole per 12,000 to 15,000 grams of protein. This was shown by performic acid oxidation followed by amino acid analysis. The presence of cysteine has also been demonstrated in CG-50 column separated pea bud histone III-IV by the same method (A. Sadgopal, personal communication). However, the reported occurrence of both nucleic acids and acidic protein in fractions prepared by these methods (Hnilica and Bess, 1965) made these observations of doubtful significance. The demonstration that properties of electrophoretically pure histone III are altered by reactions affecting disulfide and sulfhydryl groups clearly indicates the presence of cysteine residues in the histone III molecules themselves.

Hnilica and Bess (1965) have examined several electrophoretic components of oxidized histone III and on the basis of amino acid analyses and peptide maps have suggested that they represent aggregates of molecules with similar primary structure. The present data support this suggestion. To further demonstrate that the oxidative polymerization of calf thymus histone III is a non-specific reaction of identical monomeric units, the "dimer" and "trimer" components were each isolated by preparative disc electrophoresis, reduced to the monomeric form with β-mercaptoethanol, and the monomer then allowed to reoxidize

during extensive dialysis in 0.01 M tris buffer, pH 8.0. As control some native monomer was also allowed to oxidize. The complex electrophoretic patterns of the resulting materials are alike. In this particular experiment six electrophoretic components were observed in each case.

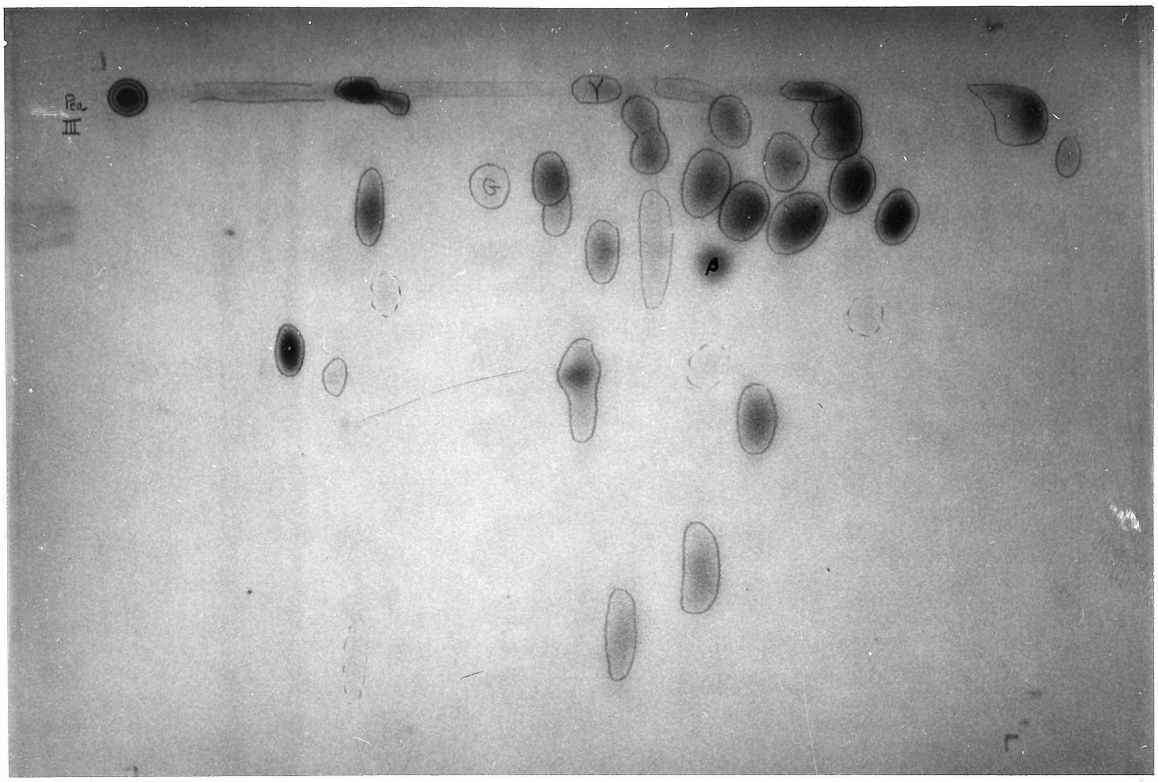
When histone is freshly prepared, most of the histone III is in the monomeric form. Before isolation of chromatin all of the histone III may perhaps be in this form. There have been several reports concerning the amount of disulfide in histone as a function of biological events in the genetic apparatus (see Stocken, 1967). The biological significance of these measurements is at present unclear.

d. Peptide maps

Peptide maps of tryptic digests of pea bud and calf thymus histone III and IV are presented in Figures 16 and 17. As evidenced by the ninhydrin positive material at the origins, not all of the tryptic peptides are soluble in the buffers used for chromatography and electrophoresis. It has been estimated for calf thymus histones f2a and f3 (Phillips, 1964) that about 27% of the protein in each case forms an insoluble precipitate of peptides upon tryptic hydrolysis.

The peptide maps of histones III and IV are entirely different. On the other hand, the peptide maps of homologous pea and calf thymus histones are extremely alike. Each map of histone III contains 29 peptides of which 26 are common to pea and calf, including one yellow and one gray staining peptide. The peptides unique to pea or calf

Figure 16. Peptide maps of the tryptic peptides of (a) pea bud histone III and (b) calf thymus histone III. The uncircled spot marked "β " is a trace of β-alanine from the electrophoresis buffer. "G" gray and "Y" yellow peptides (staining with collidine-ninhydrin reagent).


Y
G
A

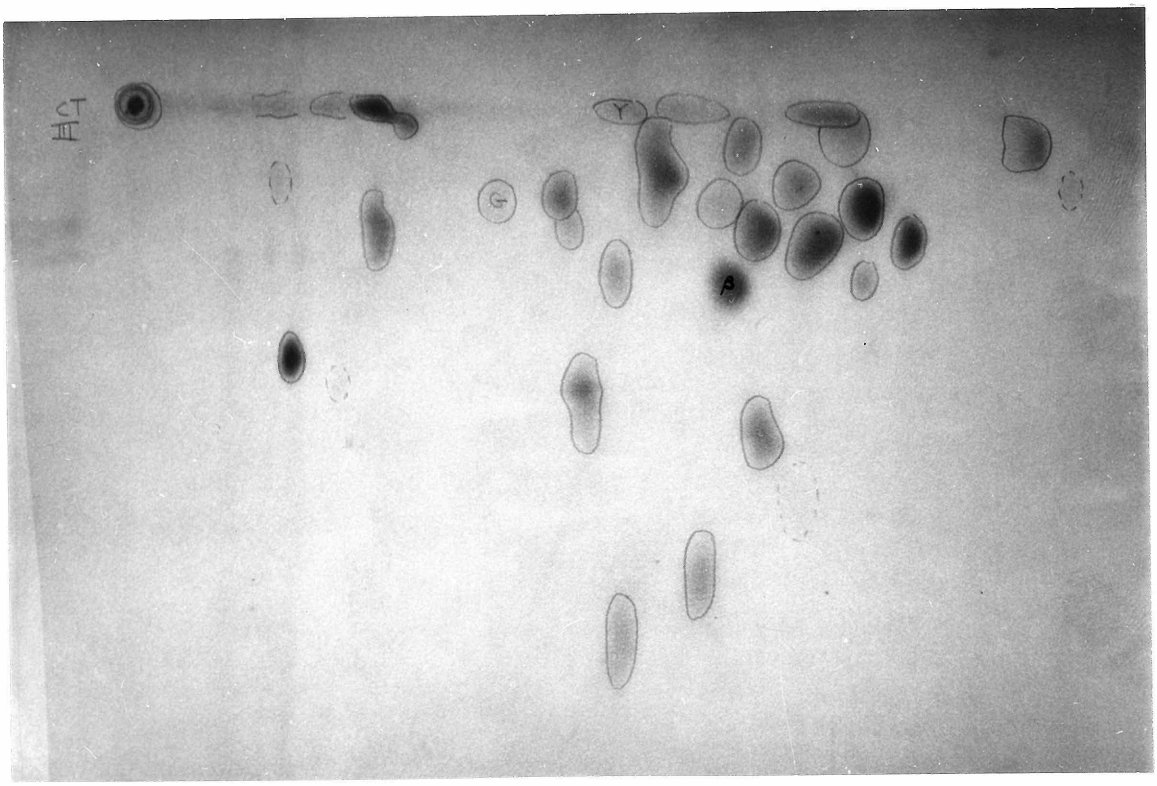
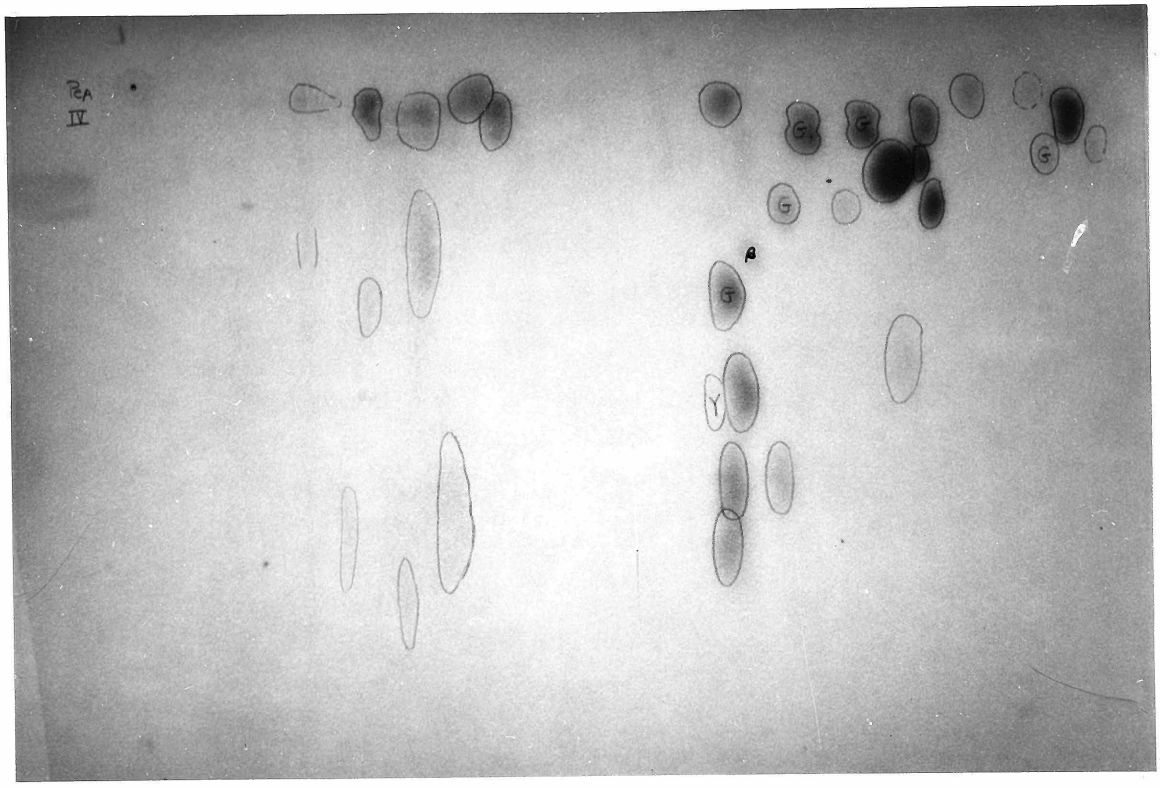
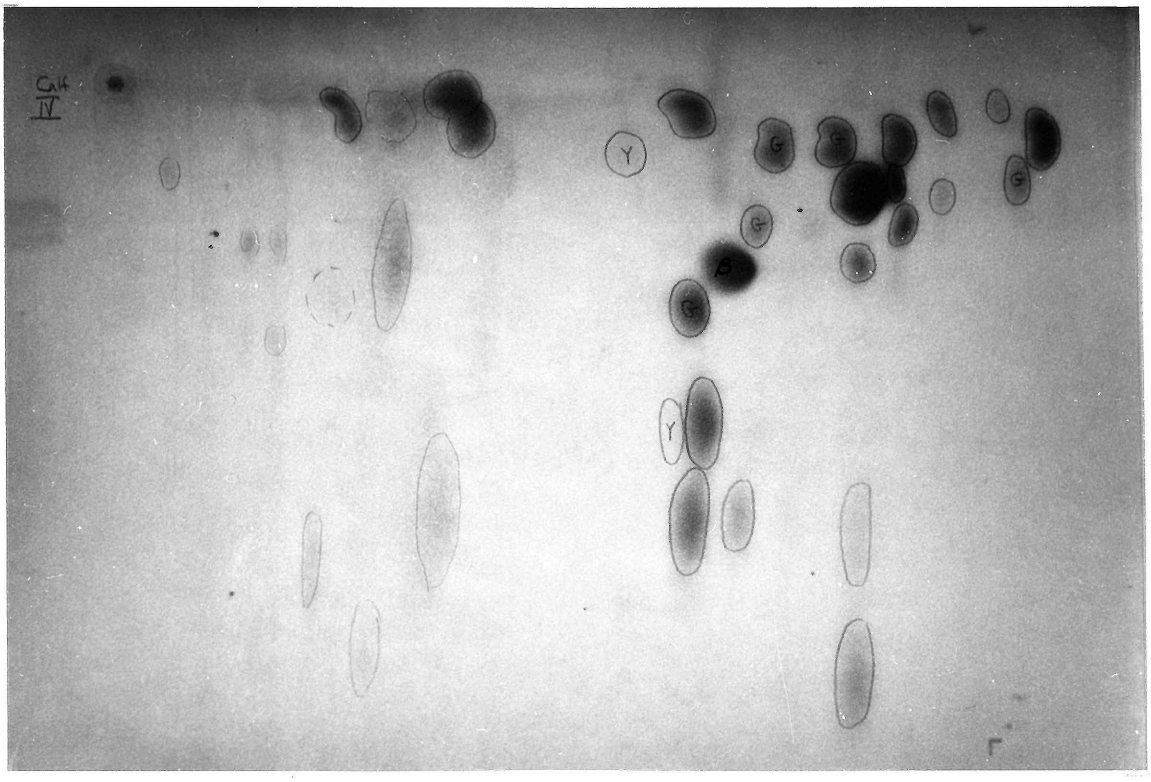

Y
G
A

Figure 17. Peptide maps of the tryptic peptides of (a) pea bud histone IV and (b) calf thymus histone IV. The uncircled spot marked "β" is a trace of β-alanine from the electrophoresis buffer. "G" gray and "Y" yellow peptides (staining with collidine-ninhydrin reagent).


PEA
IV
G
G
G
G
B
Y


CAH
IV
Y
G
G
G
G
B
Y

histone III are all weakly staining ones. There is some difference in the relative intensity of staining of corresponding peptides, suggesting that some of the spots represent two or more peptides in one case and fewer peptides in the other. Based on an estimated molecular weight of 20,000 the number of observed peptides is quite reasonable, assuming that some of the spots represent two or more peptides and that 20-30% of the peptides are at the origin.

Each map of histone IV contains 32 peptides of which 27 are common to pea and calf. These include five which stain a distinctly gray hue and another which stains yellow. Among the unique peptides in calf thymus histone IV is one which stains a bright yellow.

Discussion

Histone heterogeneity

Most histone fractions prepared to date have been heterogeneous. This is not so much an indication of histone heterogeneity, however, as it is testimony to the difficulties involved in the preparation and fractionation of histones. There are four principle sources of difficulty.

First, as illustrated in Figure 1 of this chapter, there is in many tissues an abundance of non-histone acid soluble protein which may contaminate to some extent even the purest of preparations of nucleoprotein. In the case of pea cotyledons many of these same non-histone proteins seem to be present in large quantities in the microsomal fraction and thus may well be acid soluble ribosomal proteins.

Second, when insufficient care is taken in the preparation of histones, some proteolysis of histones occurs. A case in point concerns the extremely lysine-rich $\alpha 1$ fraction obtained by Cruft et al. (1957). This fraction has not been found by other investigators. Johns (1964b) has demonstrated that the lysine-rich histones are extremely sensitive to proteolysis and has suggested that fraction $\alpha 1$ is a product of proteolysis. Degradation of lysine-rich histone during storage of calf thymus nucleohistone has often been observed. A complex electrophoretic pattern of calf thymus lysine-rich histone isolated from nuclei incubated for long periods of time at $37^{\circ}C$ has been reported by Reid and Cole (1964), and a complex chromatographic pattern of partially degraded calf thymus histone I has been observed by Kinkade (1966). Although deterioration of pea bud nucleohistone has not been observed by the present author, the elec-

phoretic pattern of some preparations of pea histone have occasionally been observed to contain a large number of bands in the histone Ia to histone IIa region together with an unusually small amount of components Ia and Ib. The occurrence of such patterns appears to be associated with the use of frozen and subsequently over-thawed tissues in the preparation of chromatin or with overheating of the tissue homogenates. Therefore such patterns are probably indicative of limited proteolysis of histone I.

Third, histones tend to aggregate both with themselves and with other proteins. Hnilica and Bess (1965) have reported the presence of acidic proteins and possibly nucleic acids in a purified f3 fraction of calf thymus histone. Cruft et al. (1958) and Mauritzen et al. (1967) have shown that the arginine-rich histones and histone f2a2 are caused to aggregate in the presence of even quite dilute solutions of NaCl at pH 5 and above. In this aggregation extensive β-conformation is presumably formed (Zubay and Wilkins, 1962). In the light of the findings reported in this thesis, it is also quite clear that in the process of precipitation of the arginine-rich histones there is also extensive polymerization of histone III by formation of disulfide bridges. Presumably such disulfide bridges can also be formed between histone III and cysteine-containing non-histone proteins. The cross contamination of histone fractions (presumably due to histone aggregation) is of very common occurrence (see, for example, Murray, 1964, 1965; Power and Butler, 1965).

Fractions of pea bud and calf thymus histones prepared by Amberlite CG-50 chromatography or by the Johns method are almost always demonstrably impure (see Figures 6, 7, 10, 11).

Fourth, at least one histone fraction is composed of two or more components of very similar primary structure. This "microheterogeneity" is, as far as is known, an exclusive characteristic of the lysine-rich histone fraction. On the basis of the peptide maps presented in Figures 12 and 13, calf thymus histones Ia and Ib appear to possess more than 90% sequence identity. The same appears to be true for pea bud histones Ia, Ib, and Ic, although the data are not so clear-cut. Despite extensive similarities in primary structure, both calf thymus and pea bud lysine-rich histones are partially separated by Amberlite CG-50 chromatography and pea bud lysine-rich histones can be separated by disc electrophoresis. There is little evidence for microheterogeneity in slightly lysine-rich and arginine-rich histone components. While such components are difficult to purify, each component has characteristics quite distinct from every other and each appears to be electrophoretically homogeneous. However, Hnilica (1965) has suggested on the basis of the kinetics and products of carboxypeptidase digestion that calf thymus histone f2b is heterogeneous. Phillips (1966) has made the same suggestion of the basis of identification of two N-terminal proline tryptic peptides. The corresponding pea bud histone IIa is definitely composed of two components, but the multiplicity of tryptic peptides from this fraction suggests that the two components are more than slightly dissimilar.

The data presented in this thesis indicate that calf thymus and pea bud histones are each composed of only a few molecular species. Each molecular species has been identified as a major component of some chromatographic fraction. Each has been identified as a single electrophoretic component in whole histone. Most components have been isolated by preparative disc electrophoresis, and are electrophoretically homogeneous upon repeated electrophoresis. Each histone component identifiable by chromatography and electrophoresis has been shown to possess a single N-terminal amino acid or to be blocked at the N-terminal end. Each has likewise been shown to possess a single C-terminal amino acid. The number of tryptic peptides obtained upon digestion of each histone component is consistent with each component being a single molecular species.

Calf thymus histone appears to be composed of six molecular species: Ia, Ib, IIb1, IIb2, III and IV. According to Kinkade (1966) histone I is composed of four molecular species. The present author has not been able to repeat his fractionation. There is, however, extensive agreement between the data of Kinkade and of the present author concerning such matters as the number of arginine and tyrosine peptides in histone I subfractions, the number of tryptic peptides obtained from such subfractions, and the occurrence of microheterogeneity. As mentioned above, it is possible that calf thymus histone IIb2 is not homogeneous, but the small number of peptides (twenty-five) separated in tryptic fingerprints of this histone (Hnilica, 1966) and the occurrence of indistinguishable histone IIb2 molecules in calf and chicken and rat argue against such

heterogeneity. The greatest apparent heterogeneity is found in histone III. Bellair and Mauritzen (1965) and Mauritzen et al. (1967) have reported isolation of many histone III components from chicken erythrocytes and calf thymus. Amino aicd analyses of these components are very similar. Hnilica and Bess (1965) have also isolated several components of histone III from calf thymus and on the basis of amino acid analyses and peptide maps suggest that these components are "aggregates of similar molecules". Data presented in this thesis (see Figure 15) confirm that histone III components are indeed complexes formed by disulfide bridges between apparently identical monomer units. This finding explains the complex electrophoretic patterns reported for the arginine-rich histones (Rasmussen et al., 1962) and the identification of large numbers of electrophoretic components in whole histone (Neelin and Neelin, 1960; Mauritzen, et al., 1967).

Pea bud histone appears to be composed of eight molecular species: Ia, Ib, Ic, IIa1, IIa2, IIb, III and IV. Of these only histones Ib, IIb, III and IV have been isolated in electrophoretically pure form. Although it is technically possible to separate histones IIa1 and IIa2 and histones Ia, Ib and Ic, these separations remain to be done. However, pea bud histone IIa and Iab appear to contain only two molecular species each.

Comparison of pea bud and calf thymus histones

Evolution of histones:

Pea bud and calf thymus contain homologous histone components which appear to be related by evolution and virtually identical in function.

This hypothesis is based upon similarities in chromatographic and electrophoretic behavior, amino acid compositions, terminal amino acids, and peptide maps of pea bud and calf thymus histone fractions. The hypothesis is further strengthened by the similarities in the strength of binding of homologous histones to DNA (see Chapter III). The principal similarities and differences between homologous calf thymus and pea bud histone components are summarized in Table V.

The evidence for homology between pea bud and calf thymus arginine-rich components is particularly convincing. The sequence homology indicated by terminal analyses and peptide maps seems much too extensive to have arisen by convergent evolution. The lysine-rich histones of pea bud and calf thymus share many characteristics such as selective extractability from nucleohistone with 0.5 M TCA and 0.5 M NaCl, very unusual amino acid compositions, identical terminal amino acids, and microheterogeneity. These similarities are also more easily explained by limited divergent than extensive convergent evolution. Calf thymus and pea bud slightly lysine-rich histones have similar chromatographic and electrophoretic properties and N-terminal amino acids. Pea bud histone IIb and calf thymus histone IIb1 are also quite similar in amino acid composition, and, as in the case of the lysine-rich histones, it appears likely that they are truly homologous. The similarities between the N-proline slightly lysine-rich components are not extensive. The two components of pea bud histone IIa may be quite dissimilar in composition and structure. Until these two components have been individually characterized, a more meaningful comparison with calf thymus histone IIb2 is not possible. On the basis

Table V

Major Similarities and Differences Between Homologous Calf Thymus and Pea Bud Histone Components*

Histone Component	Synonyms (1)	(2)	Relative Electrophoretic Mobility (3)	N-Terminal Amino Acid (4)	C-Terminal Amino Acid	Major Compositional Similarities and Other Features (5, 6)
Calf Iab	f1	α	slow	blocked	Lys	Lysine-rich histones containing 25-28% Lys, 22-24% Ala, 8-10% Pro, <1% Tyr and Phe, no Met. Microheterogeneity. Pea contains His.
Pea Iabc			slow	blocked	Lys	
Calf IIb2	f2b	γ	intermediate	Pro	Lys	Slightly lysine-rich histones containing 16-17% Lys and 6-7% Arg. Calf is serine-rich. Pea is two components.
Pea IIa1,2			intermediate	Pro	Ser,Ala	
Calf IIb1	f2a2	β	intermediate	blocked	Lys	Slightly lysine-rich histones containing 10-11% Leu and 11% Gly. Lys/Arg ratio about 1.2. All amino acids present to comparable extent in pea and calf.
Pea IIb			intermediate	blocked	Ala	
Calf III	f3	β	complex (7)	Ala	Ala	Arginine-rich histones containing e-methyl Lys, Cys, 11% Glu. All amino acids present to comparable extent in pea and calf. Extremely similar peptide maps.
Pea III			complex (7)	Ala	Ala	
Calf IV	f2a1	β	fast	blocked	Gly	Arginine-rich histones containing 15-17% Gly, <2% Pro. e-methyl Lys detected only in calf. Other amino acids present to comparable extent. Extremely similar peptide maps.
Pea IV			fast	blocked	Gly	

*See notes on following page.

Notes on Table V.

(1) Nomenclature of Johns, Phillips and Butler. See Butler (1965).

(2) Nomenclature of Cruft, Hindley, Mauritzen and Stedman (1957).

(3) Electrophoresis in polyacrylamide gel at pH 4.3.

(4) Blocked N-terminal groups are presumably N-acetylated. This is known to be the case for calf thymus histone IIb1 (Phillips, 1966) and Iab.

(5) Values for amino acid composition are in Mole Per Cent.

(6) In addition, all histone fractions lack tryptophan. This has been demonstrated by fluorometric analysis (R. Jensen, unpublished data) and by negative results from staining peptide maps with Ehrlichs reagent. All histone fractions except histone III also lack cysteine.

(7) The complex electrophoretic patterns of calf and pea histone III have been shown to be due to the formation of histone III complexes by interpeptide chain disulfide bridges. Pea histone III, containing one cysteine per molecule, can exist as monomer and dimer. Calf histone III, containing two cysteines per molecule, can exist as monomer and various sized multimers.

of the limited data at hand and by analogy with other histone fractions, an evolutionary relationship between pea bud histones IIa and calf thymus histone IIb2 seems probable.

There is some justification for inferring that the characteristics common to the histones of pea bud and calf thymus are common to the histones of all higher plants and animals. First, the histones of all tissues of an organism appear to be qualitatively identical. Crampton et al. (1957) prepared histone fractions from calf thymus, liver, and kidney by ion exchange chromatography and found a common elution pattern and virtually identical amino acid compositions of corresponding fractions. Hnilica et al., (1962) compared fractions f1, f2 and f3 of calf thymus, spleen and liver by amino acid analyses, N-terminal analyses and starch gel electrophoresis and found no tissue specificity. Similar findings have been reported for various rat tissues (Hnilica et al., 1962; Neidle and Waelsh, 1964; Hnilica, 1966) and chicken tissues (Lindsay, 1964). In Chapter II of this thesis the histones of various pea tissues are shown to be chromatographically and electrophoretically like those of pea bud.

Second, the histones of all vertebrates appear to be quite similar. Comparisons have been made between calf and guinea pig (Crampton et al., 1957), calf and rat (Hnilica et al., 1962; Laurence et al., 1963, 1966), rat, mouse and guinea pig (Neidle and Waelsch, 1964), calf, rat and chicken (Hnilica, 1966), calf and trout (Palau and Butler, 1966). These studies indicate very limited species specificity. Similarly, but on the basis of electrophoretic analysis only, it appears that the histones of all vascular plants are quite similar. The electrophoretic pattern of pea

bud histones closely resembles the electrophoretic pattern of lily histones reported by Sheridan and Stern (1967) and the electrophoretic patterns of the histones of Cestrum, ivy, onion, cucumber, tobacco and mung bean (Smart and Fambrough, unpublished observations).

Data on the histones of lower plant and animal phyla are quite limited. The report concerning the histones of Chlorella (Iwai, 1964) is quite fragmentary, and the reported occurrence of histones in yeast (Tonino and Rozijn, 1966) is of questionable validity. Cruft (1966) has reported the absence of histones from the ctenophore Beroë cucumis and refers to the work of Leaver (1964), demonstrating the absence of histones in various protozoa. The occurrence of histones in the protozoan Tetrahymena, has however, been reported (Lee and Scherbaum, 1966). Electrophoretic analysis of the histones of sea urchin embryos (Marushige and Ozaki, 1967) and sea urchin and starfish sperm (Palau and Subirana, 1966) suggest that echinoderm histones are quite like those of the vertebrates. Preliminary electrophoretic analysis similarly suggests that Drosophila histones are vertebrate-like (Griffith, unpublished observation).

Present data do not permit us to trace the origin and evolution of the histones. A common evolutionary origin of higher plant and animal histones strongly implies the existence of histones in all creatures phylogenetically related to the common ancestor of plants and animals. The limited molecular evolution especially of the arginine-rich histones will perhaps make the histones especially valuable in studying relationships between large phylogenetic groups.

The genetic code has apparently remained virtually unaltered through eons of evolution, and the processes of DNA replication, RNA synthesis and protein synthesis are thought to be virtually identical in all organisms. The intimate association of histones with the genetic material is surely a prime factor in the restriction of change in histone structure. As discussed in the "General Introduction", features common to homologous molecules of phylogenetically remote organisms are most likely features crucial to the proper function of the molecules. By this logic it can be concluded that the essential features of histone molecules are very many, and that most mutations resulting in altered primary structure of histones must be lethal. Restrictions on the mutability of the arginine-rich histones must be especially severe.

A more precise concept of histones:

No acceptable definition of histones more precise than that offered in the "General Introduction" (page 1.) has heretofore been proposed. When the function of each molecular species of histones is known, histones can then be precisely defined in terms of function. In the absence of such knowledge it is still possible to sharpen our concept of histones as more information on the nature of histones becomes available. The data presented in this thesis demonstrate that the histones of phylogenetically remote organisms are quite similar and suggest that the same molecular set found to compose vertebrate and angiosperm histones will be found to compose the histones of all higher organisms. Thus the concept of histones as basic proteins natively complexed with DNA can be refined to

include only those basic proteins which resemble in chemical and physical properties the few molecular species of calf thymus and pea bud histones described above. This concept can be ammended to include the very specialized basic proteins (the protamines) found complexed with DNA in the gametes of certain organisms.

References

Bellair, J.T. and Mauritzen, C.M. (1965), Australian J. Biol. Sci. 18, 160.

Bennett, J.C. and Dreyer, W.J. (1967), unpublished method.

Bonner, J., Chalkley, G.R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R.C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H., Olivera, B.M., and Widholm, J. (1967). Methods in Enzymology: Nucleic Acids Academic Press, New York. In press.

Butler, J.A.V. (1966) in Histones - Their Role in the Transfer of Genetic Information, Ciba Foundation Study Group No. 24, Little, Brown and Co., Boston, p.4.

Crampton, C.F., Stein, W.H., and Moore, S. (1957), J. Biol. Chem. 225, 363.

Cruft, H.J. (1966), Biochem. J. 101, 36P.

Cruft, H.J., Hindley, J.H., Mauritzen, C.M., and Stedman, E. (1957), Nature 180, 1107.

Cruft, H.J., Mauritzen, C.M., and Stedman, E. (1958) Proc. Roy. Soc. (London), Ser. B, 149, 21.

Dreyer, W.J. and Bynum, E.L. (1967), Methods in Enzymology Vol XI: Enzyme Structure, Academic Press, New York.

Edman, P. (1960), Ann. N.Y. Acad. Sci. 88, 602.

Edman, P. and Sjöquist, J. (1956), Acta Chem. Scand. 10, 1507.

Gray, W.R. (1967), Methods in Enzymology, Vol. XI: Enzyme Structure, Academic Press, New York.

Hinman, R. (1956), Anal. Chim. Acta 15, 125.

Hnilica, L.S. (1966), Biochim. Biophys. Acta 117, 163.

Hnilica, L.S. (1965), in Developmental and Metabolic Control Mechanisms and Neoplasia (The University of Texas M.D. Anderson Hospital and Tumor Institute) Williams and Wilkins Co., Baltimore. p 273.

Hnilica, L.S. and Bess, L.G. (1965), Anal. Biochem. 12, 421.

Hnilica, L.S., Edwards, L.J., and Hey, A.E. (1966), Biochim. Biophys. Acta 124, 109.

Hnilica, L.S., Johns, E.W., and Butler, J.A.V. (1962), Biochem J. 82, 123.

Iwai, K. (1964) in The Nucleohistones (J. Bonner and P.(.P. Ts'o, eds.) Holden-Day, San Francisco, p 29.

Johns, E.W. (1964b), Biochem. J. 93, 161.

Johns, E. W. (1964), Biochem. J. 92, 55.

Johns, E.W. and Butler, J.A.V. (1962), Biochem. J. 82, 15.

Johns, E.W. and Butler, J.A.V. (1962b), Biochem. J. 84, 436.

Kinkade, J. (1966), Ph.D. Thesis, University of California, Berkeley.

Kleinsmith, L.J., Allfrey, V.G., and Mirsky, A.E. (1966) Proc. Nat. Acad. Sci. (Wash.) 55, 1182.

Kostka, V. and Carpenter, F.H. (1964), J. Biol. Chem. 239, 1799.

Laurence, D.J.R., Phillips, D.M.P., and Butler, J.A.V. (1966), Archs. Biochem. Biophys. 113, 338.

Laurence, D.J.R., Simson, P., and Butler, J.A.V. (1963) Biochem. J. 87, 200.

Leaver, J.L. (1964), Ph.D. Thesis, University of Edinburgh.

Lee, Y.C. and Scherbaum, O.H. (1966), Biochemistry 5, 2067.

Lindsay, D.T. (1964), Science 144, 420.

Lowry, O.H., Rosebrough, N.J., Faar, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265.

Luck, J.M., Rasmussen, P.S., Satake, K. and Tsvetikov, A.N. (1958), J. Biol. Chem. 233, 1407.

MacGillivray, A.J. (1966), Biochem J. 101, 24P.

Marushige, K. and Bonner, J. (1966), J. Mol. Biol. 15, 160.

Marushige, K. and Ozaki, H. (1967), Dev. Biol., in press.

Mauritzen, C.M., Starbuck, W.C., Saroja, I.S., Taylor, C.W. and Busch, H. (1967), J. Biol. Chem. 242, 2240.

Murray, K. (1965), Ann. Rev. Biochem. 34, 209.

Murray, K. (1964), in The Nucleohistones (J. Bonner and P.O.P. Ts'o, eds.) Holden-day, San Francisco. p. 15.

Murray, K. (1964b), Biochemistry 3, 10.

Narita, K. (1958), Biochim. Biophys. Acta 28, 184.

Neelin, J.M. and Neelin, E.M. (1960), Canad. J. Biochem. Physiol. 38, 354.

Neidle, A. and Waelsch, H. (1964), Science 145, 1059.

Nui, C. and Fraenkel-Conrat, H. (1955), J. Am. Chem. Soc. 77, 5822.

Palau, J. and Butler, J.A.V. (1966), Biochem J. 100, 779.

Palau, J. and Subirana, J.H. (1966), Biochem. J. 101, 34P.

Phillips, D.M.P. (1963), Biochem J. 87, 258.

Phillips, D.M.P. (1965), Biochem. J. 97, 669.

Phillips, D.M.P. (1966), Biochem. J. 101, 23P.

Phillips, D.M.P. (1964), in The Nucleohistones (J. Bonner and P. O.P. Ts'o, eds.) Holden-Day, San Francisco, p. 46.

Phillips, D.M.P. and Johns, E.W. (1965), Biochem. J. 94, 127.

Rasmussen, P.S., Murray, K., and Luck, J.M. (1962) , Biochemistry 1, 79.

Rees, M.W. (1946), Biochem. J. 40, 632.

Reid, B.R. and Cole, R.D. (1964), Proc. Nat. Acad. Sci. (Wash.) 51, 1044.

Reisfeld, R.A., Lewis, N.J., and Williams, D.E. (1962), Nature 195, 281.

Satake, K., Rasmussen, P.S., and Luck, J.M. (1960), J. Biol. Chem. 235, 2801.

Sheridan, W.F. and Stern, H. (1967), Exptl. Cell Res. 45, 323.

Sjöquist, J. (1960), Biochim. Biophys. Acta 41, 20.

Stocken, L.A. (1966), in Histones - Their Role in the Transfer of Genetic Information, Ciba Foundation Study Group No. 24, Little, Brown, and Co., Boston, p. 62.

Tonino, G.J.M. and Rozijn, T.H. (1966), Biochim. Biophys. Acta 124, 427.

Zubay, G. and Wilkins, M.H.F. (1962), J. Mol. Biol. 4, 444.

CHAPTER II.

In this chapter, the histones of different pea tissue are compared both qualitatively and quantitatively in order to answer the questions: Are there histones unique to different pea tissues? and -- Are there quantitative differences in the proportions of the histone components of different pea tissues? The concept that different molecular species of histones might be found in different types of cells of the same organism arose originally from the observation that the sperm of some fish contain an unusual sort of histone, protamine. Important to the further development of this concept were the findings that a single type of cell contains several histones and that there are reproducible differences in amino acid composition between crude histone fractions of chicken erythrocytes and of other chicken tissues (Stedman and Stedman, 1950, 1951). These observations gave rise to the theory that the main function of histones is control of gene expression. Implicit in the simplest statement of this theory is the idea of gene recognition by specific histone molecules (histone specificity) and thus great histone heterogeneity.

Although several demonstrations of histones unique to specific tissues have been reported (Mauritzen and Stedman, 1959, 1960; Davis and Busch, 1959; Neelin and Butler, 1961; Lindsay, 1964; Lee and Scherbaum, 1966), more refined methods of histone analysis have failed to demonstrate the existence of different histones in different types of somatic cells or even the existence of markedly different histones

in different species (Crampton et al., 1957; Hnilica et al., 1962; Neidle and Waelsch, 1964; Laurence et al., 1963, 1966; Palau and Butler, 1966; Hnilica, 1966; Fambrough and Bonner, 1966). Further, improved methods of preparation and analysis indicate that there is only a small number of molecular species of histone (Hnilica and Bess, 1965; Butler, 1966; and see Chapter I).

The basis of most of the observations of cell specificity and heterogeneity of histones can seemingly be attributed to aggregation of histones, proteolysis, the presence of ribosomal and other "minor components", the formation of histone III complexes by the oxidative coupling of cysteine residues, and analysis of histone mixtures in which there is quantitative variation of individual components.

There are, however, convincing reports of quantitative variations in the proportions of histones. These show that the histone:DNA ratio may vary from one tissue to another(Sporn and Dingman, 1963; Dingman and Sporn, 1964; Bonner et al., 1967a) and that there is variation in the amounts of individual histone components present in different tissues (Hnilica et al., 1966).

This report demonstrates a lack of tissue specificity among pea histones and the occurrence of reproducible quantitative differences in the distribution of histones in different pea tissues.

Methods

Pea Tissues

Histones were obtained from the following tissues of Pisum sativum var. Alaska: embryos, apical buds, etiolated stems, roots, leaflets, whole leaves, flowers, pods, seeds, and cotyledons.

Embryos were prepared by germination of mature pea seeds in tap water, mechanical fragmentation of embryos from the cotyledons and seed coats by application of slight pressure, and separation from cotyledons and seed coats by filtration and buoyancy in sucrose solution. Apical buds were harvested from etiolated seedlings. Etiolated stems and roots were harvested from seedlings after removal of the apical buds. Leaflets, whole leaves, flowers, pods, and seeds were harvested from mature plants grown under field or greenhouse conditions. Pods were prepared by removal of seeds from the fruit. Cotyledons were prepared from pea seeds by removing the seed coats and embryos by hand. A method was devised for obtaining very homogeneous collections of pea seeds in different stages of maturation. After separation of pea seeds into two size classes, using a sieve with pores 0.8 cm in diameter, each lot was further subdivided on the basis of buoyant density. The seeds, contained in a wire mesh basket, were transferred to successively denser sucrose solutions, and those peas which floated were removed. Individual seeds in each density fractions which failed to resemble the majority in color and size were discarded. During maturation the seeds increase in size and decrease in density until they very nearly reach full size, and thereafter they become increasingly dense.

A possible explanation of this phenomenon is that during growth in seed size starch and/or concentrated sugar solution energy stores are consumed in the synthesis of less dense lipid and protein materials; then as mature size is approached the synthesis of denser storage protein and starch grains together with loss of water results in increasing density. Even before marked desiccation, maturing pea seeds become more dense than saturated sucrose solution. Concomitant with changes in density there are changes in seed color from an initial light yellow-green to a pale green (due to thickening of the seed coat) and then a progressive darkening to a dark bluish green as the seed coat becomes thin and tough and the cotyledons become more highly pigmented.

By observing pod morphology as a function of the ripening of the fruits and correlating these observations with determination of the maturation stages of the contained pea seeds, a rough timetable of pea seed maturation was deduced. For these studies greenhouse plants were used. Although reasonable correlation between maturation stage and pod morphology was found, often seeds in more than one stage of maturation were present in the same pod. A description of maturation stages of pea seeds is presented in Table I.

Preparation of Chromatin and Histone

For the preparation of histones minimally contaminated by non-chromosomal proteins it is necessary to use purified chromatin as the starting material for histone extraction. Purified chromatins of various pea tissues were prepared according to the method of Bonner

Table I

Maturation Stages of Pea Seeds

Stage	Cotyledon Diameter (mm)	Density of seeds	Age (days)[a]	Other Characteristics
1	4-5	1.030-1.039	19	Liquid in seeds, cotyledons elongated and not oppressed.
2	6-7	1.022-1.030	21	Liquid in seeds, cotyledons only partially oppressed and not yet rounded.
3	7	1.022	24	Little or no liquid in seeds, cotyledons approximately round.
4	8	1.022	27	Cotyledons fill seeds, seed coat thick and very lightly pigmented.
5	8-9	1.022-1.030	30	Seed coat becoming thinner, cotyledons well rounded.
6	8-9	1.030-1.039	32	Cotyledons becoming increasingly tough, seeds appear a darker green.
7	8-9	1.039-1.064	34	Seed coat becoming progressively thinner and tougher, seeds becoming a darker bluish green, cotyledons becoming quite hard.
8	8-9	1.064-1.095	36	
9	8-9	1.095-1.192	39	
10	8-9	---	45	Seeds completely dry. These seeds are soaked in water for 8-12 hr before use.

[a] Days after open flower.

et al. (1967b). Purified chromatin was extracted with 0.2 N H_2SO_4 and the residue reextracted with 0.4 N H_2SO_4. The histones were precipitated from the extract with three volumes of ethanol at -20° for 36 hr. The purified chromatin from some tissues, especially leaves, was contaminated with chloroplasts or fragments thereof. Such contamination was not removed by repeated sedimentation through 1.7 M sucrose. However, typical pea histones could be prepared by acid extraction of this material. In the case of mature cotyledons much difficulty was encountered in preparing chromatin not contaminated by storage protein and ribosomes. Thus significant amounts of acid- soluble non-histone proteins (as defined by column chromatography, disc electrophoresis and amino acid analysis) were usually present.

At least three preparations of chromatin and histone were made from each source except pod and root. Quantitative data were obtained on the proportion of each histone fraction in three tissues: bud, leaf, and cotyledon. Such data were obtained on eight preparations of pea bud and six preparations of leaf histones, and on two preparations of the histones of cotyledons in each of seven seed maturation stages.

Column Chromatography

Histones were fractionated by column chromatography on Amberlite CG-50, according to the method of Luck et al. (1958). Histones were dissolved in 8% guanidinium chloride (GuCl) applied to a 0.6 x 60 cm column, and eluted with a linear gradient of 8-13% GuCl (50 ml total

volume) followed by 40% GuCl. All the GuCl solutions were buffered to pH 6.8 with 0.1 M sodium phosphate. Protein content in the fractions was determined by turbidity (absorption at 400 mu) after precipitation of protein from a 1.1 M TCA solution (Luck et al., 1958; Bonner et al., 1967b). The turbidity is a linear function of protein concentration throughout the range of experimental determinations and is independent of the nature of the histone.

Disc Electrophoresis

Disc electrophoresis was performed, using a modification (Bonner et al., 1967b) of the method of Reisfeld et al. (1962). A pH 4.3 gel which was 15% in acrylamide and 6 M in urea was prepared by mixing 1 volume of Temed solution (48 ml of 1 N KOH, 17.2 ml of glacial acetic acid, 4 ml of N,N,N',N'-tetramethylethylenediamine, deionized water to 100 ml), 2 volumes of acrylamide solution (60 g of acrylamide, 0.4 g of N,N'methylenebisacrylamide, deionized water to 100 ml), and 5 volumes of 0.2% (w/v) ammonium persulfate in freshly deionized 10 M aqueous urea solution. Aliquots (0.9 ml) were pipetted into 6.5 cm lengths of 5 mm i.d. glass tubing and overlaid with 0.1 ml of 3 M urea for anaerobic polymerization. Each histone sample was dissolved at a concentration of 1 mg/ml in 10 M urea, and 1-20 µl applied to a gel. This solution was overlaid with tray buffer (31.2 g of β-alanine, 8 ml of acetic acid, water to 1 liter) and electrophoresed in a standard disc electrophoresis apparatus at a constant current of 4ma./tube for 1.5 hr. Gels were stained for at least 4 hr in 1% amidoschwarz-40%

ethanol-7% acetic acid aqueous solution. The gels were then destained by electrophoresis at less than 2 ma/gel and a trace of stain was added to the destaining solution -- both procedures to prevent discoloration of the protein bands. Any background color remaining in the gels after destaining was removed by dialysis against destaining solution (7% acetic acid-40% ethanol aqueous solution).

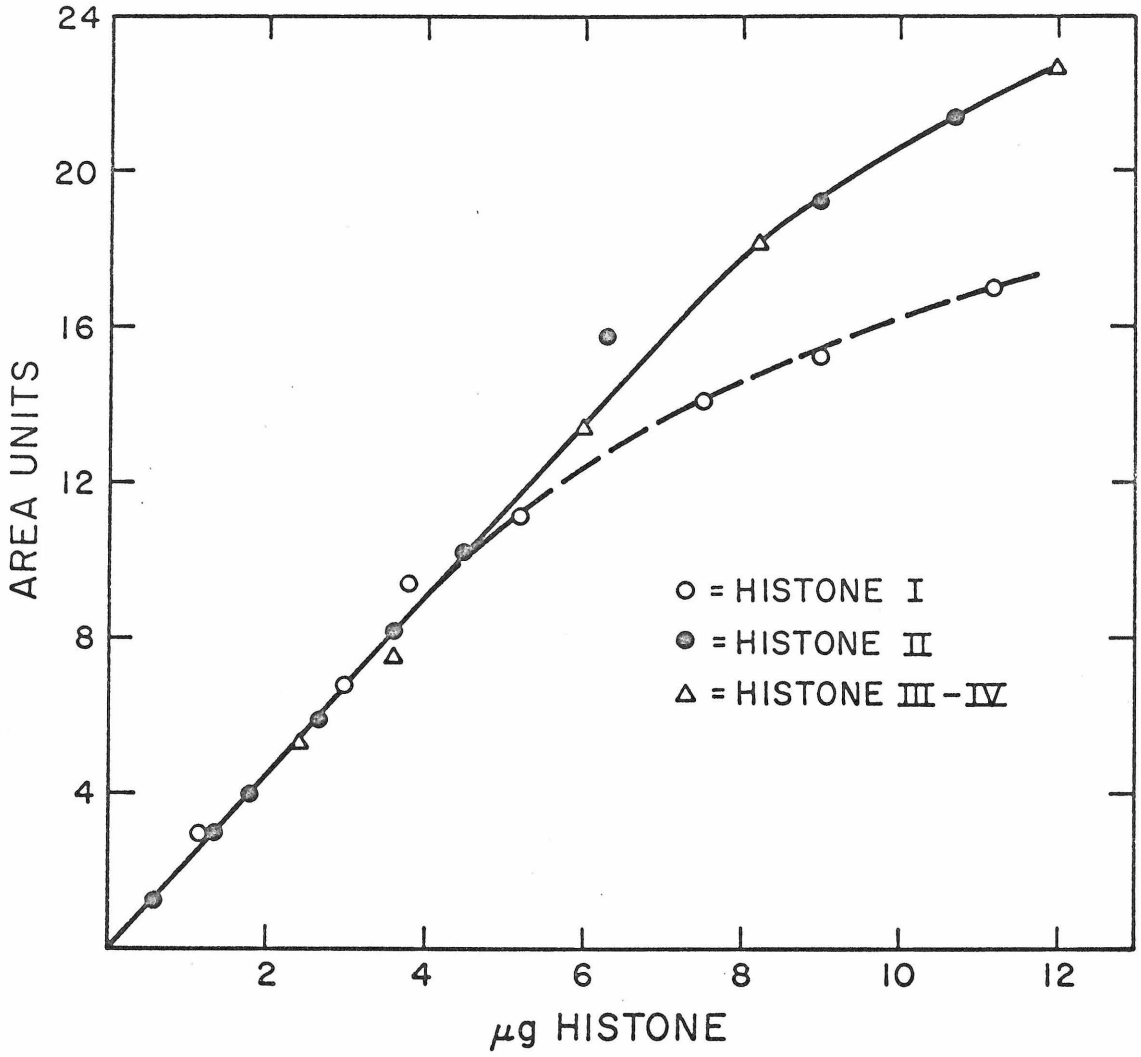
Quantification of Disc Electrophoresis

Histone fractions prepared by column chromatography on Amberlite CG-50 were used for quantification of analytical disc electrophoresis. Histone samples were dissolved in 10 M urea at a concentration of approximately 1 mg/ml. The protein concentrations were then accurately measured by the method of Lowry et al. (1951). Desired amounts of histone solution were applied to 15% polyacrylamide gels containing 6 M urea. Electrophoresis, staining and destaining were carried out as described above.

The gels were next scanned in the Canalco Model E microdensitometer. Rotation of the gels about their long axes causes no change in the densitometric tracings. All densitometric tracings were made at a constant chart speed and calibrated gain, using white light from a tungsten lamp, passed through a Wratten #39-A gelatin filter. Areas under the traces were measured either by a planimeter or by transferring the traces to a tracing paper of uniform weight, cutting out the peaks and weighing.

Figure 1 shows the relationship between histone concentration and the intensity of staining for the major histone chromatographic fractions.

Figure 1. Relation between histone concentration in electrophoretic bands and intensity of staining by amidoschwarz dye. Histone concentration is expressed as μg per polyacrylamide gel, intensity of staining as area (arbitrary units) under the curve of densitometric tracings.

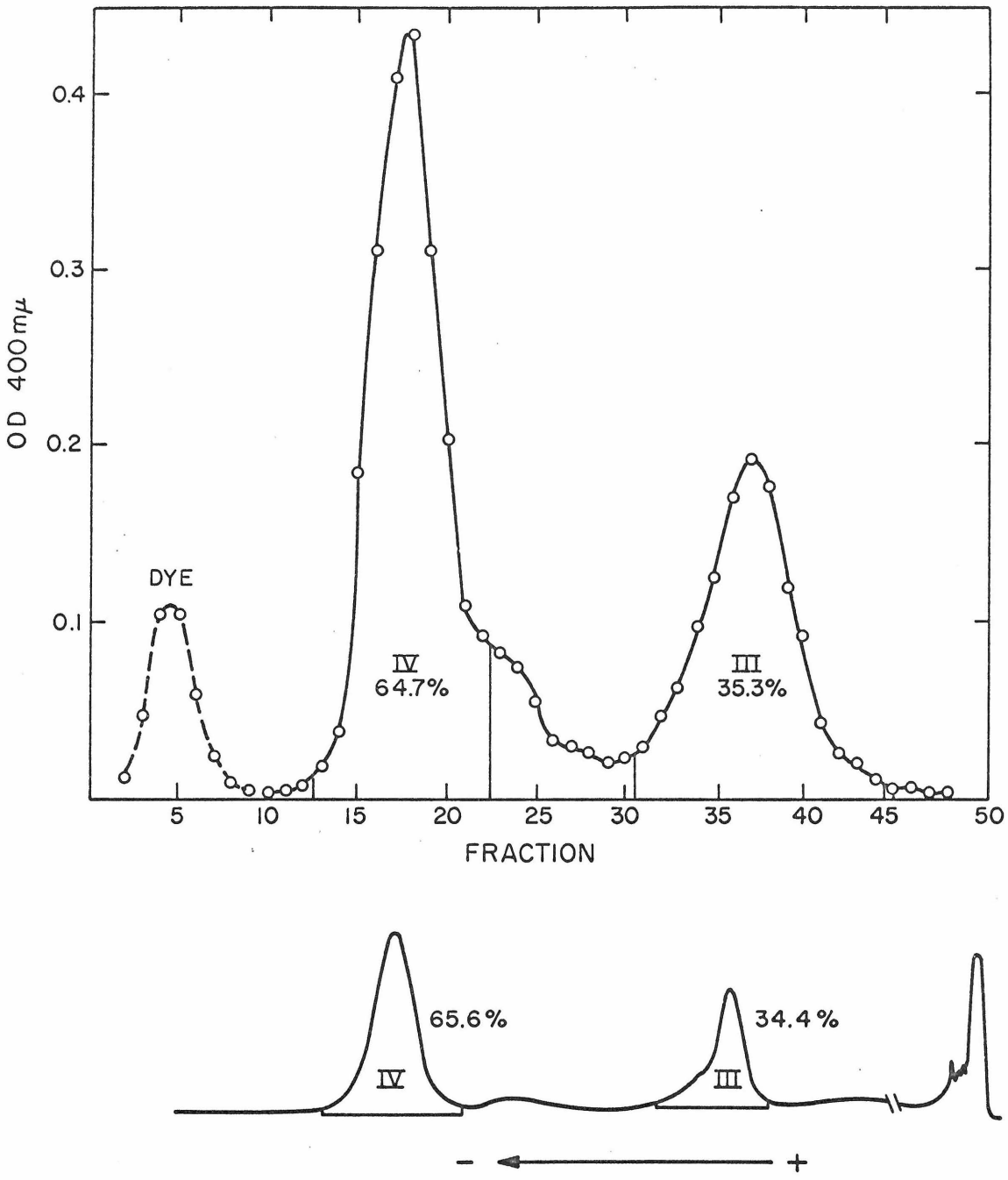

24
20
16
12
8
4
AREA UNITS
2
4
6
8
10
12
μg HISTONE
○ = HISTONE I
● = HISTONE II
△ = HISTONE III-IV

For each set of eight gels in one electrophoretic run the staining with amidoschwarz is a linear function of histone applied to the gel up to 10 μg of histone IIa and III-IV and 5 μg of histone I. Reproducibility from one set of gels to the next is quite good: the standard deviation for each point is about ± 4% (n = 5). The non-linearity of staining at higher protein concentrations may be either a direct effect of concentration on staining or the inability of the scanning device to accurately trace out the density of fine, densely stained bands. Either explanation also can account for the difference between the non-linear staining of histone I and of the other histones, for histone I electrophoretic bands are extremely fine and stain a different color than do the other histones.

With the advent of preparative disc electrophoresis it has become possible to isolate most histone components in an electrophoretically homogeneous state (see Chapter I). Use has been made of this improved method of fractionation to test the conclusion that all histone components have identical staining constants (optical density of dye bound per unit weight protein) at low protein concentration. This was done by comparing the elution profiles from preparative disc electrophoresis runs (which give a direct measure of protein concentration) with densitometric tracings of the same histone mixtures fractionated by analytical disc electrophoresis. An example comparison is presented in Figure 2.

Other examples are the analyses of histone I and II mixtures one of which had a composition of 47.6% histone I and 52.4% histone II by

Figure 2. Quantitative comparison of the profile of elution of histones III and IV from preparative disc electrophoresis and the densitometric tracing of the electrophoretic pattern of histones III and IV separated by analytical disc electrophoresis and stained with amidoschwarz. Preparative disc electrophoresis was performed using a Canalco Prep Disc apparatus with PD 2/320 upper column, a 2 cm (8 cm^3) 7.5% polyacrylamide gel, and an applied current of 40 ma. The system was cooled with tap water. Elution buffer was β -alanine-acetic acid, pH 4.3. Protein was determined by TCA precipitation and measurement of OD 400 mμ after 15 min. The contributions of histones III and IV to the areas under the peaks are indicated on the figure.


0.4
0.3
0.2
0.1
OD 400mμ
DYE
IV
64.7%
III
35.3%
5
10
15
20
25
30
35
40
45
50
FRACTION
65.6%
34.4%
IV
III
−
+

analytical disc electrophoresis and 47.3% histone I and 52.7% histone II by preparative disc electrophoresis, a second contained 66.3% histone I and 33.7% histone II by analytical disc electrophoresis and 67.6% histone I, 32.4% histone II by preparative disc electrophoresis. Assuming identical staining constants for all histone components, the estimations of the compositions of histone mixtures by analytical disc electrophoresis have always been within 2% of the values determined by preparative disc electrophoresis.

In further test experiments several individual histone molecular species were prepared by preparative disc electrophoresis and the staining constants with amidoschwarz dye of all these histone fractions are very similar.

In the experimental use of this quantification procedure, the areas under the peaks of the electrophoretic tracings are taken as measures of histone concentration. Fortunately the problem of reproducibility of staining intensity, while apparently not a severe one in any case, is not involved in most uses of quantitative electrophoretic analysis since all measurements are of the contribution of individual histone peaks to the total area under the curve for each single gel. Thus it is only important that the staining be uniform for all components in the gel. Since the linear relationship between histone concentration and staining falls off above 10 μg per gel (5 μg for histone I) it is naturally important that no gel contain more than these amounts of single histone components. The total histone applied to the gels was therefore kept in the range of 15-20 μg.

A sample electrophoretic tracing is presented in Figure 3. Because not every histone component is well separated from every other, certain components of similar mobility are grouped in the electrophoretic analyses. To separate histones IIa and IIb, a line perpendicular to the abscissa was drawn from the lowest point of the trough between the two peaks in the tracing. Histone III contains cysteine and can exist both in a reduced form as a monomer with electrophoretic mobility very close to that of histone IIb or in an oxidized form as a dimer with electrophoretic mobility approximately the same as histone Ic (see Chapter I). In all quantitative experiments the histone III was all converted to the oxidized form, so histone Ic contributes a small amount to the histone III fraction -- about 5% of total histone III concentration. This oxidation can conveniently be accomplished by dissolving histone in 8M urea and keeping it in the cold (5^{o}) for about two days.

Results

Column Chromatography

The elution patterns which result from fractionation of the histones of various pea tissues by column chromatography on Amberlite CG-50 are presented in Figures 4a-h. The peaks are identified by Roman numerals, using the nomenclature of Luck et al. (1958) as adapted for pea histones by Fambrough and Bonner (1966). Material derived from each peak of each separation was checked by analytical disc

Figure 3. Densitometric tracing of the electrophoretic pattern of whole pea bud histone. Vertical and horizontal lines indicate the partitioning of the total area under the curve among the various fractions. Histone III is present exclusively in the oxidized form and thus masks the presence of a small amount of a lysine-rich histone component Ic, which contributes about 5% to the area under this peak. When histone III is reduced to monomeric form, it possesses a mobility similar but not identical to that of histone IIb. Peak at far left is origin of gel, not protein.

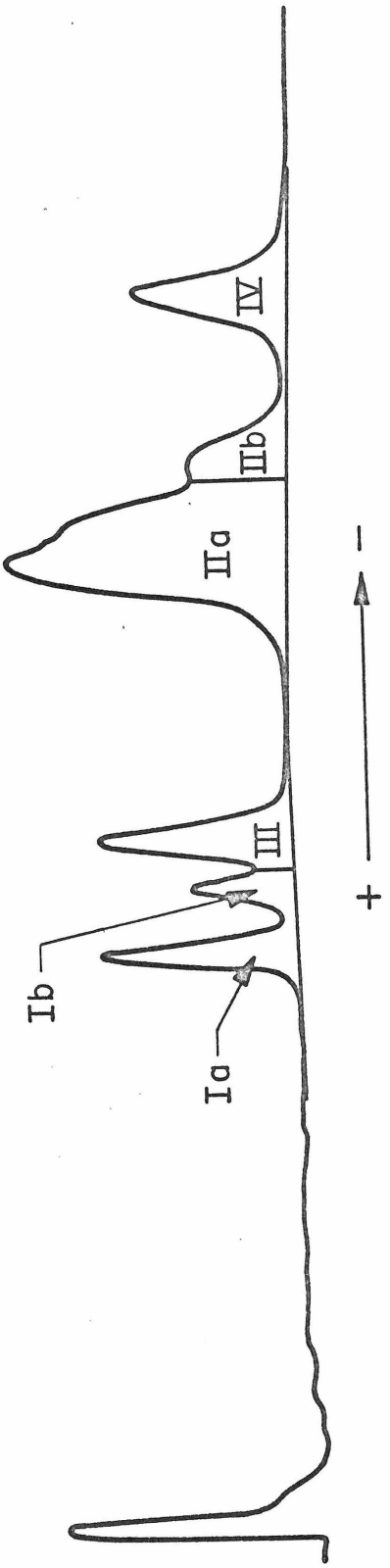
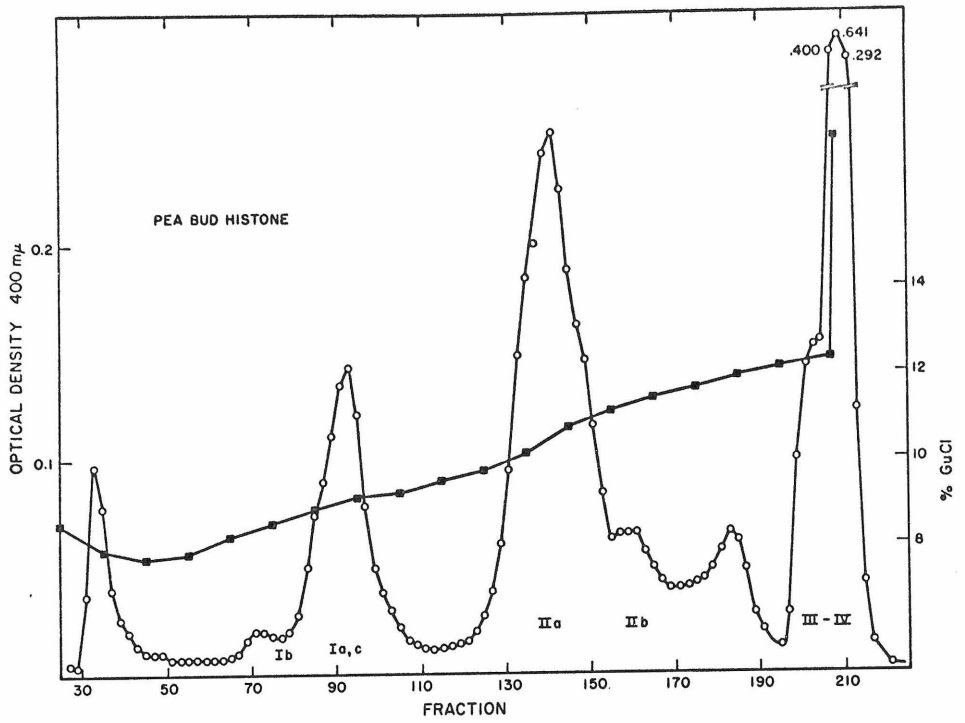
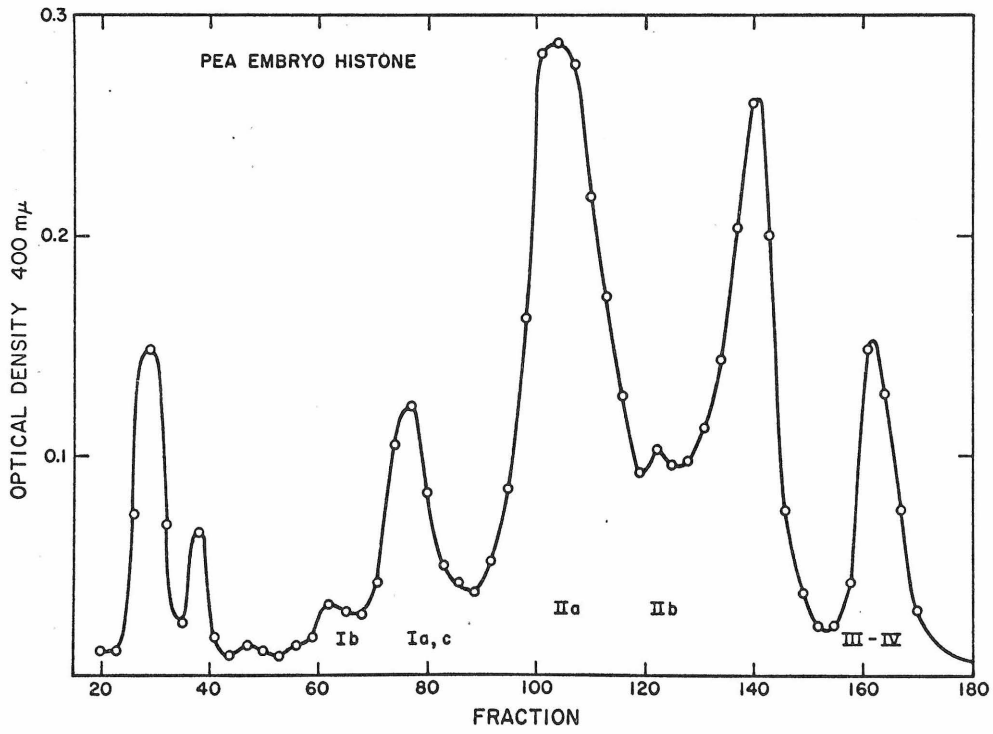
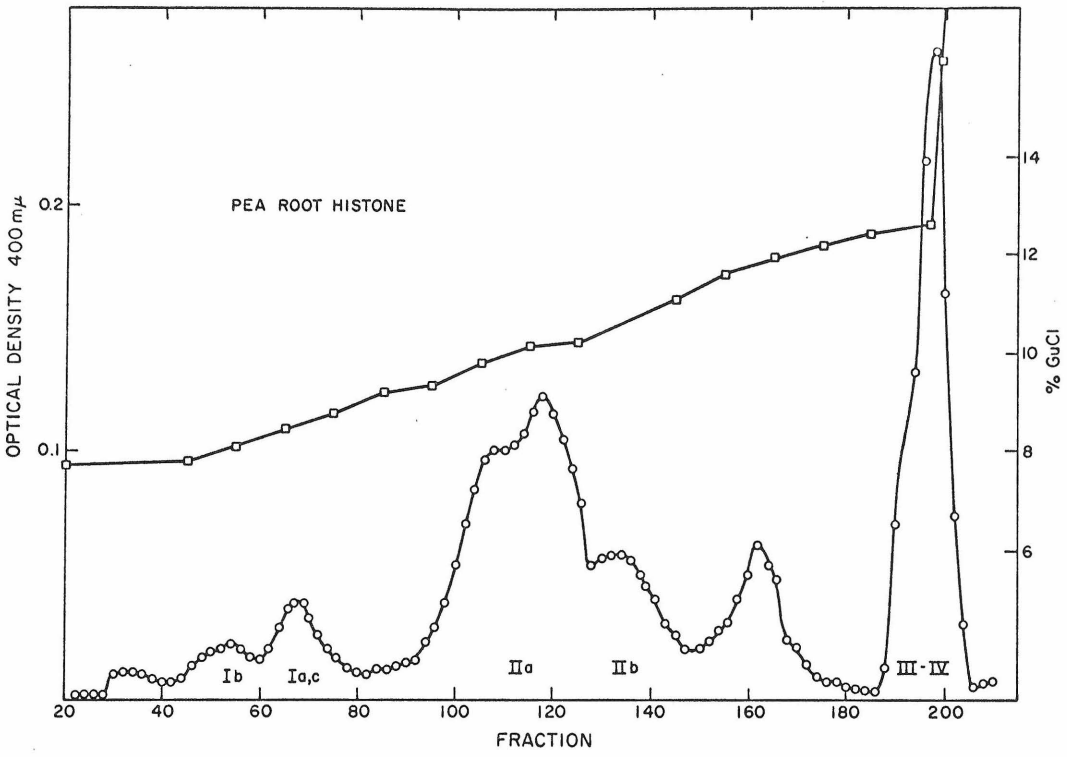
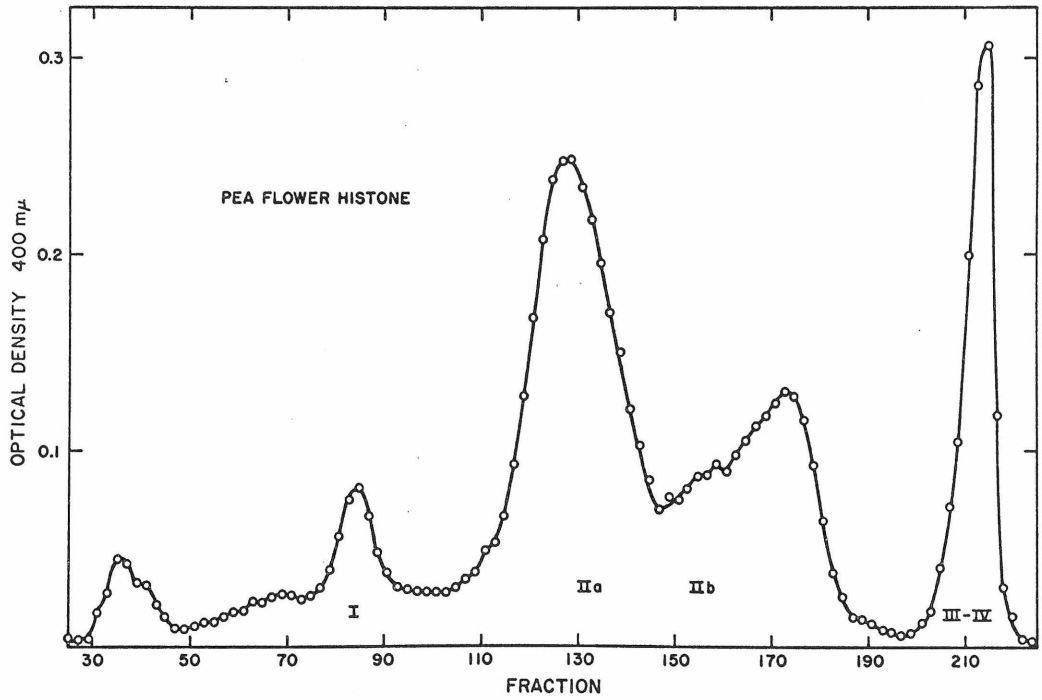

Ia
Ib
III
IIa
IIb
IV
-
+

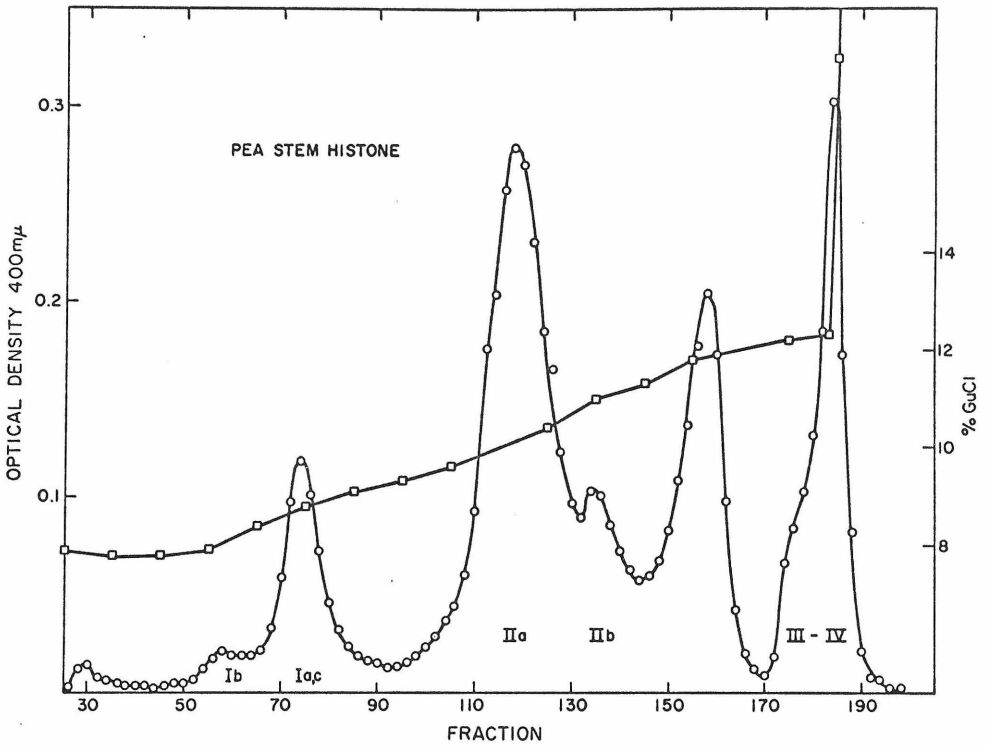
Figure 4 (a-h). Fractionation of pea tissue histones by column chromatography on Amberlite CG-50. Protein concentration in the effluent fractions was determined by optical density at 400 mu of the turbid solutions resulting from precipitation of the fractions for 15 min in 1.1 M TCA (0----0---). Concentration of guanidinium chloride in the effluent is indicated by □ ---- □ .

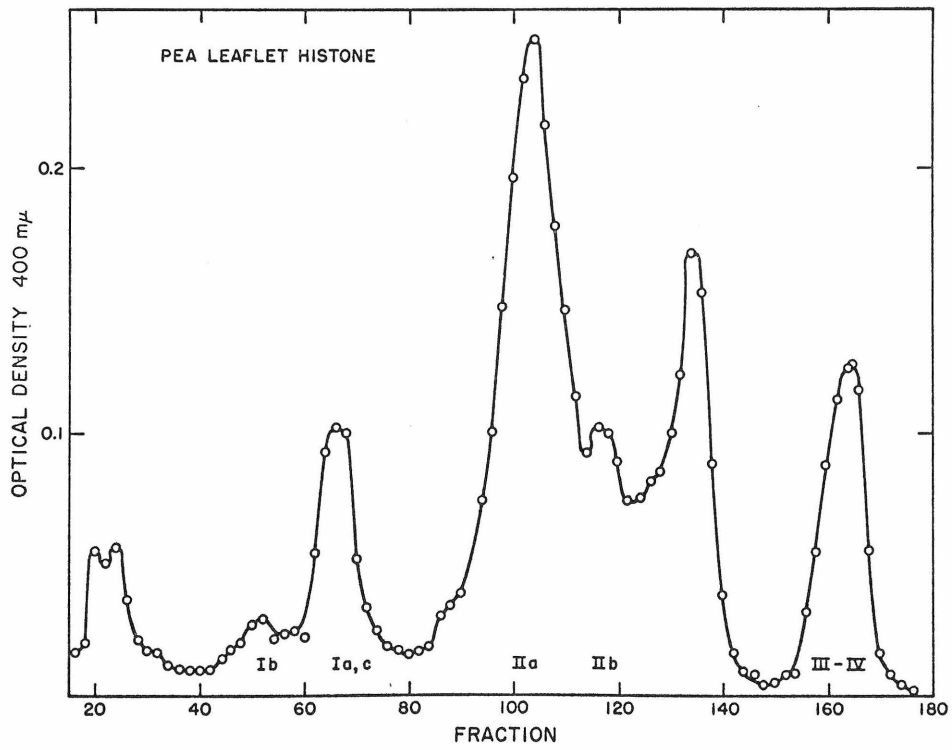

PEA BUD HISTONE
.641
.400
.292
OPTICAL DENSITY 400 mμ
0.2
0.1
% GuCl
14
12
10
8
Ib
Ia, c
IIa
IIb
III - IV
30
50
70
90
110
130
150
170
190
210
FRACTION

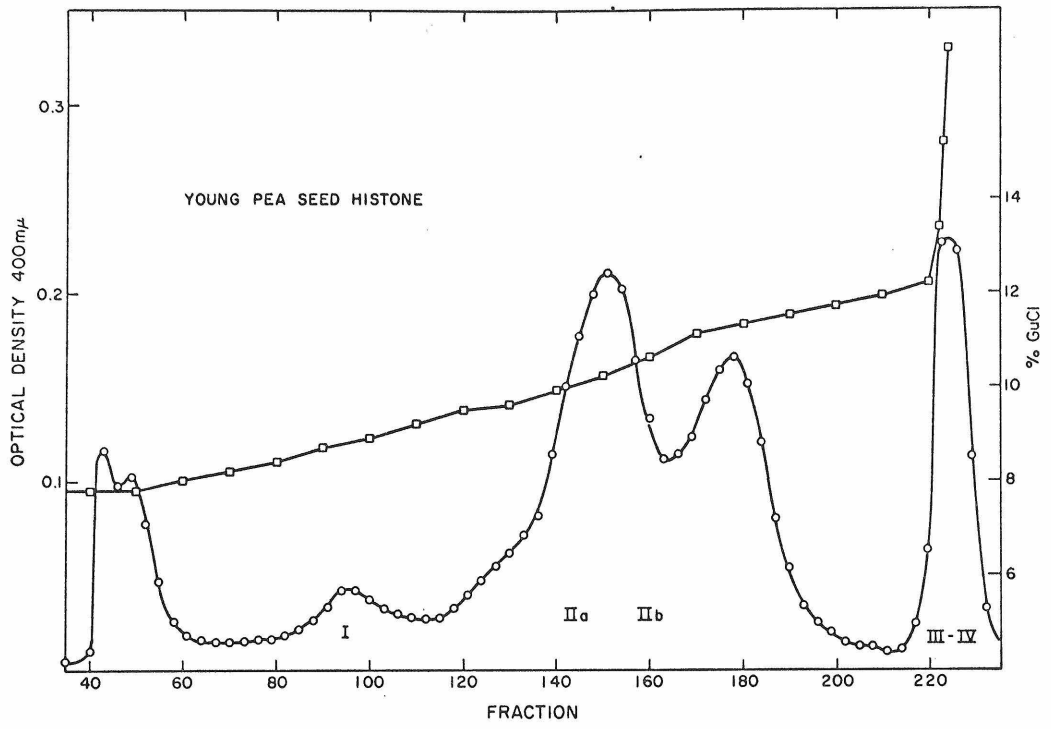

PEA EMBRYO HISTONE
OPTICAL DENSITY 400 mμ
0.3
0.2
0.1
Ib
Ia, c
IIa
IIb
III - IV
20
40
60
80
100
120
140
160
180
FRACTION

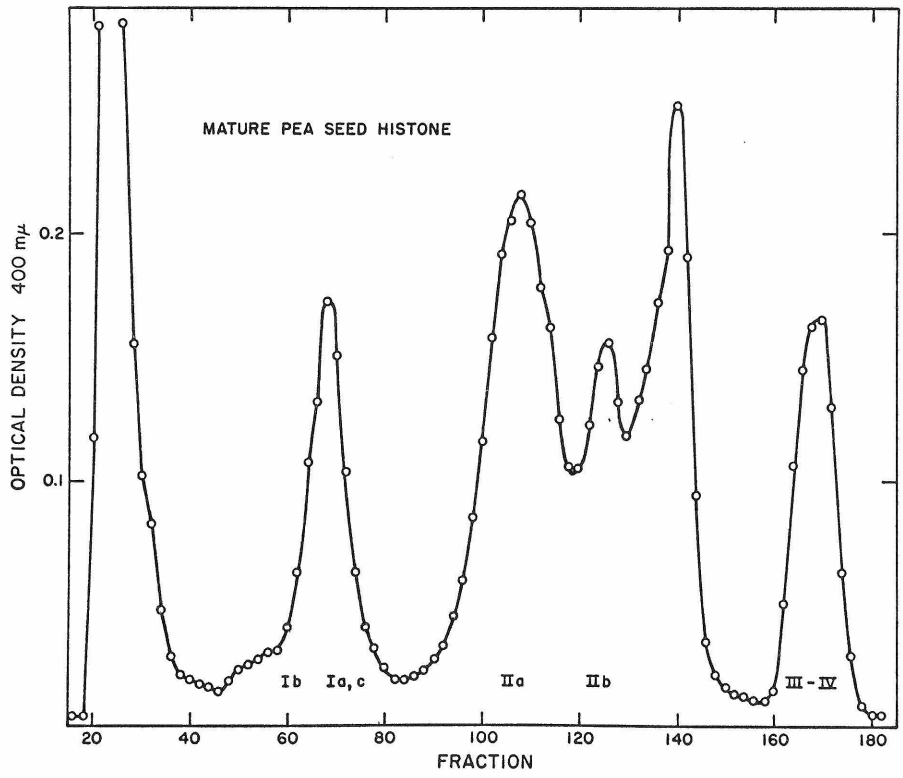

PEA ROOT HISTONE
OPTICAL DENSITY 400 mμ
0.2
0.1
% GuCl
14
12
10
8
6
Ib
Ia,c
IIa
IIb
III-IV
20
40
60
80
100
120
140
160
180
200
FRACTION


PEA FLOWER HISTONE
OPTICAL DENSITY 400 mμ
0.3
0.2
0.1
I
IIa
IIb
III-IV
30
50
70
90
110
130
150
170
190
210
FRACTION


PEA STEM HISTONE
OPTICAL DENSITY 400mμ
0.3
0.2
0.1
14
12
10
8
% GuCl
Ib
Ia,c
IIa
IIb
III - IV
30
50
70
90
110
130
150
170
190
FRACTION


PEA LEAFLET HISTONE
OPTICAL DENSITY 400 mμ
0.2
0.1
Ib
Ia, c
IIa
IIb
III-IV
20
40
60
80
100
120
140
160
180
FRACTION


YOUNG PEA SEED HISTONE
OPTICAL DENSITY 400mμ
% GuCl
FRACTION
I
IIa
IIb
III-IV


MATURE PEA SEED HISTONE
OPTICAL DENSITY 400 mμ
FRACTION
Ib
Ia, c
IIa
IIb
III-IV

electrophoresis to confirm the identifications. In the interpretation of the chromatographic data one must bear in mind that some contamination of all fractions by histone II always occurs and that some histone samples were not completely soluble in the starting buffer. For these reasons and because some subfractions are poorly resolved by this method of separation, estimates of the relative amounts of the individual components are subject to error (see Table II). Despite these limitations the column separation results suggest certain generalizations which are enumerated and discussed below.

First, the general chromatographic pattern is the same for histones of all pea tissues. Following the run-off peak, a histone I peak appears in the elution salt gradient range 8.6-9.3% GuCl. Histone IIab is next eluted by 10.1-10.8% GuCl, and is followed by a peak rich in III-IV, eluted by 10.8-12.5% GuCl. Finally, the remaining III-IV is removed from the column by 40% GuCl. There are no histone peaks specific to any tissue and no peak is completely lacking in any tissue.

Second, there are quantitative differences in elution profiles which are quite obvious in some cases. Most clear is the variation in histone I content. Table II lists the percentage of histone I in total histone from various tissues as measured by areas under the turbidity curves. Although the present estimates are rough they are closely corroborated in the cases of pea bud, leaf and cotyledons by quantitative disc electrophoresis.

Finally, there are some peripheral observations to be made about the run-off peak protein. The biological significance of protein such

Table II.

Proportion of Lysine-Rich Histone in the Total Histone of Various Plant Tissues

Pea Tissue	% Histone I by Column Chromatography	% Histone I by Analytical Disc Electrophoresis
Young seeds	6.5	7.0 ± 0.4[a]
Mature seeds	18.0	16.1 ± 0.6
Embryo	12.4	-
Bud	16.5	14.4 ± 0.5
Stem	12.7	-
Root	9.4	-
Leaflets	15.0	12.9 ± 0.7[b]
Flower	9.6	-

[a]Pea cotyledons, stage 4. (Cotyledons constitute the bulk of the seed).

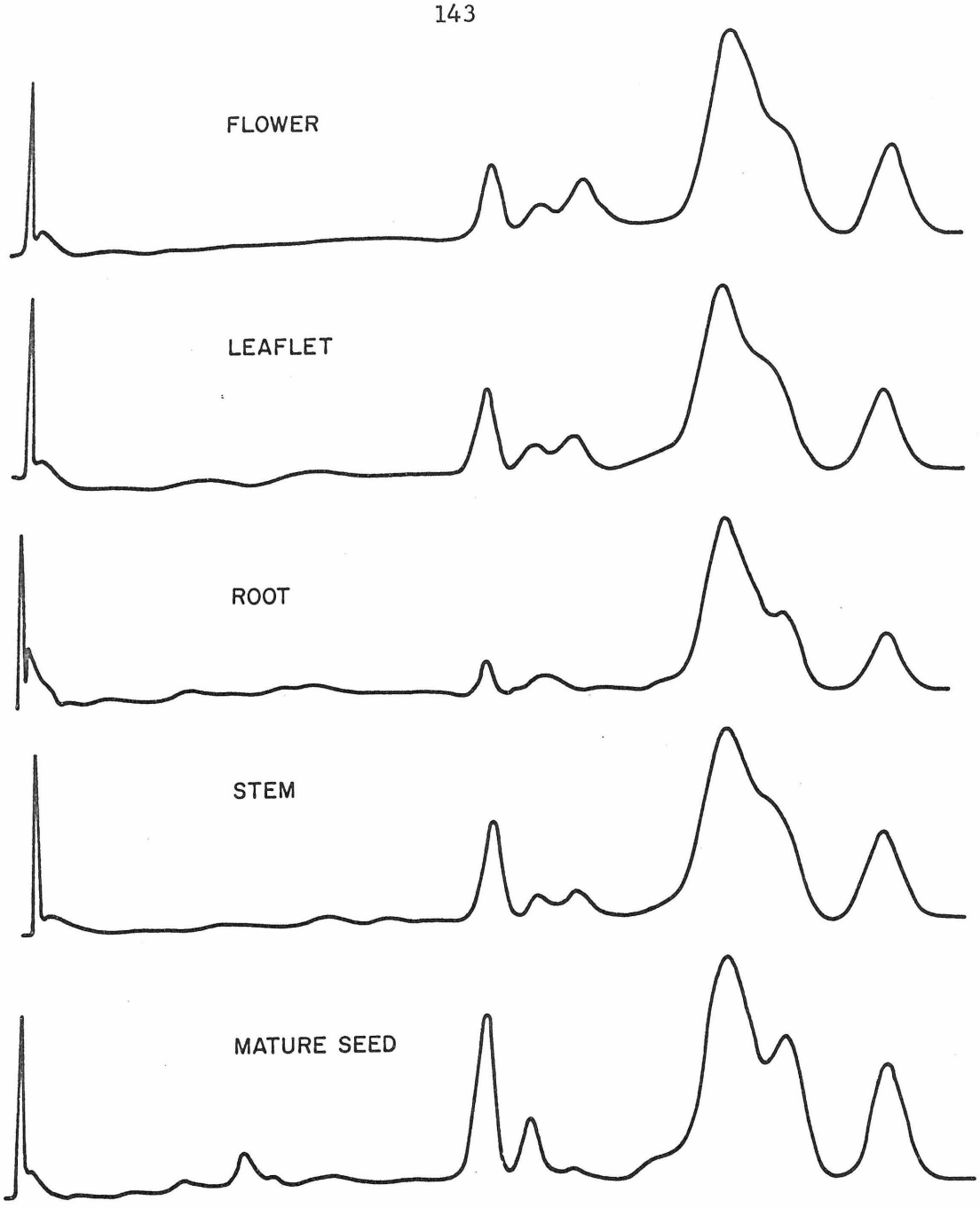
[b]Whole pea leaves.

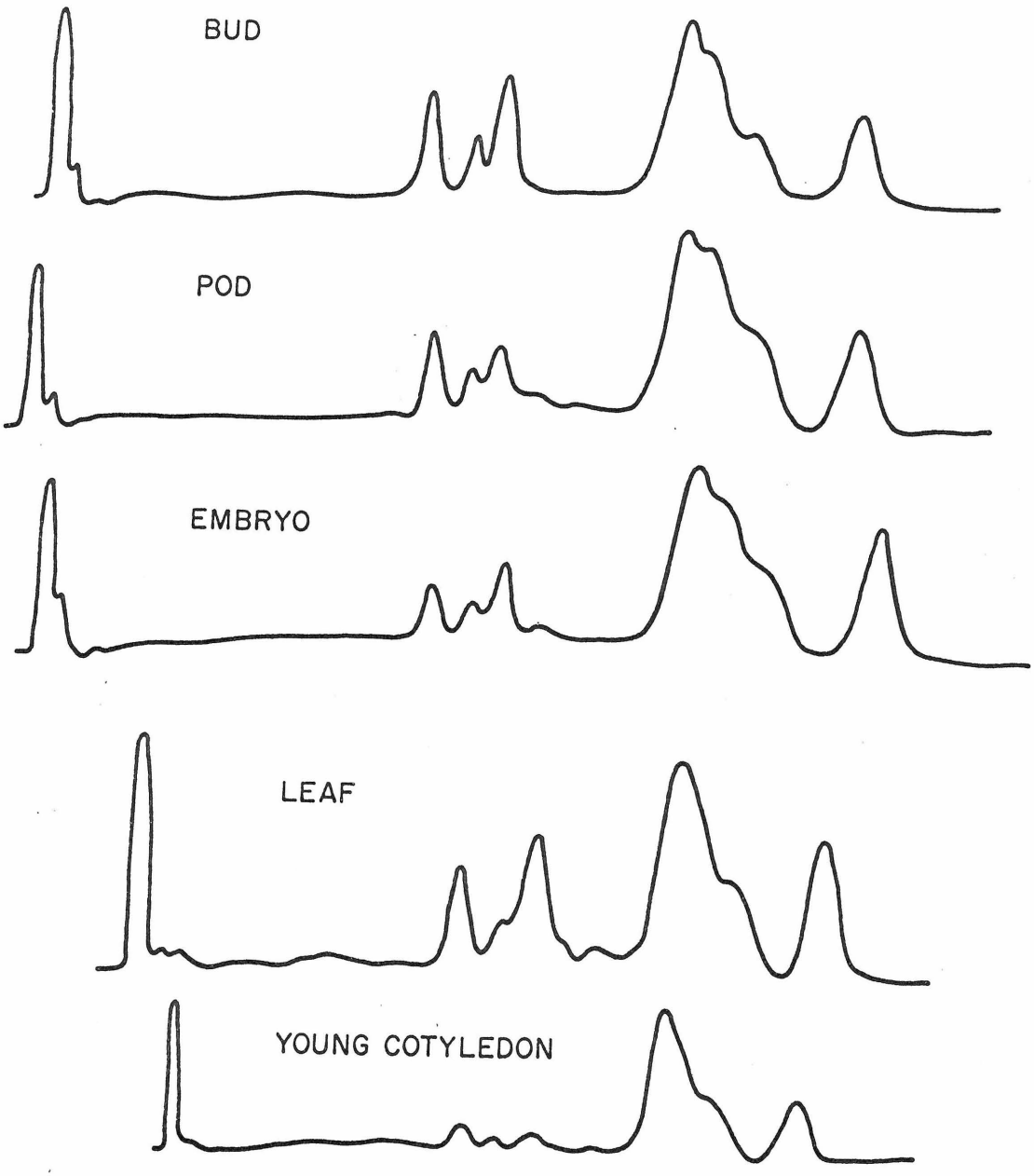
as this, which is extracted together with histones, has long been a subject of controversy between those who maintain that such proteins are bona fide elements of the genetic apparatus and those who feel that they are only artifactually associated with nucleoprotein. In the case of pea tissues it is generally true that the amount of such protein extracted with the histones is directly related to the crudeness of the chromatin preparation. Preparations which are turbid or contain very large amounts of protein relative to DNA or which contain large amounts of RNA are always rich in "run-off" protein. Substantial amounts of this protein can be removed from the chromatin by repeated centrifugations. Amino acid analyses which have been made of this material all indicate that it does not resemble histone. It is probable that the variable amount of this material in different pea tissues is at least largely an artifact of preparation.

Disc Electrophoresis

Densitometric tracings of the electrophoretic patterns of whole histone from many different pea tissues are presented in Figure 5a, b. By disc electrophoresis essentially every identified histone component can be directly visualized. The same conclusions suggested by the similarities in chromatographic patterns can also be drawn from the results of this more elegant method of histone fractionation. There is no major histone fraction unique to any specific tissue, no fraction is completely absent from any tissue, and quantitative variations in histone distribution are apparent.

Figure 5a,b. Densitometric tracings of the electrophoretic patterns of pea histones. Peaks at far left indicate origins of gels, not protein. In these histone preparations histone III is present in both oxidized and reduced forms, and thus contributes to the areas under the curve in the neighborhood of histones Ic and IIb.


FLOWER
LEAFLET
ROOT
STEM
MATURE SEED


BUD
POD
EMBRYO
LEAF
YOUNG COTYLEDON

Quantitative determinations of the histones from pea buds, leaves, young and mature cotyledons, are presented in Table III. In all cases histone III was present only in the oxidized form (see Methods), thus histone Ic contributes slightly to the values of histone III given in the table. In all cases, however, histone Ic is less than 1% of the total histone. Its contribution to the histone III peak is, therefore, not large. The data in Table III are the average of eight gels. The standard deviations are all less than 1.1% of total histone for repeated analyses of one histone preparation , and are only slightly larger when several preparations are compared. Quantitative differences well outside the range of experimental error are shown in these data. Again, the largest difference is in the content of lysine-rich histone I, present in very low amounts in young pea cotyledons.

Changes in the proportions of the histone components during maturation of pea cotyledons have been further studied by electrophoresis. In Figure 6 the histone I content of pea cotyledons is plotted as a function of maturation stage. Densitometric traces of histone extracted from cotyledons in three stages of maturation are presented in Figure 7. The histone I content of pea cotyledons seems to follow qualitatively the same chronological chart as does the density of the pea seeds: minimum histone I content is found in peas of minimum buoyant density and maximum content in very dense, mature peas. There is virtually no variation in amount of histones IIb, III and IV present in total histone during maturation of pea cotyledons. The increase in lysine-rich histone I is accompanied by a decrease in slightly-lysine rich histone IIa.

Table III.

Composition of Histones from Pea Tissues

Histone Fraction	% of Total Histone			
	Pea Bud	Pea Leaf	Mature Pea Seeds	Young Pea Cotyledons
Ia	9.9 ± .4	9.6 ± .6	10.7 ± .7	5.3 ± .3
Ib	4.5 ± .3	3.3 ± 1.0	5.4 ± .4	1.7 ± .4
Iab	14.4 ± .5	12.9 ± .7	16.1 ± .6	7.0 ± .4
IIa	46.8 ± .7	43.5 ± 1.1	38.8 ± 1.0	46.0 ± .7
IIb	6.8 ± .5	7.6 ± .4	7.6 ± .7	8.8 ± .6
III[a]	17.8 ± .7	18.7 ± .7	17.2 ± .3	18.9 ± .9
IV	14.8 ± .5	17.3 ± 1.0	20.3 ± .4	19.3 ± .8

[a]Includes histone Ic as ± 1% of total histone.

Figure 6. Lysine-rich histone content (percent of total histone) in pea cotyledons as a function of maturation of seeds.

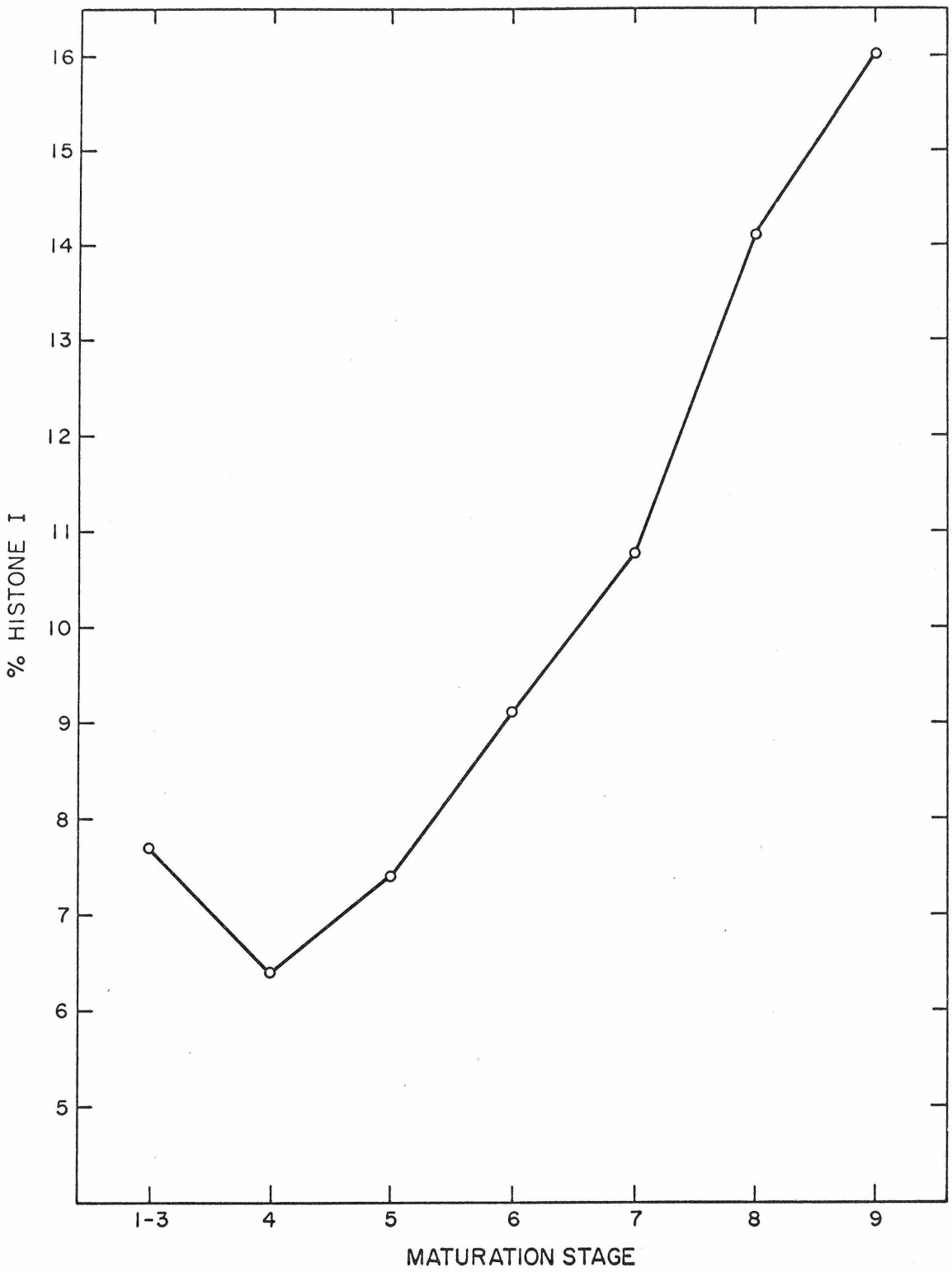
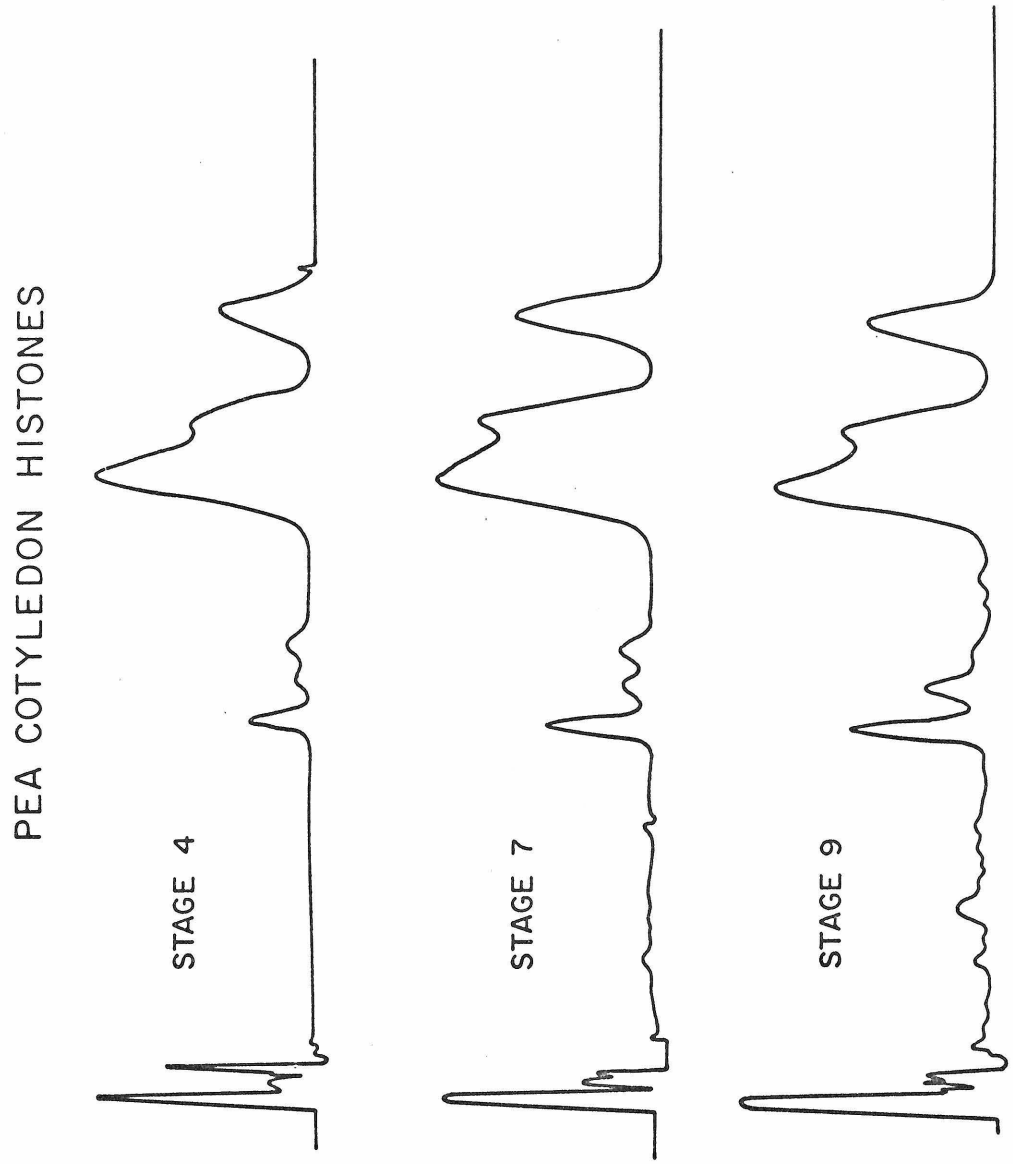

16
15
14
13
12
11
10
9
8
7
6
5
% HISTONE I
1-3
4
5
6
7
8
9
MATURATION STAGE

Figure 7. Densitometric tracings of the electrophoretic patterns of pea cotyledon histones isolated from three maturation stages of seeds. Note progressive increase in histone I components. Histone III is present primarily in the reduced form.


PEA COTYLEDON HISTONES
STAGE 4
STAGE 7
STAGE 9
+
-

Discussion

The number of histone molecular species in both pea bud and calf thymus is apparently quite small, and homologous pea bud and calf thymus fractions share many characteristics (Fambrough and Bonner, 1966, and see Chapter I). In view of the small number of, and nearly perfect 1:1 correspondence between, the histones of two organisms as different as pea and calf, one would scarcely expect to find histones unique to the much more closely related cells which make up the different organs of a single organism. The data presented in this chapter confirm this expectation: no histone specific to any pea tissue has been found.

On the other hand, there is rather large variation in the quantitative distribution of the several histone fractions in different pea tissues. The components showing most variation are lysine-rich histone I, slightly-lysine-rich histone IIa, and arginine-rich histone IV (homologous respectively to f1, f2b, and f2a1 of vertebrates). Histones IIb and III (homologous to f2a2 and f3) show practically no variation in the tissues selected for careful study.

In their study of quantitative distribution of histones in selected mammalian tissues, Hnilica et al. (1966) found most variation in histones f2b, f3 and f2a1, and practically no variation in histones f1 and f2a2. Taken together with the present study the data give no clear hint as to the underlying biological significance of histone component variability. In our case the very low content of lysine-rich histone I in pea coty-

ledons is paralleled by a quite low histone:DNA ratio of 0.76 and high template activity in support of *in vitro* RNA synthesis, 32% that of purified DNA. Pea buds, which possess a relatively high histone I content, also possess high histone:DNA ratio (1.30) and low template activity (6%) (Bonner *et al.*, 1967a).

Despite the differences in the nature of the quantitative variation of histones reported by Hnilica and by us, there are some probably meaningful consistencies in the combined data: in all mammalian and pea tissues so far examined the slightly-lysine-rich histone IIa or f2b is the major histone fraction, while histone IIb or f2a2 is always present in quite small amounts, and the arginine-rich histones III and IV or f3 and f2a1 together constitute about 30-40% of total histone. These parallels underscore the extent to which the functions of the histones specify not only their chemical natures but also their proportions in the genetic material.

References

Bonner, J., Chalkley, G.R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R.C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H., Olivera, B.M., and Widholm, J. (1967b), Methods in Enzymology: Nucleic Acids, New York, Academic Press. In press.

Bonner, J., Dahmus, M., Fambrough, D., Huang, R.C., Marushige, K., and Tuan, D. (1967a), Science. In press.

Butler, J.A.V. (1966), in Histones - Their Role in the Transfer of Genetic Information, Ciba Foundation Study Group No. 24, Little, Brown and Co., Boston, p 4.

Crampton, C.F. Stein, W.H., and Moore, S. (1957), J. Biol. Chem. 225, 363.

Davis, J.R. and Busch, H. (1959), Cancer Res. 19, 1157.

Dingman, C.W. and Sporn, M.B. (1964), J. Biol. Chem. 239, 3483.

Fambrough, D., and Bonner, J. (1966), Biochemistry 5, 2563.

Hnilica, L.S. (1966), Biochim. Biophys. Acta 117, 163.

Hnilica, L.S., and Bess, L.G. (1965), Anal. Biochem. 12, 421.

Hnilica, L.S., Edwards, L.J., and Hey, A.E. (1966), Biochim. Biophys. Acta 124, 109.

Hnilica, L.S., Johns, E.W., and Butler, J.A.V. (1962), Biochem. J. 82, 123.

Laurence, D.J.R., Phillips, D.M.P. and Butler, J.A.V. (1966), Arch. Biophys. Biochem. 113, 338.

Laurence, D.J.R., Simson, P., and Butler, J.A.V. (1963), Biochem. J. 87, 200.

Lee, Y.C. and Scherbaum, O.H. (1966), Biochemistry 5, 2067.

Lindsay, D.T. (1964), Science 144, 420.

Luck, J.M., Rasmussen, P.S., Satake, K., and Tsvetikov, A.N. (1958), J. Biol. Chem. 233, 1407.

Mauritzen, C.M., and Stedman, E. (1959), Proc. Roy. Soc.,London, Ser. B, 150, 299.

Mauritzen, C.M., and Stedman, E. (1960), Proc. Roy. Soc.,London, Ser. B, 153, 80.

Neelin, J.M. and Butler, G.C. (1961), Canad. J. Biochem. Physiol. 39, 485.

Neidle, A., and Waelsch, H. (1964), Science 145, 1059.

Palau, J., and Butler, J.A.V. (1966), Biochem. J. 100, 779.

Reisfeld, R.A., Lewis, N.J., and Williams, D.E. (1962), Nature 195, 281.

Sporn, M.B., and Dingman, C.W. (1963), Science 140, 316.

Stedman, E., and Stedman, E. (1950), Nature 166, 780.

Stedman, E., and Stedman, E. (1951), Phil. Trans. Roy. Soc., London, Ser. B, 235, 565.

CHAPTER III

In this chapter the dissociation of pea bud histone classes from pea bud DNA by increasing concentrations of NaCl is described. The dissociation of histones from calf thymus nucleohistone by NaCl has been most thoroughly investigated by Olivera (1966), Tuan (1967) and Ohlenbusch, Olivera, Tuan and Davidson (1967), who have shown that it is possible, to some extent, to selectively dissociate individual histone classes from native nucleohistone. Their studies confirm and extend the earlier reports of Daly and Mirsky (1955), Lucy and Butler (1955), Giannoni and Peacocke (1963) and Ohba (1966). The extensive chemical and physical similarities of the histones of pea buds to those of calf thymus (reported in Chapter I) suggest that homologous histones might be similarly complexed with DNA in the native state. Since the concentration of NaCl needed to dissociate a given histone-DNA complex is a measure of the strength of ionic interaction between that histone and DNA, a comparison of the dissociation of histones from pea bud and calf thymus nucleohistones by NaCl yields a measure of the comparative strengths of ionic interactions of homologous plant and animal histones with their respective DNAs. Such a comparison, based upon the present data and that of Ohlenbusch *et al.* (1967) confirms that the similarities between pea bud and calf thymus histones extend, in general, to ionic interactions of homologous histones with DNA in native nucleohistone.

The use of pea bud nucleohistone for dissociation studies is especially advantageous because individual pea histone components can be

more readily identified by disc electrophoresis than can those of calf thymus. Also pea bud nucleohistone, unlike calf thymus nucleohistone, is not prone to deterioration and selective loss of lysine-rich histones during the several days required for preparation of salt extracted nucleoprotein.

Methods

To facilitate comparison with calf thymus data, the methods adopted by Olivera and Tuan during the course of their experiments (which were observed by the present author) were used with only a few modifications to improve quantitative reproducibility.

Preparation of chromatin and nucleohistone

Pea bud chromatin was prepared as described in Chapter I. After dialysis overnight against 0.01 M Tris-HCl buffer, pH 8.0, the chromatin was sheared at 80 volts for 90 seconds in a small metal Waring Blendor (Bonner et al., 1967) and centrifuged at 10,000 x g for 30 minutes. The supernatant (nucleohistone) was removed without disturbing the pellet. The recovery of DNA in this step was about 90%. That no fractionation of histones occurred up to this point was shown by quantitatively comparing the electrophoretic patterns of histones extracted from chromatin with those extracted from nucleohistone or from the pellet produced in the preparation of nucleohistone. Histones were simultaneously extracted from these three sources, fractionated by disc electrophoresis, and quantitatively compared as described in Chapter II. The contribution

of each histone peak to the total area under each scan was the same for all electrophoretic fractionations.

Sodium chloride treatment and analyses

In all experiments chromatin or nucleohistone was first diluted with 0.01 M Tris-HCl buffer, pH 8.0, to a concentration of 50 μg DNA/ml (optical density at 260 mμ 1.5). Twenty ml aliquots were placed in thoroughly cleansed dialysis tubing and dialyzed against 1 liter volumes of NaCl solutions. All solutions were buffered with 0.01 M tris buffer, pH 8.0. Dialysis was carried out at 2-4°C, and the dialysis medium was changed at 8-12 hour intervals. After dialysis against three changes of salt solutions each nucleohistone sample was divided into two equal volumes, placed in 12 ml cellulose nitrate tubes, and underlayered with 2-3 ml cushions of 1.7 M sucrose, to give a final volume of about 12 ml per tube. The tubes were centrifuged 36-48 hours at 36,000 rpm in #40 Spinco rotors at 5°C.

After centrifugation the top 5-6 ml of each tube contents were removed for study of the dissociated histone, and the remaining solution was removed by aspiration to leave only the pellets. Pellets from the same salt treatment were combined and suspended in 0.01 M tris buffer and recentrifuged 36 hours at 36,000 rpm. The resulting washed nucleohistone pellets were extracted with 2 ml of 0.2 N H_2SO_4 and then with 1 ml of 0.4 N H_2SO_4. That the procedure extracted all of the histone was shown by failure to detect any additional protein by dissolving the acid extracted, perchloric acid hydrolyzed pellet in 1 N NaOH and

performing a protein determination by the method of Lowry et al. (1951). The combined acid extracts were mixed with three volumes of ethanol and the histones quantitatively precipitated at -20°C for 36 hours.

The acid extracted nucleohistone samples were dispersed in 2 ml of 5% PCA and heated to 100°C for ten minutes to hydrolyze the DNA. The resulting deoxyribonucleotides were quantified by the method of Dische (1955) after removal of the unhydrolyzed protein by centrifugation. For each nucleohistone sample three values for total DNA were obtained by separate Dische reactions and the mean of these values was used as the DNA content. Individual values were almost always within 3% of the mean. The ethanol precipitated histone was pelleted by centrifugation at 17,000 x g for 15 minutes and the supernatant completely removed without disturbing the histone pellet. (At this point the histone precipitate is quite sticky and adheres strongly to the glass walls of the centrifuge tube.) Eight molar urea was then added to each tube to completely dissolve the histone at a concentration of roughly 1 mg/ml. The exact protein content of each solution was obtained from three replicate determinations of protein by the method of Lowry et al. (1951). When any of these values was far from the mean, additional determinations were made. Since urea gives a slightly positive reaction in the Lowry test, a blank containing an appropriate amount of urea was always used.

Two methods were used to recover the salt-dissociated histones. First, the supernatant salt solutions were made 1.1 M in TCA and the resulting turbid solutions centrifuged to pellet the histones. Second,

the supernatant salt solutions were dialyzed against several changes of 0.1 M acetic acid and then lyophilized.

To examine the histones dissociated by the various salt concentrations and the histone still bound to the DNA, the histones were fractionated by analytical disc electrophoresis. The amidoschwarz stained gels were scanned, using a Canalco Model E Microdensitometer, and these scans were analyzed by measurement of the areas under each peak. The staining constants (intensity of staining per unit weight protein) for all of the histone components are virtually identical, and the intensity of staining is linearly related to protein concentration in the histone sample for concentrations below about 10 μg of a single histone component in one gel. Details of the quantification techniques are presented in Chapter II.

Since the amounts of histone III in the oxidized and reduced forms change with time during the interval between dissolving of the histone in urea and the start of electrophoresis (see Chapter I for details) the IIIox and IIb-IIIred peaks were combined in the calculations. Histone Ic was unavoidably included in the III-IIb fraction. However, its contribution to this fraction was always less than 5% and after salt treatments of 0.5 M and greater was presumably zero.

Results

In Figure 1 the fraction of histone remaining bound to DNA is plotted against molarity of NaCl used in the extraction. Data are normalized in this way to eliminate systematic errors in either histone or DNA determinations and to allow use of combined data from experiments employing chromatin and nucleohistone (whose histone/DNA ratios are about 1.1 and 1.35 respectively). The experiment was performed four times, once using chromatin and three times using nucleohistone. Quantitative variation between experiments is small: the values for the fraction of histones remaining bound to DNA after NaCl extraction never deviate more than 5% from the mean. Data for chromatin and nucleohistone are not significantly different.

These data indicate that no histone is dissociated from DNA by 0.3 M NaCl, that some fraction of histone is dissociated rather sharply over the range 0.3 to 0.5 M NaCl, and that the dissociation of the remaining histones is rather linear with increasing NaCl concentration over the range 0.75 to 1.5 M NaCl. Two molar NaCl dissociates more than 99% of total histone. These facts are verified by quantitative analysis of the behavior of the individual histone components.

Densitometric tracings of the electrophoretic patterns of the histones which remain bound to DNA after the various NaCl extractions are presented in Figure 2. It is clear from a simple qualitative analysis of these data that the electrophoretic pattern of the bound histones is unaltered by extraction with 0.3 M NaCl and that the two slowest electro-

Figure 1. The fraction of total histone remaining bound to DNA after extraction with 0.0, 0.15, 0.30, 0.375, 0.50, 0.75, 1.0, 1.5 and 2.0 M NaCl, buffered at pH 8.0 with tris-HCl, 0.01 M. Each point for pea bud histones is the average value from four experiments. Individual values are all $\pm$ 5% of the mean. 0----0, pea bud histone; Δ---- Δ, calf thymus histone, data of Ohlenbusch, Olivera, Tuan & Davidson (1967). The values of histone remaining bound to DNA after the salt treatments are somewhat lower for calf thymus due to the greater relative content of lysine-rich histone, which is removed by 0.5 M NaCl.

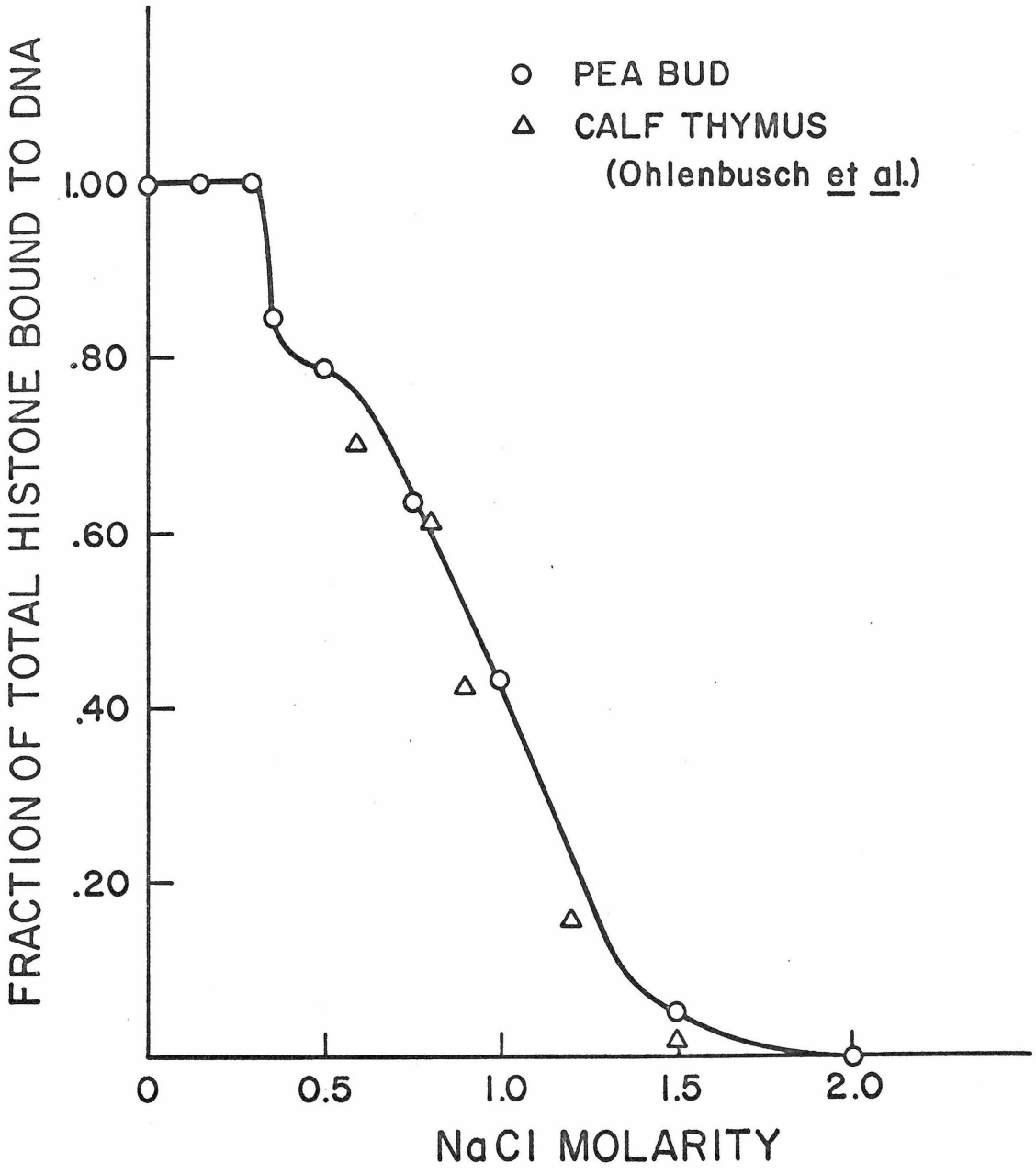
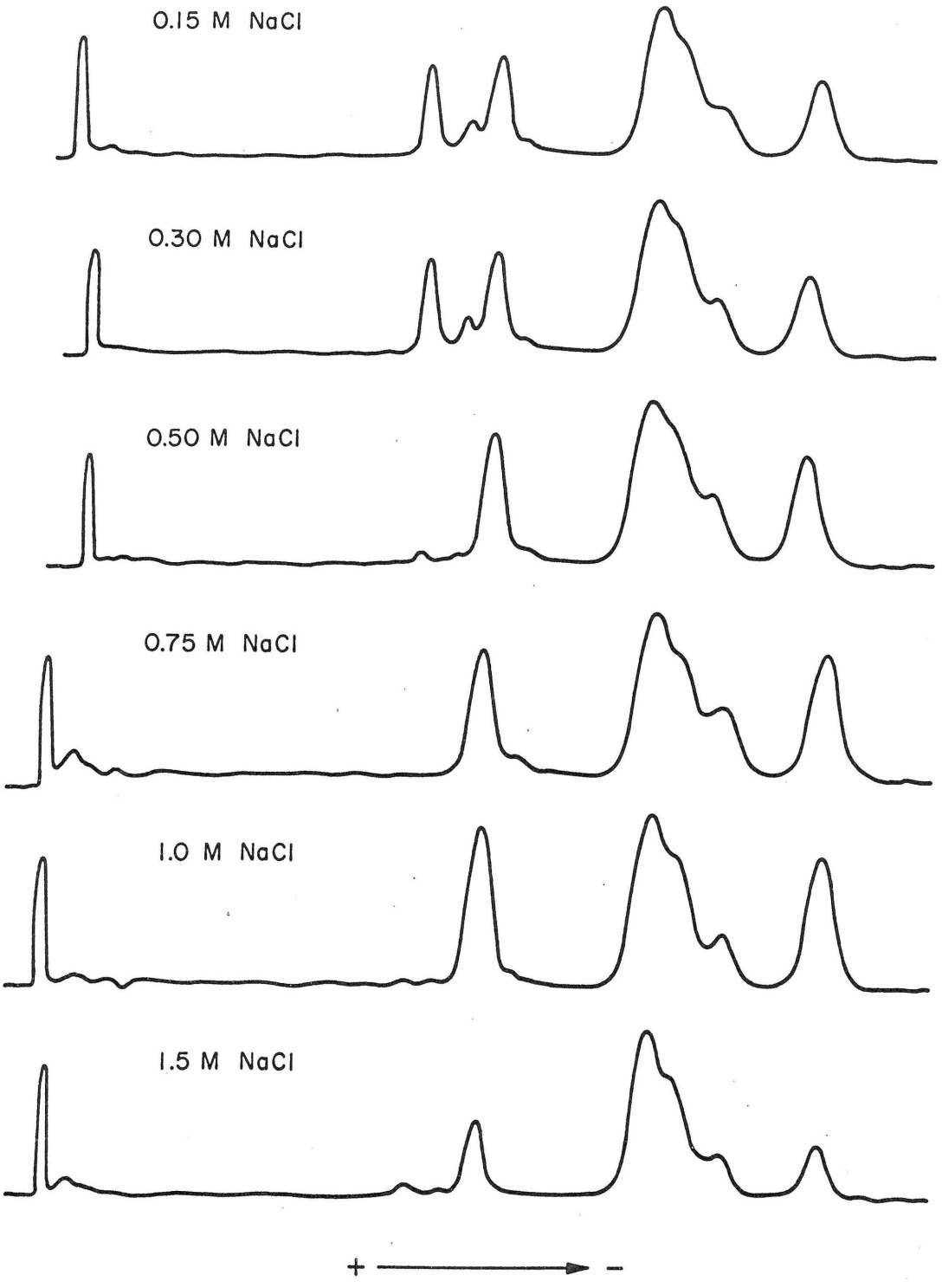

PEA BUD
CALF THYMUS
(Ohlenbusch et al.)
FRACTION OF TOTAL HISTONE BOUND TO DNA
1.00
.80
.60
.40
.20
0
0.5
1.0
1.5
2.0
NaCl MOLARITY

Figure 2. Densitometric tracings of the electrophoretic patterns of pea bud histones remaining bound to DNA after extraction with NaCl. The molarity of NaCl used in each extraction is indicated on the figure.


0.15 M NaCl
0.30 M NaCl
0.50 M NaCl
0.75 M NaCl
1.0 M NaCl
1.5 M NaCl
+
-

phoretic components are selectively removed by extraction with 0.5 M NaCl. It also appears that histones III and IV are slightly less readily extracted by 0.75 and 1.0 M NaCl than is histone IIa, although the residual 2% histone after 1.5 M NaCl extraction seems to be predominantly IIa. The trace of histone I in this latter material indicates some contamination by dissociated histones.

Quantification of the electrophoretic data combined with measurements of the amount of total histone remaining bound to DNA after each NaCl extraction permits straightforward calculation of the dissociation of each histone component from the nucleoprotein as a function of NaCl concentration. The results of these calculations are presented graphically in Figure 3. One such calculation is presented below not only to illustrate the method of computation but also to demonstrate the sort of variability encountered in the experiments. Further measurements are summarized in Table II.

Consider the data for the dissociation of histones from chromatin and from nucleohistone in 1.0 M NaCl. Two electrophoretic fractionations of histone were made for each of the four experiments and the densitometric tracings of these electrophoretic patterns were quantified to express the area under each peak as a percentage of the total area under the curve (Table I).

The average values from the above table were next multiplied by 0.436 (the fraction of total histone remaining bound to DNA after 1.0 M NaCl extraction) to yield the amount of each histone component remaining

Figure 3. The fraction of individual histone components remaining bound to DNA after extraction with various concentrations of NaCl. Lysine-rich histone I: ● ; slightly-lysine-rich histone IIa: □; slightly-lysine-rich histone IIb and arginine-rich histone III: 0; and arginine-rich histone IV: Δ . These fractions are homologous to mammalian histones f1, f2b, f2a2 and f3, and f2a1 respectively.

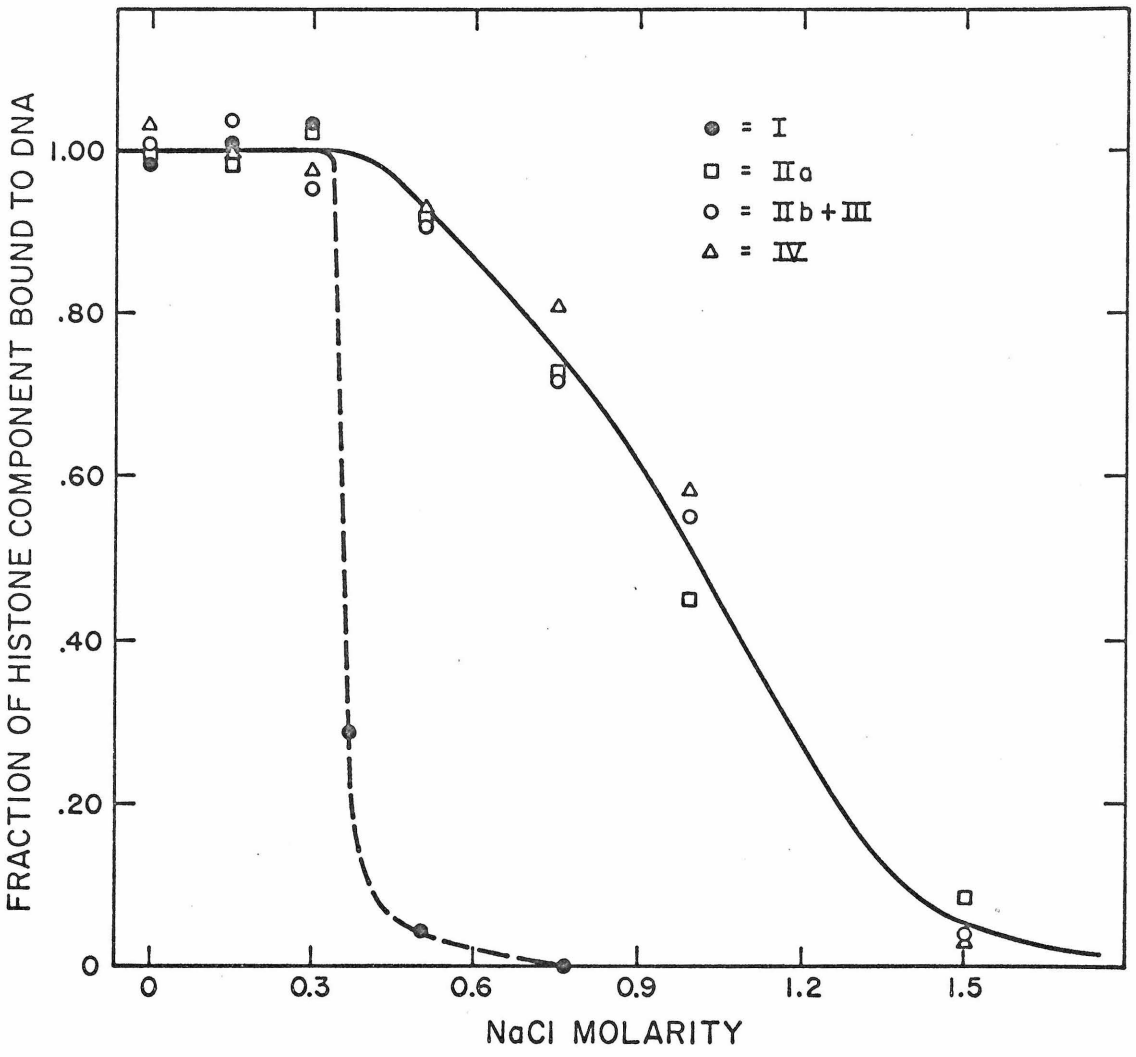

FRACTION OF HISTONE COMPONENT BOUND TO DNA
1.00
.80
.60
.40
.20
0
0
0.3
0.6
0.9
1.2
1.5
NaCl MOLARITY
● = I
□ = IIa
○ = IIb+III
△ = IV

Table I

Amount of Histone Component as a Per Cent of Total Histone Remaining Bound to DNA after 1.0 M NaCl Extraction and Results of Calculations

Experiment	Histone I	IIa	IIb-III	IV
1	0	40.0	34.7	25.3
	0	46.3	30.9	21.2
2	0	54.2	27.9	17.9
	0	58.4	25.3	16.4
3	0	49.8	31.7	18.5
	0	49.0	31.6	19.4
4	0	45.5	33.5	21.2
	0	48.0	31.2	21.0
Average $\pm$ s.d. (n-8)*	0	48.8±5.7	30.8±3.0	20.1±2.7
% Relative to Total Histone	0	21.3	13.4	8.8
Composition of Total Histone	14.4	46.8	24.6	14.8
Fraction of Component Remaining	0	0.46	0.55	0.59

*Standard deviations are calculated assuming each measurement independent of the others. These calculations are merely intended to reflect the degree of variability in the measured values.

Table II

Composition of Histone Bound to DNA after Extraction with Various Concentrations of NaCl

NaCl Molarity	Iab	IIa	IIb and III	IV
0.0	13.9 ± 1.1	46.7 ± 4.1	24.1 ± 3.1	15.4 ± 1.9
0.15*	14.3 ± 0.6	46.1 ± 3.4	24.8 ± 1.9	14.7 ± 1.7
0.30*	14.5 ± 2.0	48.0 ± 4.7	22.8 ± 2.4	14.5 ± 1.1
0.375**	4.9	50.8	26.9	17.5
0.50	1.0 ± 0.9	55.0 ± 2.8	26.9 ± 1.7	17.3 ± 1.4
0.75	0	52.9 ± 5.1	27.0 ± 2.7	18.9 ± 2.4
1.00	0	48.8 ± 5.7	30.8 ± 3.0	20.1 ± 2.7
1.50	0	70.6 ± 5.9	15.2 ± 5.3	9.2 ± 1.5

Values are ± standard deviation (n = 8) except as indicated.

*n = 6

**n = 2

bound to DNA after 1.0 M NaCl extraction relative to the total histone in untreated nucleohistone. The resulting numbers were then divided by the amount of each histone component in untreated nucleohistone. The resulting numbers are the fractions of each histone component remaining bound to DNA after 1.0 M NaCl extraction. Results of these calculations are also presented in Table I. In this example 0.46 of histone IIa remains bound to DNA together with 0.55 of histones IIb and III and 0.59 of histone IV.

Electrophoretic analysis of dissociated histones supports several of the conclusions reached above. No histones can be demonstrated in the 0.15 and 0.3 M NaCl extracts and the 0.375 and 0.5 M NaCl extracts contain nearly pure histone I. However, quantitative agreement between analyses of bound and dissociated histones has not been found. The recovered salt-dissociated histones are always richest in lysine-rich histone components. This phenomenon is associated with low yields of recovered histone. Both of these results can be understood in terms of well-documented behavior of histones in salt solutions. Under conditions of high NaCl concentration and neutral pH the more arginine-rich histone fractions IIb, III and IV are selectively aggregated and in the centrifugal fields employed in these experiments should sediment to the region of the salt solution -- sucrose solution interface where they would be unavailable for subsequent recovery (Cruft et al., 1958). Lysine-rich histones are not aggregated by high salt concentrations. Thus, although analysis of bound histone shows that about 25% of histones IIa, IIb and III and IV are dissociated by 0.75 M NaCl, analysis of dissociated histones

does not indicate the presence of any histone IV and only a trace of these other histones. This point is especially important in the comparison of the present data with data on the dissociation of calf-thymus histones and will thus be cited in the Discussion

Non-histone protein

When pea bud chromatin, which has a "histone/DNA" ration of 1.34 is extracted with 0.15 or 0.3 M NaCl its "histone/DNA" ratio drops to 1.05. Electrophoretic analysis of both the bound and dissociated histones reveal that no histone is dissociated from DNA at these concentrations of NaCl. The explanation for this apparent paradox is that the "histone" measured in the chemical analysis of chromatin is actually the acid soluble protein fraction, which is well-known to contain non-histone components (see Chapter I). The protein dissociated at low NaCl concentrations migrates in disc electrophoresis as a large number of faint bands of lower mobility than characteristic of histones. When nucleohistone is extracted with 0.3 M NaCl no acid soluble protein is dissociated. It may be that the protein dissociated from chromatin in 0.15 or 0.30 M NaCl is an artifactual component of chromatin or that it is a natural component of chromatin but one which is lost in the preparation of nucleohistone, either due to the disruptive shear forces employed or due to the fractionation which accompanies the process. There are two lines of evidence which suggest that fractionation of the nucleoprotein may be involved. a) The preparation of nucleohistone seems to involve loss of the template-active regions of the pea bud genetic material

(Bonner and Huang, 1963) and b) template active stretches of chromatin seem to possess a larger compliment of non-histone proteins (Marushige, unpublished data; and see also Frenster, 1965; Lezzi, 1967ab).

In the usual chemical analysis of pea bud nucleohistone no non-histone protein is detected (Huang and Bonner, 1963). However, it was noted in the present experiments that after acid extraction of histones and hydrolysis of DNA (5% perchloric acid for 10 minutes at 100°C) a significant amount of insoluble material remains. Therefore in a separate experiment 350 OD_{260} mμ of pea bud nucleohistone was prepared and the histone and DNA removed. The remaining material was found to be almost totally soluble in 1% sodium dodecylsulfate, 8 M urea and could be purified by precipitation with ammonium sulfate, a method developed for the study of the protein of rat liver nucleohistone (Marushige, unpublished method; my thanks to Dr. K. Marushige for his aid in this experiment). This material was estimated to amount to 1/4 to 1/3 the weight of the DNA from which it was isolated. The same sort of residue was found in the perchloric acid hydrolysates regardless of the NaCl concentration employed in prior extraction. Thus it may be inferred that at least a large portion of this residue is non-histone protein which is not dissociated from DNA by extraction with 2 M NaCl.

Solubility of nucleohistone in NaCl solutions

As observed by many other investigators (see Phillips, 1961), dialysis of deoxyribonucleoprotein against 0.15 or 0.3 M NaCl results in virtually complete precipitation of the DNP complex. After dialysis of pea bud

nucleohistone against 0.5 and 0.75 M NaCl only a few small aggregates are formed and in higher NaCl concentrations no visible precipitation occurs.

Discussion

Comparison of Pea Bud and Calf Thymus Data

In general, the dissociation of histones from pea bud nucleohistone by NaCl resembles dissociation of histones from calf thymus nucleohistone. In both cases no histone is dissociated by 0.3 M NaCl, the lysine-rich histones are selectively extracted by 0.5 M NaCl, and the remaining histones are dissociated over a much broader range of higher NaCl concentrations. It appears that histone removal as a function of NaCl concentration over the range 0.6-1.5 M NaCl follows the same slope for pea bud and calf thymus (Figure 1). The difference in the absolute values can be attributed to a higher concentration of histone I in calf thymus (see Chapter II and Hnilica et al., 1966), which is removed by 0.5 M NaCl.

The dissimilarities between the dissociation behavior of pea bud and calf thymus nucleohistones are difficult to precisely evaluate. Qualitatively there is no detectable dissociation of calf thymus histones II, III and IV from DNA below 0.8 M NaCl while there is slight dissociation of pea bud histones II, III and IV even at 0.5 M NaCl. Quantitatively, calf thymus histone I seems to be slightly more difficult to dissociate than pea bud histone I, and calf thymus histones II, III and IV (bands B2, B1 and A of Ohlenbusch, et al., 1967) are, to some extent, selectively dissociated

while there appears to be virtually no selectivity in dissociation of pea bud histones. Pertinent quantitative data are presented in Table III.

One source of difference in the analyses of pea bud and calf thymus histones is that the calf thymus fraction IIb contains components IIb1 and IIb2 while pea bud histone IIb (homologous to calf thymus IIb1) is lumped with pea bud histone III in the analyses. Many of the differences, however, can perhaps best be attributed to differences in methods of analysis. In analysis of the calf thymus data Ohlenbusch et al. (1967) rely heavily on a supposed material balance between bound and dissociated histones. Their quantitative analysis is based upon visual estimation from electrophoretic patterns of the distribution of each histone component between bound and dissociated histones. This method of analysis suffers from two obvious pitfalls. 1) There is no evidence that such a material balance was achieved, expecially after extraction at high NaCl concentrations, and in the case of pea histones no such material balance could be attained. 2) No care was taken to avoid overloading of gels, so the staining of individual calf thymus histone components was not necessarily linearly related to protein concentration. Therefore the conclusion that no calf thymus histone II, III or IV is dissociated by 0.7 M NaCl, for example, may only mean that none of these histones could be demonstrated in the NaCl extract. Only a trace of pea histone II and no IV can be demonstrated in a similar extract at 0.75 M NaCl. Thus the true difference between dissociation of these histones from pea bud and calf thymus nucleohistone may be much less significant than it at first appears. Pertinent to this possibility, too, is the fact that extraction

Table III

Comparative Data on the Dissociation of Some Pea Bud and Calf Thymus Histone Fractions from DNA by NaCl

	C IIb	P IIa	C III	P III-IIb	C IV	P IV
Approximate molarity of NaCl required to dissociate 10% of histone	0.8	0.5	0.8	0.5	0.8	0.5
Approximate molarity of NaCl required to dissociate 95% of histone	1.6	1.5	1.2	1.5	2.0	1.5

of calf thymus nucleohistone with 0.6 M NaCl removes 31% of total histone (Ohlenbusch et al., 1967), while histone I accounts for only about 20% of total histone (Johns, 1964; Hnilica et al., 1966).

Comparative analysis is further complicated by one disparity between the data of Ohlenbusch et al.,and that of Tuan (1967) as to the extent of selectivity in the removal of histones IIb, III and IV. According to Ohlenbusch et al., histone III is completely dissociated from nucleohistone by 1.2 M NaCl. Some of the data of Tuan clearly show that histone III is not completely dissociated from DNA even by 1.6 M NaCl, a finding more in agreement with the pea bud data. In preliminary experiments by the present author, using calf thymus nucleohistone, this finding was corroborated.

The difference in dissociation behavior of calf thymus and pea bud histone I, while small, seems to be quite real and reasonable. About 80% of pea bud histone I is dissociated from nucleohistone by 0.375 M NaCl while only about 20% of calf thymus histone I is dissociated in 0.4 M NaCl. This difference is consistent with the differences in chemical nature (discussed in Chapter I) Briefly, the pea histone I contains fewer basic residues (in mole %) and probably more acidic ones than calf thymus histone I. Some of the other reasons for the difference in electrophoretic properties of pea bud and calf thymus histone I may also apply to the strength of ionic binding between these histones and DNA.

Conclusions

Despite lingering uncertainties concerning the true differences in the details of dissociation of calf thymus and pea bud histones from their respective DNAs, it is clearly possible to selectively dissociate the lysine-rich histones by 0.5 M NaCl in both cases. From a utilitarian standpoint, it is this rather clean, selective dissociation that is particularly useful in the preparation of well-defined native nucleoprotein complexes lacking specific histone components. Such preparations should play an important role in the elucidation of histone functions. Toward this end, several other methods for selective dissociation of histones from DNA have been or are being developed. These include the use of sodium perchlorate (Ohlenbusch, 1966; Ohlenbusch et al., 1967), guanidinum chloride (Bonner et al., 1967) sulfuric acid (Murray, 1967) and sodium dodecylsulfate (Smart, unpublished data). In these cases the dissociating forces are presumably more complex than in the case of NaCl. The similarities between dissociation of pea bud and calf thymus histones from DNA by NaCl together with the chemical and physical similarities of pea bud and calf thymus histones suggest that these other procedures for selective dissociation might also be applicable to both plant and animal nucleohistones. In the light of these similarities the other procedures are currently being applied to plant and animal nucleohistones alike. Preliminary results from experiments on selective removal of histone components other than the lysine-rich, using guanidinium chloride and sodium dodecylsulfate (Smart, Bekhor, unpublished data) indicate the general applicability of these procedures to plant and animal

material. These findings further emphasize the extensive similarities between homologous plant and animal histones.

References

Bonner, J., Chalkley, G.R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R.C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H., Olivera, B.M., and Widholm, J. (1967), Methods in Enzymology: Nucleic Acids, New York, Academic Press. In press.

Bonner, J. and Huang, R.C. (1963), J. Mol. Biol. 6, 169.

Cruft, H.J., Mauritzen, C.M. and Stedman, E. (1958), Proc. Roy. Soc. (London), Ser. B, 149, 21.

Daly, M.M. and Mirsky, A.E. (1955) J. Gen Physiol. 38, 405.

Dische, Z. (1955) in The Nucleic Acids Vol. I (E. Chargaff and J.N. Davidson, eds.) Academic Press, New York, N.Y.

Frenster, J.H. (1965), Nature 206, 680.

Giannoni, G. and Peacocke, A.R. (1963), Biochim. Biophys. Acta 68, 157.

Hnilica, L.S., Edwards, L.J., and Hey, A.E. (1966), Biochim Biophys. Acta 124, 109.

Johns, E.W. (1964), Biochem. J. 92, 55.

Lezzi, M. (1967a), Chromosoma 21, 72.

Lezzi, M. (1967b), Chromosoma 21, 89.

Lowry, O.H., Rosebrough, N.J., Faar, A.L., and Randall, R.J. (1951), J. Biol. Chem. 193, 265.

Lucy, J.A. and Butler, J.A.V. (1955), Biochim. Biophys. Acta 16, 431.

Murray, K. (1966), J. Mol. Biol. 15, 409.

Ohba, Y. (1966), Biochim. Biophys. Acta 123, 76.

Ohlenbusch, H.H. (1966), Ph.D. Thesis, California Institute of Technology.

Ohlenbusch, H.H., Olivera, B.M., Tuan, D., and Davidson, N. (1967), J. Mol. Biol. 25, 299.

Olivera, B.M. (1966), Ph.D. Thesis, California Institute of Technology.

Phillips, D.M.P. (1961) Progr. Biophys. Biophys. Chem. 12, 211.

Tuan, D. (1967), Ph.D. Thesis, California Institute of Technology.

Appendix

Young pea cotyledons contain less than half as much histone I as do mature pea cotyledons. During maturation there is a steady increase in the amount of histone I present in total pea cotyledon histone (see Chapter II). This appendix describes exploratory experiments on the relation of these phenomena to other macromolecular parameters: chemical compositions, spectra, melting profiles, and template activities of pea cotyledon chromatins as well as to histone and DNA synthesis.

Methods

Preparation of chromatin

Chromatin was prepared from pea seeds and pea cotyledons of different maturation stages, using the method described in Chapter I for the preparation of chromatin from pea buds.

Chemical analyses

DNA and RNA were fractionated by the Schmidt-Tannhauser procedure as modified by Ts'o and Sato (1959). DNA was then determined by the method of Dische (1955) and RNA by the method of Dische and Schwarz (1937). Histone was extracted with 0.2 N HCl, precipitated with 20% TCA and assayed by the method of Lowry *et al.* (1951). The protein not extracted with 0.2 N HCl (non-histone protein) was determined by

the method of Lowry *et al.* (1951) after extraction of histones and subsequent removal of nucleic acids by heating (95°C for 15 min in 10% TCA).

Template activities

The ability of isolated chromatin to serve as template for the *in vitro* synthesis of RNA using *E. coli* RNA polymerase was measured by the method of Marushige and Bonner (1966) without modification. RNA polymerase fraction 3 (F3) was prepared from frozen log phase *E. coli* strain B cells, using the method of Chamberlain and Berg (1962) as modified by Bonner *et al.* (1967). Incubations were performed at 37°C for 10 min, using template limiting conditions. Under these conditions RNA synthesis is approximately linear with DNA concentration and with time for all experimental conditions used.

Melting profiles

The change in absorption accompanying heat denaturation of chromatin was measured, using a Gilford Spectrophotometer with automatic heating assembly. A heating rate of approximately 0.5°C/min over the range 25°C to 100°C was used. Chromatin was dissolved in 2.5×10^{-4} M sodium EDTA buffer at pH 8.0.

Synthesis experiments

To study histone and DNA synthesis in maturing pea cotyledons, the cotyledons were thoroughly washed and placed round side up in small

pietri dishes at room temperature in three ml of 0.05 M Tris-HCl buffer pH 8.0, containing 60,000 units penicillin and either ^{14}C lysine and arginine or ^{14}C thymidine (usually 1 μC/ml incubation fluid). In control experiments it was found that incorporation of ^{14}C lysine and arginine into protein (TCA precipitable material which was not hydrolyzed by 20% TCA, 20 min at 100°C) was linearly related to length of incubation time and to quantity of labelled amino acids in the incubation mixture for all experimental circumstances. This is shown in Figure 1. The incorporation of lysine and arginine separately into total protein is shown in Table I. There is an approximately tenfold difference between the lysine and arginine incorporation. The bulk protein of pea seeds is richer in arginine than in lysine (Hanson and Koch, 1952). This suggests different permeabilities of cotyledon cells to lysine and arginine or a large difference in the pool sizes of lysine and arginine in the cells. Whichever is true is of little consequence in the interpretation of the histone synthesis data.

Subsequent to incubation, chromatin was prepared from the cotyledons and histones extracted from it. DNA was prepared by the Schmidt-Tannhauser procedure, determined by the method of Dische (1955) and its specific activity measured. RNA (alkali sensitive nucleic acid) was virtually unlabelled, with specific activity less than 1% that of DNA.

For the fractionation of C^{14} labelled histone, chromatography on Amberlite CG-50 and disc electrophoresis were used. Chromatography was performed both with and without carrier histones, and alternate fractions were collected in scintillation counting vials. To these were added

Figure 1. Net incorporation of ^{14}C lysine and arginine into TCA precipitable material of stage 7 pea cotyledons. Incorporation is expressed as cpm per cotyledon (average weight 120 mg). Insert: incorporation of ^{14}C lysine and arginine into pea cotyledons during 16 hours incubation with lowest (1 μC/ml) and highest (3 μC/ml) concentrations of radioactive amino acids.

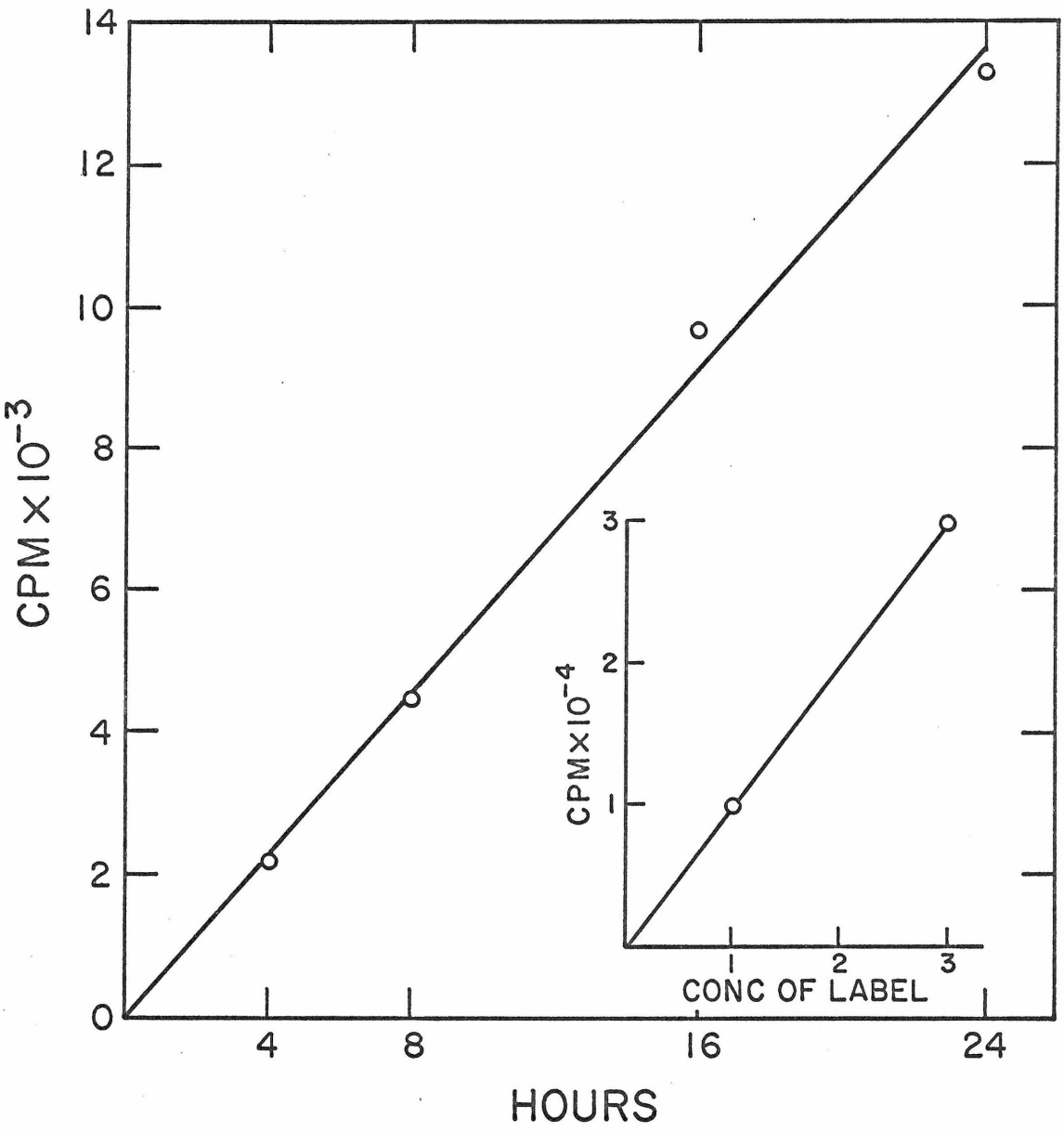

14
12
10
8
6
4
2
0
CPM X 10⁻³
4
8
16
24
HOURS
3
2
1
CPM X 10⁻⁴
2
3
CONC OF LABEL

Table I.

Incorporation of ^{14}C-labelled Lysine and Arginine into Pea Cotyledon Protein

Maturation Stage	CPM in Protein per gram Cotyledons		
	Lysine*	Arginine*	Lysine + Arginine*
1	---	24,000	170,000
2	550,000	37,000	170,000
3	1,320,000	93,000	1,050,000
4	1,050,000	87,000	635,000
5	1,400,000	134,000	1,500,000
6	2,810,000	215,000	1,960,000
7-9	3,350,000	210,000	2,640,000

*Incubation mixtures included 1 μC/ml of each labelled amino acid.

15 ml aliquots of scintillation counting fluid (see Chapter I for composition) containing, in addition, 20 g/liter Cab-o-Sil. After vigorous shaking, the vials were counted in a Packard Scintillation counter. As reported elsewhere,(Bonner et al., 1967) there is no significant change in quenching with the changing salt concentrations which result from the salt gradient. Alternate fractions were analyzed for protein by TCA precipitation and measurement of OD_{400} mμ. When electrophoretic separation was used, gels were stained with amidoschwarz and scanned in the Canalco microdensitometer. The bands were then cut from the gels, hydrolyzed 3 hours in 0.5 ml aliquots of 30% hydrogen peroxide, and counted in 10 ml scintillation counting fluid.

Results

Properties of chromatins

The chemical compositions and template activities of pea cotyledon chromatins are presented in Table I. Two features of the chemical composition data are of particular interest: first, the histone/DNA ratio of chromatin from young pea cotyledons is unusually low. For comparison with other pea tissue chromatins or with chromatin from other organisms see Bonner et al.(1967b). No other pea tissue chromatin has histone/DNA ratio less than 1.1. Second, the quantity of acid soluble protein in the chromatins of more mature stages is increasingly large and, as shown by column chromatography, consists in part of non-histone run-off protein (see, for example, the elution patterns for

young and old pea cotyledon histones presented in Figure 4gh of Chapter II). This protein interferes with a reliable determination of histone/DNA ratio.

Representative spectra of pea cotyledon chromatins are presented in Figure 2. The spectra of chromatin from older pea cotyledons show greater turbidity (OD 320 mμ). Such spectra are not significantly changed by three centrifugations of chromatin through 1.7 molar sucrose.

The thermal denaturation curves for chromatins from young pea cotyledons have a pronounced two-step character with T_m's of 66° and 84°C in 2.5×10^{-4} M EDTA. This two-step character is suggested in the melting profiles of chromatins from other pea sources but in no other is it so pronounced. The melting profiles of chromatin from two maturation stages are presented in Figure 3. The differences between these two melting profiles are small, so small, in fact, that although they can be consistently demonstrated when two chromatins are melted simultaneously, they are not always detectable when melting curves from different experiments are compared. No useful data on the thermal denaturation of more mature pea cotyledon chromatins could be obtained. During melting of these chromatins, changes in turbidity take place over the same temperature range as does melting, thus obscuring the hyperchromic changes in the solutions. The errors involved are too great to warrant correction for the turbidity changes. The small shifts in melting profiles accompanying the earlier maturation stages are in the direction of more stabilization of the DNA against denaturation. It is known that histones, especially the lysine-rich histones, stabilize

Figure 2. Spectra of chromatin from pea cotyledons of maturation stages 1-4, 6, and 10.

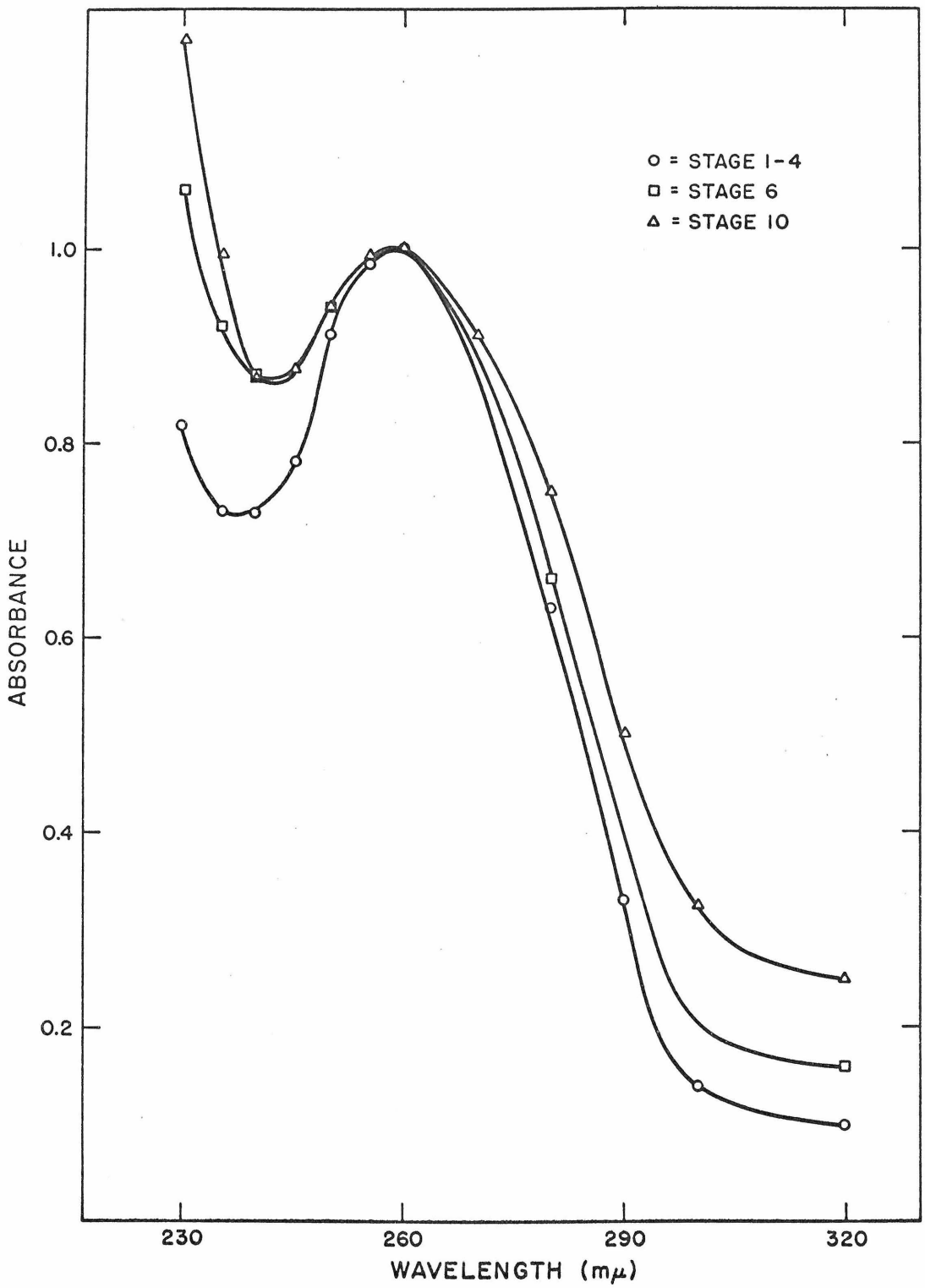

o = STAGE 1-4
□ = STAGE 6
Δ = STAGE 10
ABSORBANCE
1.0
0.8
0.6
0.4
0.2
230
260
290
320
WAVELENGTH (mμ)

Figure 3. Melting profiles of chromatin from pea cotyledons of maturation stages 2 and 5 in 2.5 x 10^{-4} M sodium EDTA, pH 8.

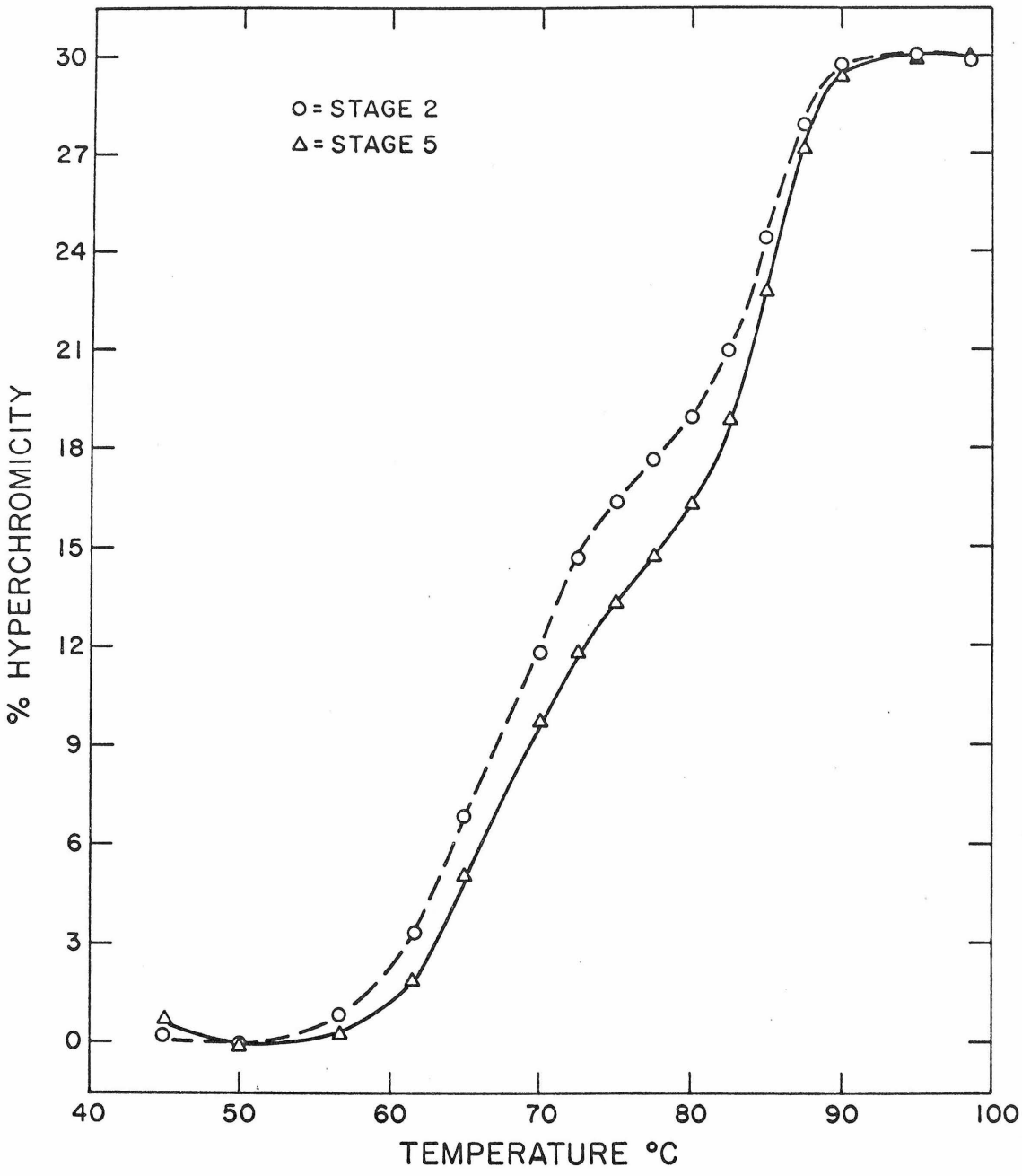

O = STAGE 2
Δ = STAGE 5
% HYPERCHROMICITY
TEMPERATURE °C
0
3
6
9
12
15
18
21
24
27
30
40
50
60
70
80
90
100

Table II.

Chemical Composition and Template Activity of Chromatin From Various Cotyledon Maturation Stages

Maturation Stage	DNA	RNA	Acid soluble Protein	Residual Protein	% Histone I	Template Activity
1	1.00	0.10	0.91	0.35	- 7.6	34 %*
2	1.00	0.08	1.13	0.26		34 %
4	-	-	-	-	6.5	28 %*
5	1.00	0.14	1.13	0.48	7.4	34 %
6	1.00	0.13	1.14	0.50	10.1	36 %
7	-	-	-	-	10.7	34 %
10	1.00	0.02	1.49	0.65	16.0	20 %

*Single determination. Others are the averages of two determinations which differ by less than ± 3% from the average.

DNA against thermal denaturation. Since there are increases both in total histone and in the amount of the lysine-rich components during maturation, increasing stabilization of the DNA is to be expected.

As shown in Table II there is no significant change in the template activity of chromatin during the early maturation stages although nearly a doubling in histone I content occurs in the same period of maturation. Chromatin from dried peas supports RNA synthesis at a rate only about two thirds that of the chromatin from young pea cotyledons. It is not clear to what extent the aggregated nature of the less active chromatin affects its priming ability. This chromatin was, however, visibly precipitated in the incubation mixture.

Histone synthesis

Analysis of the quantitative distribution of histone fractions in pea cotyledons left unanswered the questions: by what means is the histone distribution changed during maturation? Are all histones synthesized or only the lysine-rich components? Is histone synthesis coupled to DNA synthesis in this case? Both turnover of histones in the absence of DNA synthesis (Chalkley and Maurer, 1965; Sadgopal, personal communication, 1967) and histone synthesis coupled rather strictly with DNA synthesis (Robbins et al., 1967; Byvoet, 1966; Comings, 1967) have been reported.

To answer some of these questions, pea cotyledons of various maturation stages were incubated in ^{14}C lysine and arginine, the chromatins isolated, and the histone extracted and fractionated by column

chromatography and disc electrophoresis. Elution profiles of the labelled histones are presented in Figure 4a-c. Some disc electrophoresis data are presented in Table III.

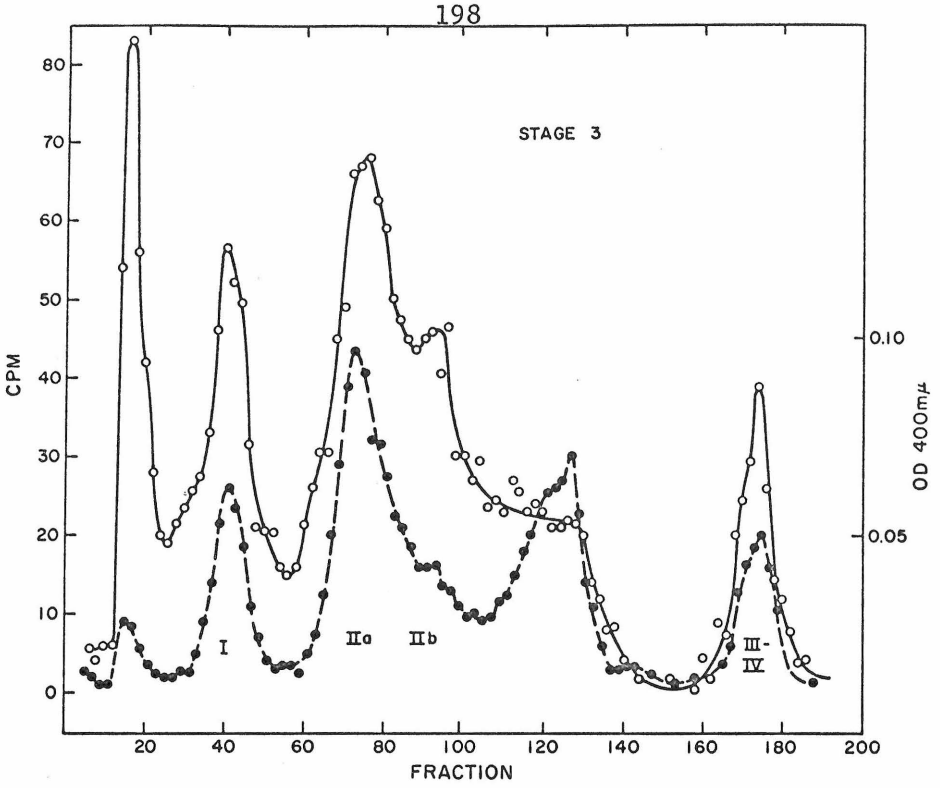
Both the chromatographic and electrophoretic data show that synthesis of all histone components takes place during all stages of maturation, even in fully matured cotyledons. It appears that during the later stages of maturation less histone II is being added to the nucleoprotein than during the early stages. This is consistent with the decline in relative histone II content (but no in histone III-IV content) during maturation.

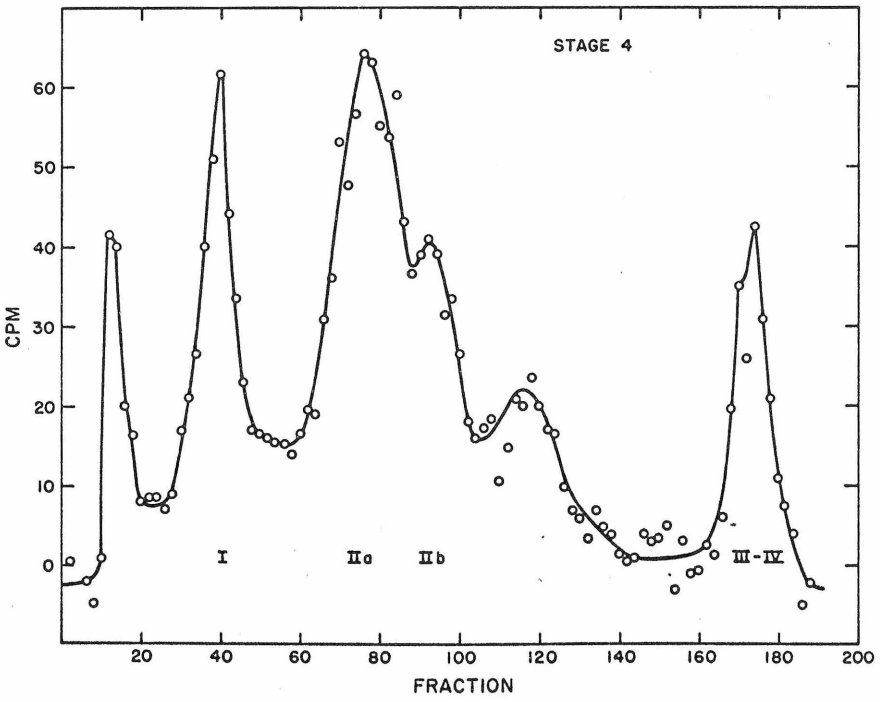
Quantitative analysis of the synthesis data cannot yield meaningful results. At least four factors are involved in the determination of the specific activities of the histone fractions: rates of synthesis, rates of degradation, rates of interaction of newly synthesized histones with the DNA and dilution of newly synthesized histones by histones already bound to the DNA. And each of these factors may be quite complex in its dependence upon other circumstances in the cells.

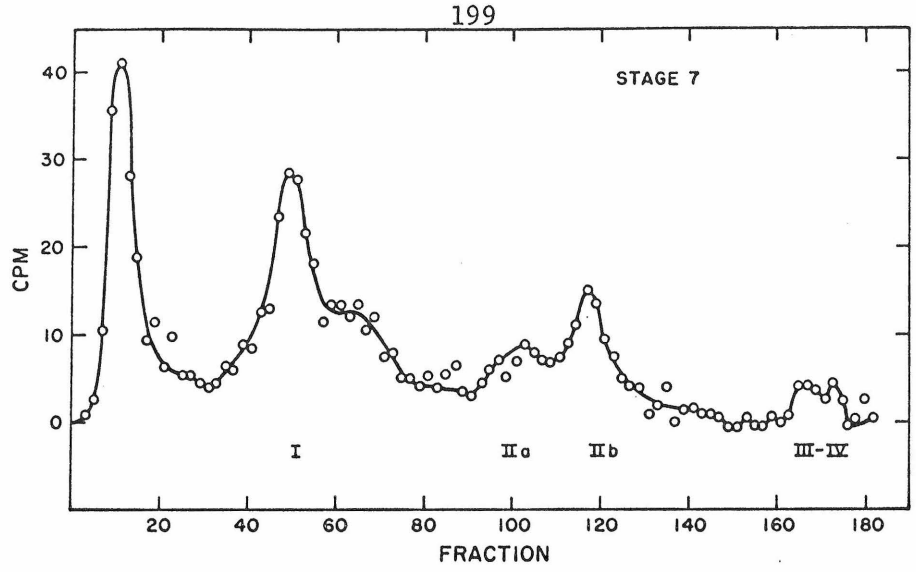
DNA synthesis

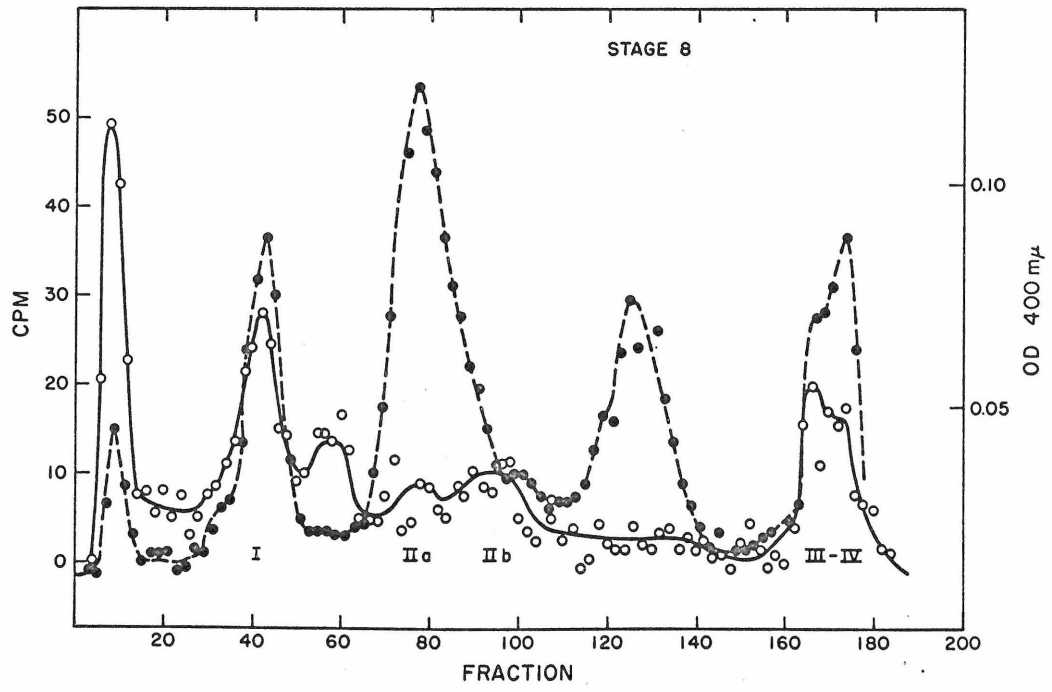
DNA synthesis as a function of maturation was measured by the incorporation of ^{14}C thymidine into pea cotyledons, isolation of the nuclear DNA, and determination of specific activity. The purity of the DNA was tested by buoyant density centrifugation -- no satelite band was found. Therefore the contamination by chloroplast and mitochondrial DNAs was judged to be low. Figure 5 describes the changes

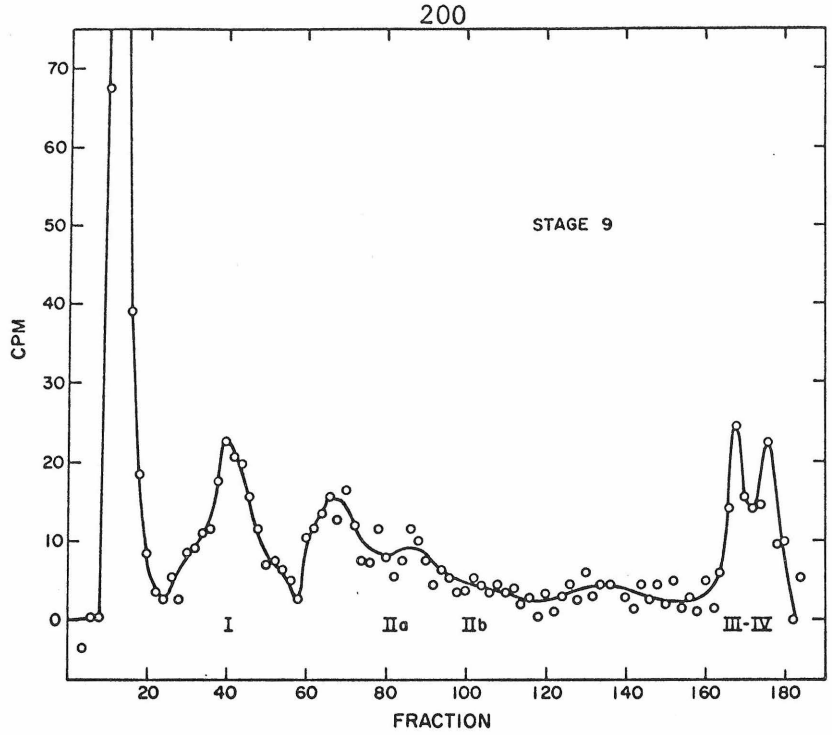
Figure 4. Column chromatography of ^{14}C lysine and arginine labelled histones from pea cotyledons in maturation stages (a) 3 and 4 (b) 7 and 8 (c) 9 and 10. Radioactivity --0--0--. Protein --●--●--.


STAGE 3
CPM
OD 400mμ
FRACTION
I
IIa
IIb
III-IV


STAGE 4
CPM
FRACTION
I
IIa
IIb
III-IV


STAGE 7
CPM
FRACTION
I
IIa
IIb
III-IV


STAGE 8
CPM
OD 400 mμ
FRACTION
I
IIa
IIb
III-IV


STAGE 9
CPM
FRACTION
I
IIa
IIb
III-IV

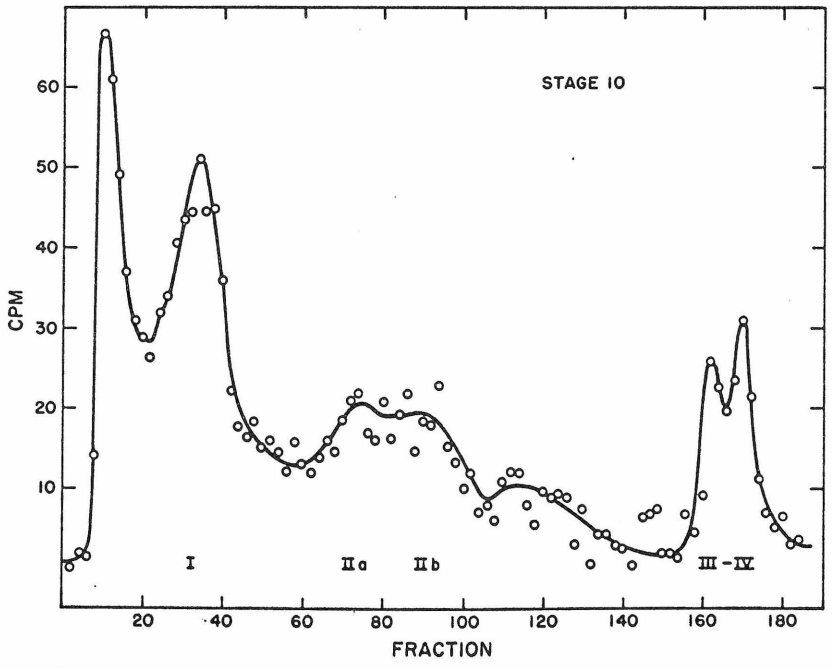
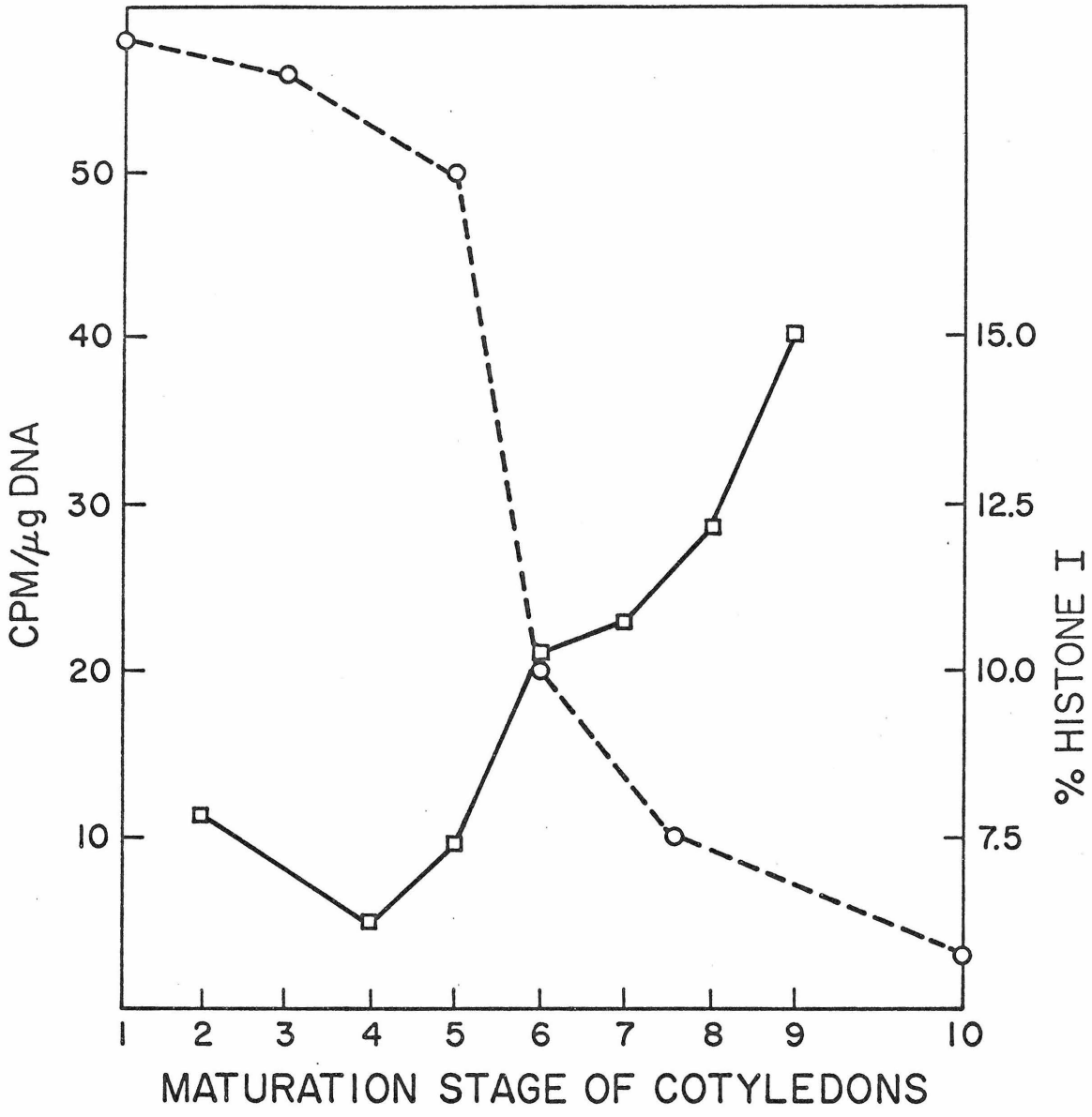

STAGE 10
CPM
FRACTION
I
IIa
IIb
III-IV

Table III.

Incorporation of ^{14}C-Labelled Lysine and Arginine Into Electrophoretic Fractions of Pea Cotyledon Histones

Histone Fraction	Maturation Stage 4		Maturation Stage 7		Maturation Stage 9	
	cpm	specific activity	cpm	specific activity	cpm	specific activity
Ia	55	4.4	28	6.7	30	5.4
Ib	100	8.0	34	21.8	52	17.2
Ic plus IIIox	70	1.2	37	11.8	39	13.8
IIa	305	2.3	57	1.5	87	2.2
IIb plus III red	60	1.9	27	1.8	28	2.1
IV	80	1.0	17	1.0	17	1.0

Figure 5. Net incorporation of ^{14}C thymidine into DNA of pea cotyledons of various maturation stages --0--0-- and histone Iab content (per cent of total histone) in pea cotyledon chromatin --□--□--.


50
40
30
20
10
CPM/μg DNA
15.0
12.5
10.0
7.5
% HISTONE I
1
2
3
4
5
6
7
8
9
10
MATURATION STAGE OF COTYLEDONS

in DNA synthesis as a function of maturation and the relation of these changes to the increase in histone I. The relatively high rate of DNA synthesis in the early maturation stages was unexpected because generally cell division in plants is virtually complete before cell enlargement takes place. The level of DNA synthesis in the youngest cotyledon stages was comparable to that of germinating embryos incubated under the same conditions. Whether or not the DNA synthesis in pea cotyledons is related to cell division is not known. It could represent repair of existing DNA or proliferation of small DNA segments. It is clear that much of the histone I synthesis accompanies a relatively low rate of DNA synthesis. It is not known whether or not any biologically meaningful relation exists between the two.

References

Bonner, J., Chalkley, G.R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R.C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H., Olivera, B.M. and Widholm, J. (1967a). Methods in Enzymology: Nucleic Acids, Academic Press, New York, in press.

Bonner, J., Dahmus, M., Fambrough, D., Huang, R.C., Marushige, K., and Tuan, D. (1967b), Science, in press.

Byvoet, P. (1966), J. Mol. Biol. 17, 311.

Chalkley, G.R. and Maurer, R. (1965), Proc. Nat. Acad. Sci. (Wash.) 54, 498.

Chamberlain, M. and Berg, P. (1962), Proc. Nat. Acad. Sci. (Wash.) 48, 1588.

Comings, D. (1967), Science, in press.

Dische, Z. (1955), in The Nucleic Acids, Vol. I (E. Chargaff and J.N. Davidson, eds.), Academic Press, New York. p 285.

Dische, Z. and Schwarz, K. (1937), Mikrochim. Acta 2, 13.

Hanson, H. and Koch, R. (1952), Pharmazie 7, 655.

Lowry, O.H., Rosebrough, N.J., Faar, A.L. and Randall, R.J. (1951), J. Biol. Chem. 193, 265.

Marushige, K. and Bonner, J. (1966), J. Mol. Biol. 15, 160.

Robbins, E. and Borum, T.W. (1967), Proc. Nat. Acad. Sci. (Wash) 57, 409.

Ts'o, P.O.P. and Sato, C. (1959), Explt. Cell Res. 17, 227.