inhibition constants determined by initial velocity and product inhibition techniques could be the result of this failure. We have used simulation techniques to study in some detail the properties of mechanism 7, assuming various rate constants, and studying cases where the central step was and was not rate limiting. The approach used was to have a digital computer calculate initial velocities as a function of substrate and product concentrations, and then to fit these true velocities to eq 2-4, as appropriate, and compare the numerical values of constants from simulated initial velocity and product inhibition experiments. In no case could really significant differences be found. Further it was obvious that for a random mechanism such as mechanism 7, initial velocities are predicted very accurately by eq 2, 9, and 10, which are derived from eq 8, even though the rapid equilibrium assumption is completely invalid. The observed discrepancies are thus probably the result of experimental difficulties.

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Quantitative Distribution of Histone Components in the Pea Plant*

Douglas M. Fambrough, Frank Fujimura, and James Bonner

ABSTRACT: Histones, prepared by acid extraction of purified chromatin from many different organs of the pea plant, are fractionated by column chromatography and by quantitative analytical disc electrophoresis. By these criteria there is no histone fraction specific to any pea tissue. The chromatins of different pea organs do, however, exhibit reproducible quantitative differences in the representation of the several histone fractions.

The histones are basic proteins which occur bound to DNA in the chromosomes of higher organisms. 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.

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Although several demonstrations of histones unique to specific organs 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 histones (Hnilica and Bess, 1965; Butler, 1966; Fambrough, 1968). One possible exception to this statement is the reported occurrence of a slightly lysine-rich histone specific to erythrocyte nuclei (Hnilica, 1964; Neelin *et al.*, 1964). This fraction has not been well characterized except by amino acid analysis (Neelin *et al.*, 1964; Bellair and Mauritzen, 1964). Bellair and Mauritzen have isolated this same fraction from chicken erythrocyte, liver and spleen, and from calf thymus (J. T. Bellair, personal communication,). Thus this fraction, too, is not organ or species specific.

There are, however, convincing reports of quantitative variations in the proportions of histones. These show that the histone:DNA ratio may vary from one organ 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).

In the present report, the histones of different pea organs are compared both qualitatively and quantitatively in order to answer the questions: Are there histones unique to different pea organs? Are there quantitative differences in the proportions of the histone components of different pea organs?

Methods

Pea Tissues. Histones were obtained from the following organs of *Pisum sativum* var. Alaska: embryos, apical buds, etiolated stems, roots, leaflets, whole leaves, flowers, pods, seeds, and cotyledons. Embryos (seedling axes) were prepared by germination of mature pea seeds in tap water, mechanical fragmentation of embryos from the cotyledons and seed coats by application of light pressure, and separation from cotyledons and seed coats by filtration and buoyancy in sucrose solution. Apical buds (the terminal 1 cm of pea shoots) 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 fraction 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 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 nonchromosomal proteins it is necessary to use purified chromatin as the starting material for histone extraction. Purified chromatins of various pea organs were prepared according to the method of Bonner *et al.* (1967b). Purified chromatin was extracted with 0.2 N H_2SO_4 and the residue was 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 nonhistone 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 applied to a 0.6 × 60 cm column and eluted with a linear gradient of 8–13% guanidinium chloride (50-ml total volume) followed by 40% guanidinium chloride. All the guanidinium 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 mμ) after precipitation of protein from a 1.1 M TCA[1] 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.

Analytical 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

[1] Abbreviation used: TCA, trichloroacetic acid.

TABLE I: Maturation States of Pea Seeds.

Stage	Cotyledon Diam (mm)	Density of Seeds	Age (days)[a]	Other Characteristics
1	4–5	1.030–1.039	19	Liquid in seeds, cotyledons elongated and not appressed
2	6–7	1.022–1.030	21	Liquid in seeds, cotyledons only partially appressed and not yet rounded.
3	7	<1.022	24	Little or no liquid in seed, 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.

prepared by mixing one 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, and deionized water to 100 ml), two volumes of acrylamide solution (60 g of acrylamide, 0.4 g of *N,N′*-methylenebisacrylamide, and deionized water to 100 ml), and five 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, and water to 1 l.) and electrophoresed in a standard disc electrophoresis apparatus at a constant current of 4 ma/tube for 1.5 hr. Gels were stained for at least 4 hr in 1% Amido Schwarz–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).

Preparative Disc Electrophoresis. Chromatographic fractions of pea histones were further purified by preparative disc electrophoresis in polyacrylamide gel. A Canalco Prep Disc apparatus was used with the PD2/320 upper column with central cooling and eluting tubes. Acrylamide gel solution (8 ml of 7.5%) containing 6 M urea was polymerized in the column under a layer of 3 M urea to form a gel 2 cm high. A solution of the sample in about 1 ml of 8 M urea was applied to the gel surface and electrophoresis was performed at 35–40 ma. Except for using 7.5% acrylamide gels the reagents and buffers are the same as for analytical disc electrophoresis. A flow rate of eluting buffer of about 30 ml/hr was generally used. The maximum sample load was about 3 mg. Greater loads resulted in marked tailing of the bands. The fractions were assayed by turbidity measurements performed as described under Column Chromatography above. Histones were recovered by precipitation with 1.1 M TCA and centrifugation at 17,000*g* for 15 min. Histone pellets were washed with acidified acetone (0.5 ml of HCl/l. of acetone) and twice with acetone, dissolved in 0.1 M acetic acid, and lyophilized.

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. Figure 1 shows a photograph of the gel electrophoretic pattern of pea histones and a densitometric tracing of the gel. Rotation of the gels about their long axes causes no change in the densitometric tracings. All densitometric tracings were made at 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 2 shows the relationship between histone con-

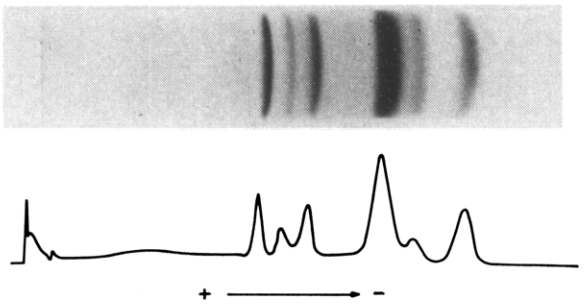


FIGURE 1: Photograph of the gel electrophoretic pattern of pea histones (above) and densitometric tracing of the same pattern (below). Compare resolution of histone components in the gel with the densitometric tracing thereof.

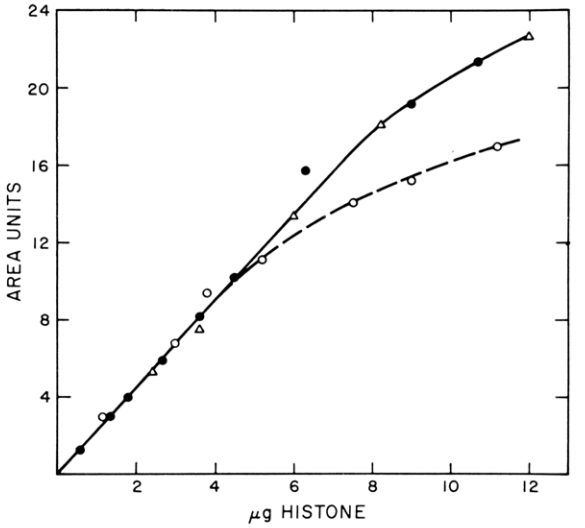


FIGURE 2: Relation between histone concentration in electrophoretic bands and intensity of staining by Amido Schwarz dye. Histone concentration is expressed as micrograms per polyacrylamide gel, intensity of staining as area (arbitrary units) under the curve of densitometric tracings. (○) Histone I; (●) histone II; (△) histone III–IV.

centration and the intensity of staining for the major histone chromatographic fractions. For each set of eight gels in one electrophoretic run the staining with Amido Schwarz 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 nonlinearity 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 nonlinear 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 (Fambrough, 1968). 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 of protein) at low protein concentration. This was done by comparing the elution profiles from preparative disc electrophoresis runs (which gave 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 3. Assuming identical staining constants for all histone components, the estimations of the composition of histone mixtures by analytical disc electrophoresis have always been within 1% of the values determined by preparative disc electrophoresis. In further test experiments several individual histone molecular species were prepared by preparative disc electrophoresis and their staining constants with Amido Schwarz dye 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/gel (5 μg for histone I) it is naturally important that no gel contain more these amounts of single histone components. The total histone applied to the gels was therefore kept in the range of 15–20 μg.

Application of Quantitative Method. The Problem of Histone III Dimerization. A sample electrophoretic tracing is presented in Figure 4. 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 (Fambrough, 1968). (Histone Ic is a minor component of the lysine-rich histones, with mobility greater than the major components Ia and b.) 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 8 M urea and keeping it in the cold (5°) for about 2 days.

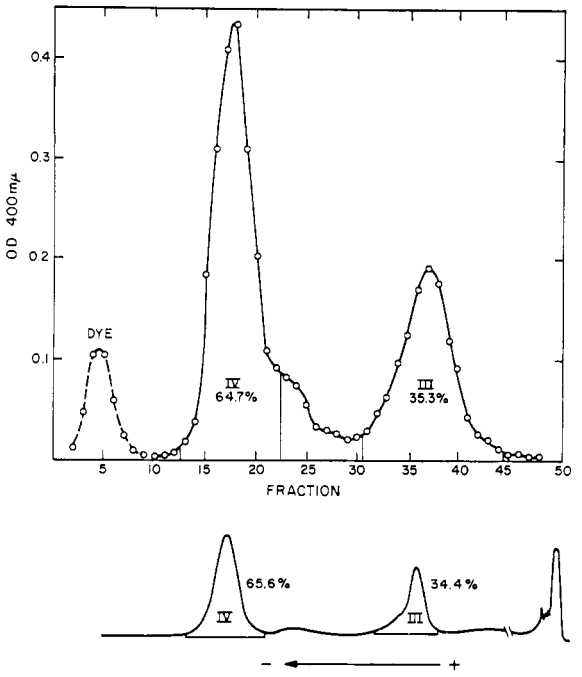


FIGURE 3: Quantitative comparison of the profile of elution of histones III and IV from preparative disc electrophoresis and the densitometric tracing of the electrophoretic pattern of histones III and IV separated by analytical disc electrophoresis and stained with Amido Schwarz. Preparative disc electrophoresis was performed using a Canalco Prep Disc apparatus with PD 2/320 upper column, a 2 cm (8 cm³) 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 optical density 400 mμ after 15 min. The contributions of histones III and IV to the areas under the peaks are indicated on the figure.

Amino Acid Analyses. Histone samples in 1 ml of constant-boiling HCl were sealed in evacuated tubes and hydrolyzed for 22 hr at 105°. Amino acid analyses were performed using a Beckman–Spinco Automatic amino acid analyzer.

Results

Most pea histone fractions have been obtained in electrophoretically pure form. N- and C-terminal analyses suggest that each electrophoretic component is a single molecular species (Fambrough, 1968).[2] Amino acid compositions of the major histone fractions are presented in Table II.

Column Chromatography. The elution patterns which

[2] A full report of the characterization of pea histones is in preparation. The characterization of pea bud histones has been reported in part (Fambrough and Bonner, 1966).

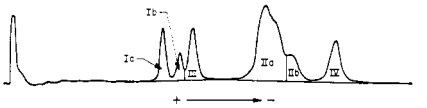


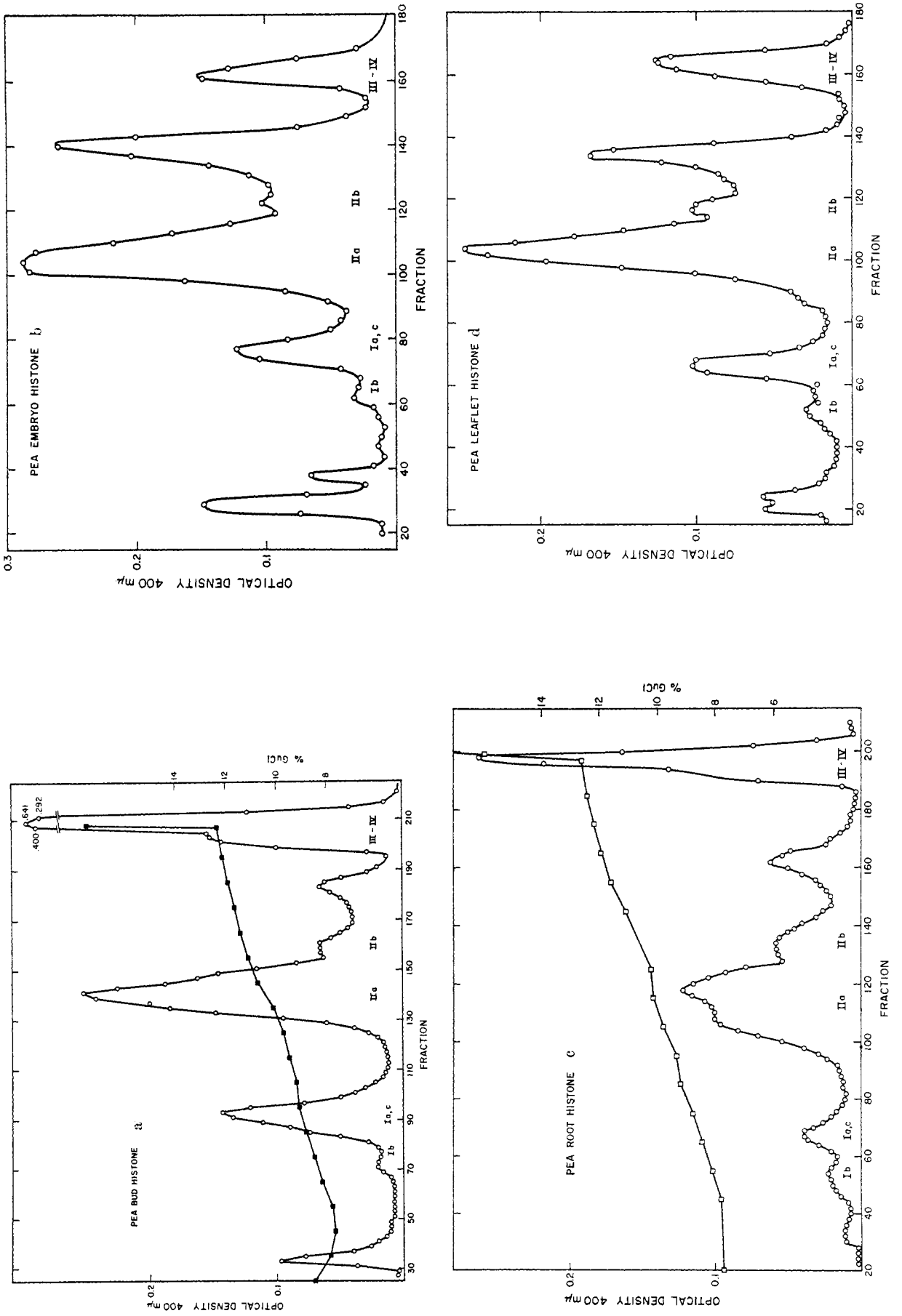
FIGURE 4: Densitometric tracing of the electrophoretic pattern of whole pea bud histone. Vertical and horizontal lines indicate the partitioning of the total area under the curve among the various fractions. Histone III is present exclusively in the oxidized form and thus masks the presence of a small 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 with that of histone IIb. Peak at far left is origin of gel, not protein.

result from fractionation of the histones of various pea organs by column chromatography on Amberlite CG-50 are presented in Figure 5a–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 electrophoresis to confirm the identifications. In the interpretation of the chromatographic data one must

TABLE II: Amino Acid Compositions of Pea Bud Histone Components.[a]

Amino Acid	Histones				
	I	IIa	IIb	III[b]	IV
Lys	25.5	16.1	10.6	8.6 (1.6)[c]	8.5
His	1.1	1.1	1.6	2.1	2.4
Arg	2.8	6.5	9.0	13.1	15.6
Asp	2.3	6.0	6.1	4.5	5.6
Thr	4.0	4.8	4.1	6.6	7.3
Ser	4.9	6.7	5.6	4.1	2.2
Glu	7.3	8.0	6.6	10.8	6.2
Pro	9.9	6.7	7.1	4.5	1.4
Gly	2.3	8.8	11.4	6.6	17.2
Ala	22.8	12.3	12.8	12.9	7.5
Val	5.3	6.7	7.9	5.2	6.6
Met		0.5		Trace	Trace
Ile	1.9	4.5	3.1	5.1	6.3
Leu	4.1	7.9	10.6	9.4	7.6
Try	0.4	1.7	1.9	1.0	3.0
Phe	0.4	1.9	1.6	3.9	2.7

[a] Each histone has been shown by analytical disc electrophoresis to be free of contamination by other fractions. Compositions are expressed as moles/100 moles of amino acids. No corrections have been made for hydrolytic losses. [b] Also contains 1 mole of cysteine/23,000 g of protein (Fambrough, 1968). [c] Value in parenthesis is ε-methyllysine in addition to lysine.


PEA BUD HISTONE a
PEA EMBRYO HISTONE b
PEA ROOT HISTONE c
PEA LEAFLET HISTONE d
OPTICAL DENSITY 400 mμ
% GuCl
FRACTION
Ia, c
Ib
IIa
IIb
III-IV
.641
.292
.400

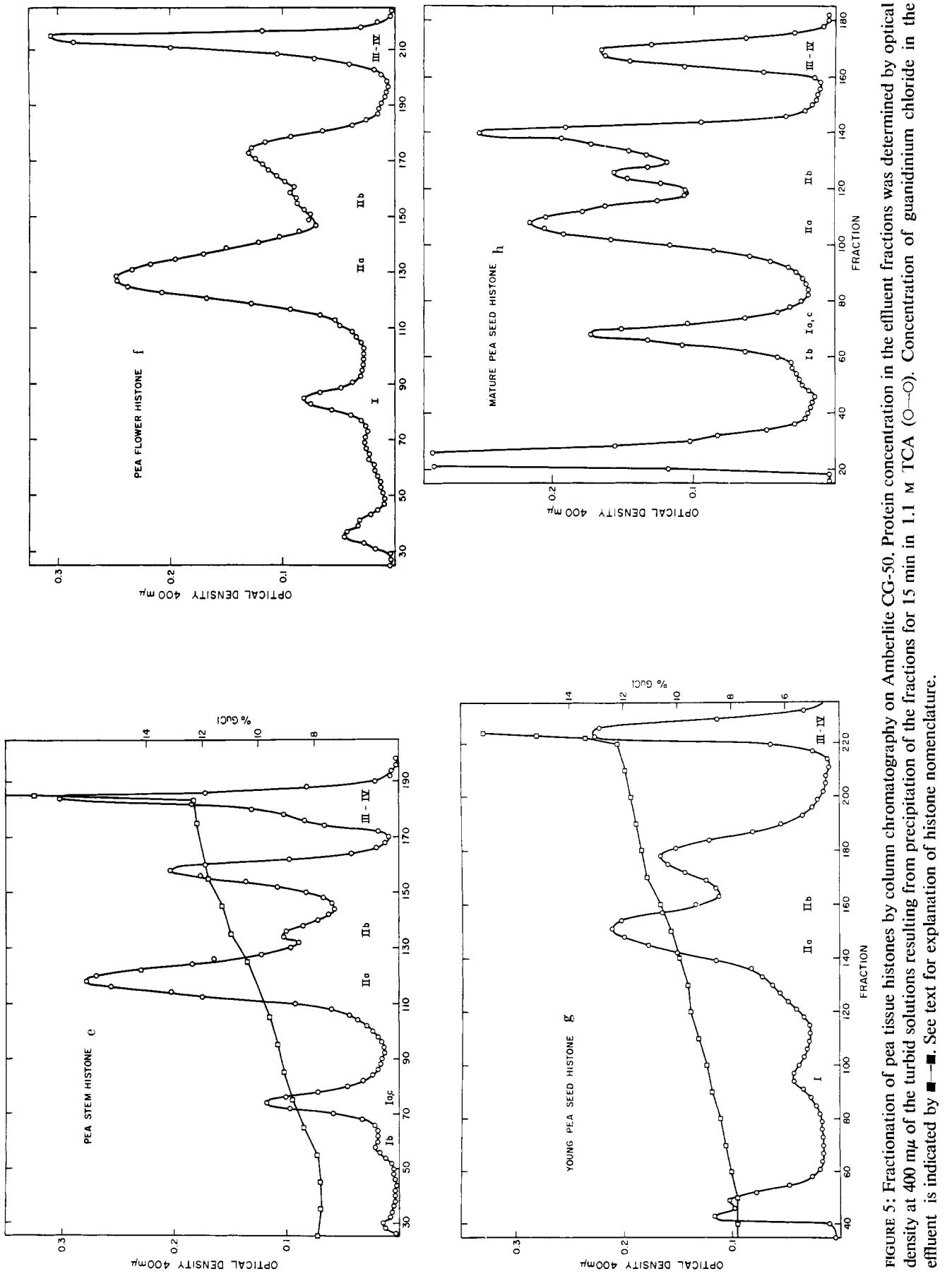


FIGURE 5: Fractionation of pea tissue histones by column chromatography on Amberlite CG-50. Protein concentration in the effluent fractions was determined by optical density at 400 mμ of the turbid solutions resulting from precipitation of the fractions for 15 min in 1.1 M TCA (○—○). Concentration of guanidinium chloride in the effluent is indicated by ■—■. See text for explanation of histone nomenclature.

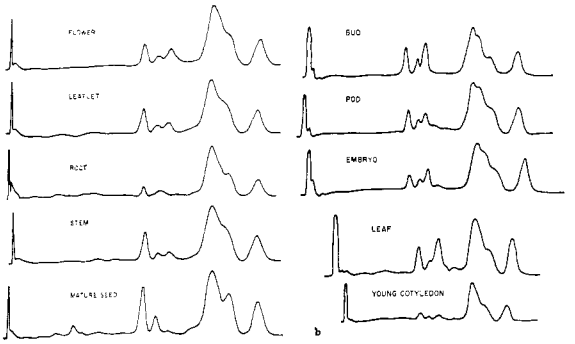


FIGURE 6: Densitometric tracings of the electrophoretic patterns of pea histones. Peaks at far left indicate origins of gels, not protein. In these histone preparations histone III is present in both oxidized and reduced forms, and thus contributes to the areas under the curve in the neighborhood of histones Ic and IIb.

TABLE III: Proportion of Lysine-Rich Histone in the Total Histone of Various Plant Organs.

Pea Tissue	% Histone I by Column Chromatography	% Histone I by Anal. 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.

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 III). 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 organs. Following the run-off peak, the lysine-rich histone I peak appears in the elution salt gradient range 8.6–9.3% guanidinium chloride. Histone I is composed of three electrophoretic components which elute in order Ib, Ia, and Ic. They possess similar primary structures. The slightly lysine-rich histones IIa and b are next eluted by 10.1–10.8% guanidinium chloride. Then follows a peak which contains traces of all histone components but is usually richest in histones III and IV. It is believed to result from histone aggregation, and it poses a major obstacle to the use of column chromatography for quantitative purposes. Finally, the arginine-rich histones III and IV are removed from the column by 40% guanidinium chloride. There are no histone peaks specific to any organ and no peak is completely lacking in any organ.

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 III lists the percentage of histone I in total histone from various organs 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 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 organs is at least largely an artifact of preparation.

Disc Electrophoresis. Densitometric tracings of the electrophoretic patterns of whole histone from many different pea organs are presented in Figure 6a,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 organ, no fraction is completely absent from any organ, and quantitative variations in histone distribution are apparent.

Quantitative determinations of the histones from pea buds, leaves, and young and mature cotyledons are presented in Table IV. 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 IV 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

TABLE IV: Composition of Histones from Pea Organs as Measured by Quantitative Disc Electrophoresis.

Histone Fraction	% of Total Histone			
	Pea Bud	Pea Leaf	Mature Pea Seeds	Young Pea Cotyledons
Ia	9.9 ± 0.4	9.6 ± 0.6	10.7 ± 0.7	5.3 ± 0.3
Ib	4.5 ± 0.3	3.3 ± 1.0	5.4 ± 0.4	1.7 ± 0.4
Iab	14.4 ± 0.5	12.9 ± 0.7	16.1 ± 0.6	7.0 ± 0.4
IIa	46.8 ± 0.7	43.5 ± 1.1	38.8 ± 1.0	46.0 ± 0 7
IIb	6.8 ± 0.5	7.6 ± 0.4	7.6 ± 0.7	8.8 ± 0.6
III[a]	17.8 ± 0.7	18.7 ± 0.7	17.2 ± 0.3	18.9 ± 0.9
IV	14.8 ± 0.5	17.3 ± 1.0	20.3 ± 0.4	19.3 ± 0.8

[a] Includes histone Ic as $\leq 1\%$ of total histone.

are shown in these data. Again, the largest difference is in the content of lysine-rich histone I, present in very low amount 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 7 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 8. 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.

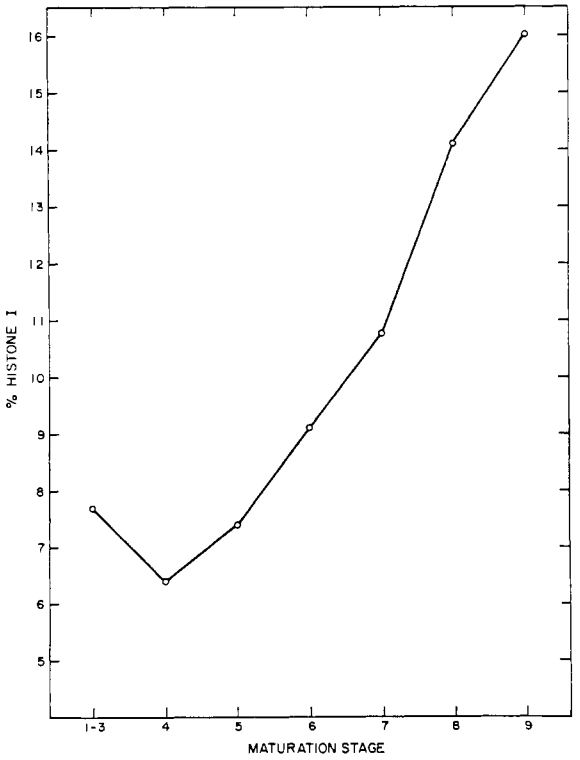


FIGURE 7: Lysine-rich histone content (per cent of total histone) in pea cotyledons as a function of maturation of seeds.

Discussion

It has been demonstrated that isolated chromatins from various pea organs contain their gene regulatory

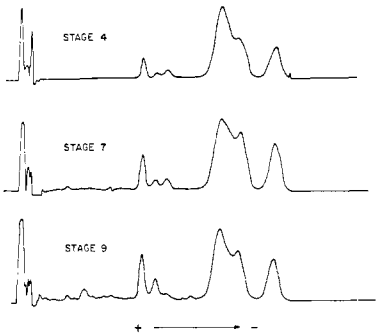


FIGURE 8: Densitometric tracings of the electrophoretic patterns of pea cotyledon histones isolated from three maturation stages of seeds. Note progressive increase in histone I components. Histone III is present primarily in the reduced form. Contamination by a large number of minor basic proteins mostly of low electrophoretic mobility is evident in stage 9 histone preparation. These minor bands are also observed in preparations of the acid-soluble ribosomal proteins from pea cotyledons and presumably represent contamination of chromatin by ribosomes.

apparatus intact (Bonner *et al.*, 1963). A large body of data suggests that some components of histone are the molecules regulating gene expression in higher organisms (Bonner *et al.*, 1967a; Huang and Bonner, 1962; Bonner and Huang, 1963; Allfrey *et al.*, 1963; Huang *et al.*, 1964; Littau *et al.*, 1965; Marushige and Bonner, 1965). In this report it is demonstrated that the same histone fractions are present in different pea organs. Thus the mechanism of gene regulation must not involve organ-specific histones. Supporting this conclusion is the finding that 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; Fambrough, 1968). 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 report show that there is rather large variation in the quantitative distribution of the several histone fractions in different pea organs. 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 organs selected for careful study. In their study of quantitative distribution of histones in selected mammalian organs, Hnilica *et al.* (1966) found most variation in histones f2b, f3, and f2a1, and practically no variation in histones f1 and f2a2. These data suggest an alternative to the notion that specific histones regulate specific genes, that is, the same few histone fractions may repress different genes in different cells. Some biological properties of the chromatins of some pea organs show an interesting correlation with the quantitative distribution of histones.

The very low content of lysine-rich histone I in pea cotyledons 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). The molecular basis of the specificity necessarily involved in gene regulation in higher organisms must, however, be sought in a mechanism auxiliary to the histones themselves.

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Exhaustive Hybridization and Its Application to an Analysis of the Ribonucleic Acid Synthesized in T4-Infected Cells*

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ABSTRACT: The method of hybridization with deoxyribonucleic acid (DNA) immobilized on nitrocellulose membrane filters has been applied to the problem of "exhaustive hybridization." Under the proper conditions it is possible to take up into DNA–RNA complexes virtually all of the T4 messenger ribonucleic acid (mRNA) present in an RNA sample from infected cells; thus making this technique very useful for the assay of RNA components present in small amounts. Exhaustive hybridization has been applied to an analysis of the RNA synthesized after infection of *Escherichia coli* B with bacteriophage T4. It has been estimated that the phage-specific RNA accounts for 2.8–4.9% of the total cellular RNA. The value observed depends on the time after infection at which the sample is taken. As late as 3 min after infection a small but significant amount of the [^{3}H]uridine incorporated during a 2-min pulse is found in host-specific RNA. The relative amount of mRNA in this host-specific RNA is estimated to be considerably less than that found in a corresponding 2-min pulse of uninfected cells.

A recent refinement in hybridization methodology, which makes use of DNA immobilized on NC[1] membranes, has been shown to possess many attractive features (Gillespie and Spiegelman, 1965). We wish to report here the application of the procedure of "exhaustive hybridization" and its use in the analysis of RNA synthesis following T4 phage infection. We have coined this expression to distinguish it from "saturation hybridization" which measures the relevant DNA cistrons by saturation with their complementary species of RNA (Yankofsky and Spiegelman, 1962). The object of the exhaustive hybridization experiment is the complete uptake, into hybrid complexes, of all the RNA which is relevant (complementary) to a specific group of DNA molecules.

The previous techniques available for exhaustive hybridization were either difficult to apply to experiments involving many samples at the analytical level (McCarthy and Bolton, 1964; Bautz and Hall, 1962) or they were subject to the complication of DNA–DNA interactions (Nygaard and Hall, 1964). The latter difficulties are particularly important since exhaustive hybridizations are carried out under conditions which maximize DNA–DNA interactions. The use of DNA immobilized on membranes obviates this problem by eliminating DNA renaturation. Furthermore, at the end of the incubation removal of the DNA-bearing membrane eliminates the hybridized RNA and all of the DNA (both hybridized and unhybridized) from the reaction mixture. The unreacted RNA left behind can then be challenged with fresh filters bearing the same or a different type of DNA.

In the present investigation we have used exhaustive hybridization to analyze the RNA synthesized after infection of *Escherichia coli* B with phage T4. The experiments reported here were designed to answer specifically the following questions. What per cent of the total RNA in the infected cell is phage specific? Can the synthesis of any host-specific RNA be detected following phage infection?

Materials and Methods

Bacterial Strains and Bacteriophage. E. coli B and phage T4 were used for all of the experiments reported here. *E. coli* Q13, a mutant lacking RNase I (Gesteland,

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[1] Abbreviations used: NC, nitrocellulose; K, kieselguhr; MAK, kieselguhr coated with methylated albumin; 1X DNA, denatured DNA.