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THE METABOLISM OF CHROMATIN-ASSOCIATED PROTEINS IN VICIA FABA AND THEIR RESPONSES TO GROWTH REGULATORS.

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THE METABOLISM OF CHROMATIN-ASSOCIATED PROTEINS IN *VICIA FABA* AND THEIR RESPONSES TO GROWTH REGULATORS

by

Barbara Suzanne Hauser Grahek

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GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by Barbara Suzanne Hauser Grahek

entitled THE METABOLISM OF CHROMATIN-ASSOCIATED PROTEINS IN VICIA FABA AND THEIR RESPONSES TO GROWTH REGULATORS

be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

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After inspection of the final copy of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:*

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SIGNED: Barbara Suzanne Hauser Cantek

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ABSTRACT

Evidence was sought for the role of chromatin-associated proteins in gene activation during the development of Vicia faba. Chromatin-associated proteins were defined on the basis of their solubilities during chromatin fractionation. A method was defined which permitted extraction of one class of nonhistones in 8 M urea-0.14 M mercaptoethanol prior to acid extraction of histones and a second class in tris-SDS following acid extraction of histones. Comparisons of histones and nonhistones extracted by other methods showed the method used in this study to have important advantages.

Changes in the distributions of chromatin-associated proteins in different cell types and changes in the metabolic activities of chromatin-associated proteins during gene activation suggested that chromatin-associated proteins represent regulators of gene activity. This conclusion is drawn from the following experimental results:

1. Some types of tissue exhibited characteristic nonhistone/DNA ratios, although the ratios do not appear to be correlated to any defined feature of the tissue. An increased proportion of species presumed to be histone f1 was observed in root meristem and leaf tissues as compared to mature

lateral root and stem tissues and in cotyledons at one stage of development. Chromatin-associated proteins presumed to be histones were not shown to vary, either in protein/DNA ratio or in banding patterns on SDS gel electrophoresis from tissue to tissue. Some urea-soluble protein species varied in different tissues. Tris-SDS-soluble proteins exhibited extreme variability.

2. With labelled-leucine, the highest specific activities for all classes of chromatin-associated proteins in excised epicotyls occurred between 12-24 hr germination. Fractions of nonhistones more tightly bound to DNA were more highly labelled. Alleged histones exhibited the lowest specific activities. Not all species of each class of chromatin-associated proteins were labelled to the same degree during 12-24 hr germination. The urea-soluble protein species exhibited characteristic patterns of synthesis throughout the germination period.

3. Different classes of chromatin-associated proteins of excised epicotyls exhibited separate and distinctive responses to plant growth regulators in double-label radioisotope experiments. In nonhistone fractions stimulation of labelled-leucine incorporation was observed in response to auxin. Presumed histone fractions responded to kinetin with stimulated leucine incorporation and to both gibberellic acid and cyclic AMP with repressed leucine incorporation.

In addition, gibberellic acid and cyclic AMP appeared to repress incorporation of leucine into total cellular protein. It was inferred that cyclic AMP may be a second messenger for gibberellin effects on histone synthesis.

4. Not all nonhistone species responded identically to auxin. Individual species of the urea-soluble protein class of nonhistones exhibited distinctive degrees of increased or repressed labelled-leucine incorporation.

CHAPTER 1

INTRODUCTION

The experiments presented in this dissertation intend, as their common purpose, to examine the role of chromatin-associated proteins in the development of a higher plant with respect to gene activation.

Gene Activation

Since the definition of gene activation is the production of a new species of RNA molecules that were not synthesized prior to a stimulus (Baserga and Stein 1971), the first problem is to define aspects of a biological system which constitute gene activation. To measure results of differential gene activation, varying features expressed by different tissues of an organism may be examined. It is generally accepted that protein constituents of a cell are ultimately determined by the genome or DNA of the cell, and that these gene products vary for cells of different tissues. However, by applying available methods one finds that the entire genome is present in nearly every cell of an organism and is thereby led to the conclusion that, from tissue to tissue, different portions of the genome are expressed. It is

therefore assumed that expressed portions of the genome have, at some time in the development of the organism, been activated or derepressed and that portions of the genome not expressed have been repressed or not activated. The general belief is that in eukaryotes, as in prokaryotes, gene repression, derepression and activation occur.

A second way of looking at gene activation is to examine various characteristics of a cell at the time of gene activation. Two instances generally accepted as examples of gene activation for higher plants are events occurring during early germination and events occurring in response to plant growth regulators, or plant hormones.

The basic question asked in studies of gene activation concerns the mechanism by which the stimulus activates the genome. From the studies of Jacob and Monod and others with microorganisms (recent reviews include those by Martin 1969 and Reznikoff 1972), it is currently believed that a mechanism operates in eukaryotes that is similar to that of prokaryotes, namely, the genome is repressed, derepressed or activated by regulatory molecules attached to DNA (Comings 1972).

Chromatin-associated Proteins

Eukaryotic DNA characteristically has large amounts of protein associated with it. These proteins are generally considered to be in two classes, histones and nonhistones.

Massive research has been done with histones and many recent reviews are available summarizing the efforts in this field, including those of Georgiev (1969), Stellwagen and Cole (1969), DeLange and Smith (1971), Elgin et al. (1971), Hnilica, McClure and Spelsberg (1971), Wilhelm, Spelsberg and Hnilica (1971), McClure and Hnilica (1972), and the recent book edited by Phillips (1971). Most authorities believe the primary function of histones is generalized repression. Histones prevent transcription of genes, but appear to lack specificity necessary for fine control.

During the last few years, much effort has been expended on nonhistone proteins associated with chromatin and the accumulated evidence indicates their involvement in regulation of gene activity. Some of their features which make them likely candidates for regulatory proteins are the following. They are a group of considerably heterogenous proteins ranging in molecular weight from 5000 to 100,000 daltons, with tissue and species specific distributions. They occur in higher concentrations in active tissues than in inactive tissues and in higher concentrations in active chromatin than in inactive chromatin. They restore histone-inhibited RNA synthesis in vitro, increase in vitro transcription of inactive chromatin and of active repressed loci, and interact with DNA

in vitro, thus modifying transcription so as to produce RNA products that are characteristic of the tissue from which the nonhistones were extracted. Nonhistone proteins are synthesized more rapidly than histones and, unlike histones, are synthesized throughout the cell cycle. Their biosynthesis is characteristically stimulated by hormones and other stimuli to gene activation and they may undergo secondary modifications in response to such stimuli. Nonhistones bind specifically to DNA and include many enzymes of nucleic acid and protein metabolism such as deoxyribonuclease (Howk and Wang 1970), methylase (Benjamin 1971), RNA polymerase (Hardin and Cherry 1972, Mondal, Mandal and Biswas 1972) and proteases (Garrels, Elgin and Bonner 1972). The characteristics of nonhistones are the subject of recent reviews by Paul (1970), Allfrey (1971), Elgin et al. (1971), Stein and Baserga (1971), McClure and Hnilica (1972) and Spelsberg, Wilhelm and Hnilica (1972).

Hypotheses to be Tested

If chromatin-associated proteins represent regulators of gene activity, changes would be expected in their distributions and metabolic activities in different cell types and in the same cell type at times of gene activation. Based on stated assumptions, this dissertation examines the following hypotheses concerning chromatin-associated proteins of Vicia faba:

1. Tissues of various states of differentiation and metabolic activity can be isolated from V. faba. It is proposed that some nonhistones are tissue specific but all histones are constant in type and in amount among these tissues.

2. It has been suggested that excised epicotyls of V. faba are capable of initiating developmental events during early germination (Stanley 1972). It is proposed that nonhistones and histones undergo characteristic and distinguishable metabolic patterns during this time.

3. Many examples of specific synthesis of chromatin-associated proteins to animal hormone stimuli have been reported. It is proposed that such phenomena occur in higher plants as well, and that nonhistones and histones exhibit separate and distinctive responses to various plant growth regulators.

CHAPTER 2

GENERAL MATERIALS AND METHODS

For all experiments, seeds of Vicia faba L. (var. Longpod) obtained from W. Allee Burpee Company were used. Harvested plant material was routinely frozen with liquid nitrogen and stored at approximately -15 C.

Chromatin Isolation

The procedure used for isolation of chromatin was modified from Huang and Bonner (1962) for V. faba (Stanley 1972). All operations were performed at 0-4 C. Centrifugations were carried out using an International centrifuge, model PR-J (with head model 269 or high-speed attachment head model 296) or using an International centrifuge, model HT (with head model 856). The latter was kept in a cold room at approximately 15 C.

Plant tissue was homogenized for one min in 25-150 ml grinding buffer (0.25 M sucrose, 0.001 M $MgCl_2$, 0.05 M tris, pH 8.0) in a Waring blender. The homogenate was filtered through four layers cheesecloth and through two layers Kimwipe to remove tissue fragments and debris. The filtered homogenate was centrifuged 10 min at 4000 x g to pellet nuclei. The pellet was washed by resuspension in

approximately 20 ml grinding buffer and repelleted as before.

The resulting pellet was homogenized in approximately 15 ml of 0.01 M tris, pH 8.0, using a Potter-Elvehjem homogenizer with a hand-driven ground-glass or Teflon pestle. The chromatin was removed from suspension by centrifugation for 10 min at 10,000 x g. Following centrifugation, chromatin accumulated on top of a layer presumed to be starch. The chromatin was scraped from the starch layer and resuspended in 0.01 M tris, pH 8.0. This process was repeated for a total of five tris washes. No visible starch was present upon completion of the washes.

The chromatin pellet was then homogenized by hand in a smooth-wall, heavy-duty-glass centrifuge tupe with a close-fitting Teflon pestle, in 1 x SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) to precipitate the chromatin. The chromatin was recovered by centrifugation for 10 min at 4000 x g. The chromatin was washed in 1 x SSC to a total of three times. The final chromatin pellet was routinely fractionated for protein components as described below.

Chromatin Fractionation

Proteins associated with chromatin were differentially extracted by a procedure modified from Gronow and

Griffiths (1971). The chromatin pellet, prepared as above, was homogenized in 0.5-1.0 ml urea buffer (8 M urea, 0.14 M 2-mercaptoethanol, 0.05 M sodium phosphate, pH 7.6). For this and all following homogenations the glass centrifuge tube with the close-fitting Teflon pestle, described above, was used. The urea buffer-chromatin suspension was allowed to remain at room temperature for 15 min. Chromatin was removed from suspension by centrifugation at 20,000 x g for 20 min. The supernatant which contained the urea-soluble chromatin-associated proteins (USP) was saved and pooled with a second supernatant from a re-extraction, performed exactly as above. The USP solution was dialyzed overnight at room temperature against dialysis buffer (8 M urea, 0.1% v/v 2-mercaptoethanol, 0.1% w/v sodium dodecyl sulfate, 0.01 M sodium phosphate, pH 7.15).

The chromatin pellet remaining after extraction of USP was washed by homogenization in 5-10 ml urea buffer and recovered by centrifugation as before. The resulting pellet was subsequently washed once in 1 x SSC.

In order to remove the histones, the pellet was homogenized in 1-2 ml of 0.4 N H_2SO_4 and the suspension was allowed to remain on ice for 15 min. The chromatin was pelleted by centrifugation at 10,000 x g for 15 min. The supernatant which contained acid-soluble proteins, (hereafter referred to as HIS) was collected for use without

further treatment. The pellet was washed twice by homogenization in 5-10 ml of 0.25 N HC and recovered by centrifugation at 4000 x g for 10 min.

The recovered pellet was homogenized in 1-2 ml of 0.05 M tris, pH 8.0, to which 1% (w/v) sodium dodecyl sulfate (SDS) had been added, and the suspension was allowed to remain overnight at room temperature. The remaining chromatin components were pelleted by centrifugation at 20,000 x g for 20 min. The supernatant which contained the residual proteins remaining after urea and acid extraction (hereafter referred to as RES) was collected for use without further treatment.

The resulting pellet which contained DNA and some tightly-bound proteins was discarded or saved for extraction of DNA, according to the plan of the experiment.

DNA Extraction

For experiments in which it was desirable to estimate the amount of DNA present, the pellet remaining after protein isolation was washed with 5 ml cold 10% (w/v) trichloroacetic acid (TCA) and re-pelleted by centrifugation at 4000 x g for 10 min. To this pellet, 2.0 ml of 5% (w/v) TCA were added, and the mixture was heated for 20 min in a boiling water bath. The mixture was allowed to cool and was then centrifuged at 4000 x g for 10 min. The resulting

supernatant which contained deoxyribonucleotides was collected for measurement of DNA concentration. The final pellet was discarded.

DNA and Protein Determination

DNA concentrations were estimated colorimetrically by the diphenylamine method (Dische, 1954) using calf thymus DNA (Nutritional Biochemicals Co.) as a standard.

Protein concentrations were estimated colorimetrically by the Folin phenol method (Lowry et al. 1952) using bovine serum albumin (Nutritional Biochemicals Co.) as a standard. Since mercaptoethanol in the USP samples gave a positive reaction with the Folin phenol test, a dialysate standard was also measured for each test. The absorbance of this standard was subtracted from the absorbance of the USP sample to give net absorbance for USP. Calculation of USP concentration was based on the value of the net absorbance. Since SDS gave a slight positive reaction with the Folin phenol test, which diminished with time in the alkaline-copper-carbonate Lowry reagent, all samples were allowed to react with the reagent for at least 30 min before addition of the phenol reagent. Acid-extracted HIS samples were routinely neutralized with an equivalent amount of base as NaOH before protein determinations were made.

Protein/DNA ratios represent total mg protein extracted/total mg DNA extracted.

Protein Electrophoresis

Electrophoresis of all protein classes employed SDS electrophoresis (Weber and Osborn 1969, Shapiro, Vinuela and Maizel 1967).

Plexiglass tubing (3/8-inch inside diameter) was cut to 9-cm lengths for supports for the polymerization of polyacrylamide gels. Tubes were covered with Parafilm at the bottom before they were placed vertically into rubber supports for gell polymerization. The bottoms of the tubes were covered with a piece of fine-mesh nylon netting, secured with a rubber band, to prevent gels from sliding out during electrophoresis.

Polyacrylamide gels were made with 10% (w/v) Cyanogum 41 (E-C Apparatus Corp.) in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1% (w/v) SDS. The gel solution was prepared by dissolving 3.0 g Cyanogum 41 in 25 ml buffer. To a separate 5 ml buffer was added 0.02 g ammonium persulfate and 20 µliter N,N,N,'N'-tetramethyl-ethylenediamine (TMED). The Cyanogum solution was added to the catalyst solution with stirring. Gel tubes were filled immediately with the mixture to a depth of 8 cm. The gel solution was then overlaid with distilled water. Complete polymerization occurred within 30 min or less.

Polymerized gels were prerun for 30-60 min during the afternoon preceding the day they were scheduled for use. The gels were allowed to remain overnight at room temperature in the assembled apparatus with buffer in both chambers.

The running buffer for protein electrophoresis and for gel prerun was identical to the gel buffer. This buffer was used in both upper and lower electrode chambers. The buffer used for prerun was exchanged for fresh buffer just before protein electrophoresis.

Protein electrophoresis and prerun were performed at room temperature, at a current of 5 ma/tube (approximately 2 volts/tube) with a regulated high-voltage power supply (Heathkit, Model IP-17). Since all proteins complexed with SDS migrate as anions, the anode was the lower electrode.

Proteins were not frozen before electrophoresis. HIS were allowed to remain overnight at room temperature in capped vials. USP were used directly after removal from the overnight dialysis, and RES were used directly after recovery of the supernatant from the overnight extraction with tris-SDS. Ultra-pure sucrose (Schwarz/Mann) was added to 0.4 ml protein samples to a final concentration of approximately 10% (w/v). In addition, 5 µliter of 1% (w/v) bromophenol blue in distilled water were added to each 0.4 ml sample of protein solution immediately prior

to electrophoresis. Samples, 200 µliter of protein-sucrose-bromophenol blue solution, were added to each gel under the running buffer.

Electrophoresis was performed for approximately 8 hours.

Gels were removed from their tubes under pressure supplied by a rubber bulb attached to the origin end. Gels were cut with a razor blade at the running front of the bromophenol blue marker and placed into staining solution (0.25% w/v Coomassie blue, Colab Laboratories, Inc., in 7% v/v acetic acid) for approximately one hour. Excess stain was removed by diffusion in an excess of 7% (v/v) acetic acid over a period of one week. Gels were stored in 11 x 100 mm glass test tubes capped with corks and further destaining occurred during storage.

Analysis of Gels

Stained gels were scanned at 555 nm with a Gilford spectrophotometer (model 240) equipped with a linear transport (model 2410) and a chart recorder (Honeywell, model Y143X). Full-scale absorbance was set at optical density equals 1.000 and ratio was set at 0.1. On some figures relative optical density of gel scans are indicated as percent of full-scale absorbance. USP gels were scanned at 1.0 cm/min and HIS and RES gels were scanned at 2.0 cm/min.

Since gels were scanned at different times after staining, variable amounts of destaining had taken place.

For experiments in which it was desirable to compute the proportion of each protein species, gel scans were Xeroxed and the relative area under each peak was determined by cutting out the peak and weighing the paper. The sum of the weights of all peaks in a scan was calculated and the proportion of each protein was expressed as a percent of that value. This procedure was performed only for HIS species due to high background staining in USP and RES gels.

A permanent record was made by photographing each gel with transmitted light (supplied by a 35-mm slide sorter) on 35-mm Kodak Improved High Contrast Copy Film. Prints were made on Kodabromide F3 or Kodak Medalist F3 paper. Gels were routinely compared to the prints to establish that every band visible to the eye was also visible on the print.

Radioisotopes

For all experiments employing radioisotopes, ^{14}C-L-leucine in 0.01 N HCl solution (uniformly labelled, specific activity 312 mc/mmole; Schwarz/Mann) and L-leucine (4,5-^{3}H) in 0.01 N HCl solution (specific activity 15,000 mc/mmole; Schwarz/Mann) were used. Amounts used are specified in relevant chapters.

Scintillation Counting

Radioactive samples were counted in a Packard Tricarb (model 3320) liquid scintillation spectrophotometer. Samples were counted in standard polyethylene vials with 10 ml cocktail or in minivials (Nuclear Associates, Inc.) using 5 ml cocktail. The cocktail used contained 0.55% (w/v) 2,5-diphenyloxazole (PPO) and 0.01% (w/v) 1,4-bis-(5-phenyloxazolyl-2)-benzene (POPOP) in a solution of one part Triton X-100 (Rohm and Haas) and two parts toluene.

Since labelling was generally low, all radioactive samples were routinely counted for a minimum of 10 min. Counting for periods of 50 min was more common. All data are expressed as counts or cpm above background.

Aliquots of protein samples to be counted were pipetted onto Whatman no. 3 filter paper discs and air- or oven-dried. In order to determine the radioactivity of specific protein species, USP gels were sliced throughout their stained length into approximately 1.25-mm slices and each slice was placed into a scintillation vial. To each gel slice was added 0.2 ml of 30% hydrogen peroxide and the slices were digested overnight at 50 C. Stained bands were cut from HIS and RES gels and digested for counting as above, but in 0.5 ml of peroxide.

CHAPTER 3

ANALYSIS OF THE EXTRACTION PROCEDURE

The literature has been literally flooded with reports of experiments involving chromatin-associated proteins and few of these are comparable in any sort of rigorous manner, since variations in methodology are almost as numerous as researchers in this area. Since my work involves the application of a relatively new procedure which has not formerly been applied to plant tissue, and since this procedure appears to be a promising one, I have felt it important to go to length in defining this procedure.

Review of Literature

Chromatin Extraction

Several reviews exist on the traditional methods for chromatin extraction and fractionation and among the most comprehensive are those by Busch (1965, 1968), Bonner et al. (1968a), Johns (1971) and Spelsberg et al. (1972).

Experiments with different methods of isolating nuclei have demonstrated that the chromatin obtained is dependent to some degree on nuclear isolation. Chromatin can also be obtained directly without prior isolation of

nuclei; it is initially recovered from grinding buffer at low speed centrifugation since, at speeds of 1000-4000 x g, the chromatin is pelleted but mitochondria and other cytoplasmic organelles are left in solution (Bonner et al. 1968a).

Methods of chromatin purification are fairly standard. Tris and SSC washes function to remove nuclear membranes, ribosomes and ribonuclearproteins, and nuclear sap proteins (Frenster, Allfrey and Mirsky 1963; Busch 1965, 1968; Arbuzova et al. 1968). Nearly all investigators use chromatin purified by this technique.

Chromatin Fractionation

The greatest procedural variation is chromatin fractionation. The chromatin may be solubilized in 1 M or 2 M NaCl and histones extracted with acid from this solution (Busch 1965); or histones and DNA precipitated out of solution, leaving some nonhistones in solution (Wang 1967). Histones may be extracted directly with acid or salt solutions, as is the usual case, or chromatin may be pretreated with dilute HCl to remove tryptophan-containing nonhistones (Arbuzova et al. 1968). Chromatin is often dissociated in concentrated solutions of urea, NaCl or CsCl and various components are isolated by column fractionation (Elgin and Bonner 1972, MacGillivray et al. 1972) or by centrifugation (Huang and Bonner 1962). It has recently

become a common practice to solubilize nonhistones in SDS solutions with (Wilhelm et al. 1972) or without mercaptoethanol (Shirey and Huang 1969, Stein and Borun 1972, Elgin and Bonner 1972, Cognetti, Settineri and Spinelli 1972). Techniques used less commonly include fractionation of chromatin components on the basis of SSC solubility (Marushige and Bonner 1971) and a new thermal fractionation devised by McConaughy and McCarthy (1972). Only a few of the many approaches to chromatin fractionation are cited here. The bulk of the literature conveys the impression that, for the most part, interesting results can be obtained from nearly any method, and often the same conclusions are derived with widely divergent techniques.

The Gronow-Griffiths Technique for Chromatin Fractionation

The development of the technique of isolating nonhistones directly from chromatin prior to acid-extraction by Gronow and Griffiths (1971) represents a significant improvement in methodology for the study of nonhistone proteins. Originally developed for use with mammalian tissue, it has not yet been widely applied (however, see Barrett and Gould 1973) and to date there has been no report of its application to plant tissue. The method's advantages include avoidance of acid pretreatment to chromatin, the requirement of only small amounts of material and the

rapidity and simplicity of the procedure. Furthermore, it appears to extract a much greater proportion of nuclear protein than most other methods commonly used. Further discussion of this latter aspect of the method will be found in the discussion section of this chapter.

Materials and Methods

Growth and Harvest of Plant Tissue

For each experiment, approximately 350 seeds of V. faba were surface sterilized by soaking 30 min in a 10% (v/v) Clorox solution with approximately 0.1% (w/v) of Tide detergent added as a wetting agent (Scalera and Ward 1971). Seeds were germinated for 5 days in running tap water with continuous aeration. The water temperature was approximately 18-26 C.

Comparative Fractionation Procedure

Chromatin was extracted as described in Chapter 2. For the final homogenation in SSC, the pellet was suspended in 4.0 ml SSC and following homogenization, the suspension was divided into two equal parts. Two pellets were recovered by centrifugation at 4000 x g for 10 min. One pellet was subjected to the routine fractionation procedure described in Chapter 2, and USP, HIS and RES were recovered. The other pellet was extracted initially with 0.4 $N_2H\ SO_4$

and acid-soluble proteins were recovered in the supernatant following centrifugation at 10,000 x g for 15 min. The pellet, after two washes with 0.25 N HCl, was washed once each with a 2-ml solution of chloroform:methanol (1:1 followed by 2:1) and recovered by centrifugation at 4000 x g for 10 min. The purpose of the chloroform:methanol washes was to remove lipoproteins. The pellet was subsequently washed once with ether to remove traces of chloroform and was then extracted with urea buffer to solubilize remaining USP. These USP were recovered in the supernatant following centrifugation at 20,000 x g for 15 min. The supernatant from a second urea extraction was pooled with that from the first.

In addition, chromatin extracted from V. faba epicotyls was fractionated by the methods of Teng, Teng and Allfrey (1971) to recover phenol soluble nuclear acidic proteins (PSNAP).

In further experiments, following acid-extraction, chromatin was extracted with 0.5 N NaOH or 0.05 N NaOH for 150 min and base-soluble proteins (BSP) were recovered in the supernatants following centrifugation at 20,000 x g for 15 min. Prior to electrophoresis, SDS was added to BSP samples to a final concentration of 0.1% (w/v).

Protein samples were subjected to electrophoresis as described previously in Chapter 2.

Analysis of Gels

Photographic records of gels were compared to establish the number and approximate positions of bands present. In addition, gel analysis as described for tissue specificity in Appendix A, was performed to compare gels of USP, acid-soluble proteins, HIS and RES. Figures A-8, A-9 and A-10 illustrate the comparative gel scans from which final gel drawings were derived.

The method described in Appendix A was also used to establish band variability among USP species based on gels for excised epicotyls from experiments described in Chapter 5.

Results and Discussion

Identification and Variability of USP Species

The routine chromatin fractionation yielding USP, HIS and RES was surprisingly reproducible, even among different tissues (discussed in Chapter 4) and from different stages of germination (discussed in Chapter 5). Repetitive extractions demonstrated that USP and HIS gel patterns were constant for repeated electrophoresis of a sample, and for repeated extraction from one batch of material and from different batches of material. From the large series of gels generated by experiments on synthesis of USP as well as early experiments on reproducibility, the identification

and variability for each USP band was established. Figure 1 contains a photograph of a typical USP gel with the bands numbered from the origin. The same numbering system is shown for gel scans and drawings in Fig. A-3 (Appendix A). Table 1 describes the variability observed for the USP bands.

Identification and Variability of HIS Species

A photograph of a typical HIS gel is shown in Fig. 1 with the bands lettered in order from the origin. The lettering system for HIS species is shown on a gel scan in Fig. A-9. The probable identification of the bands can be deduced by assuming the same order of migration as that observed by Panyim and Chalkley (1971) who also used an SDS system for histone electrophoresis. The probable identification of each HIS band is given in Table 2. The molecular weights of calf thymus histone fractions on the basis of mobility in SDS electrophoresis determined by Panyim and Chalkley (1971), are also listed in this table. Bands c and d did not always resolve into two bands, depending on the length of the electrophoretic run, thus data on the proportions of HIS subfractions discussed in later chapters shows pooled values for bands c and d.

No variability was observed in HIS bands with the exception that occasionally a very light band appeared at

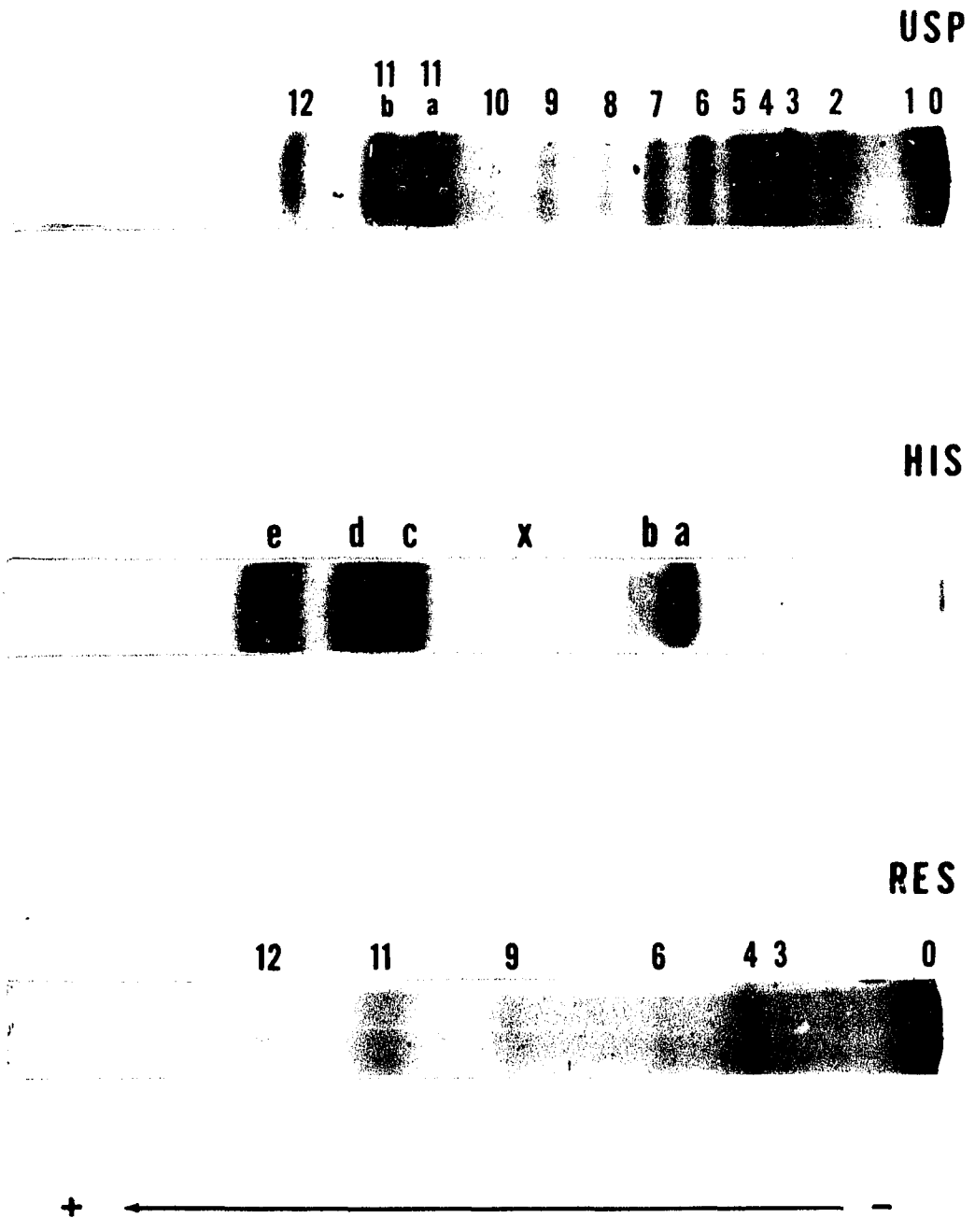

Figure 1. Electrophoretic separation of proteins on gel. -- Arrow indicates direction of migration.

Table 1. Characteristics of USP species.

Band Number	Presence	Position	Depth of Stain	Comments
0	variable	constant	medium	
1	variable	slightly variable	medium	
2	variable	variable	medium	sometimes resolved into two bands
3	constant	constant	very dark	
4	variable	constant	medium	may be a component of band 3 or 5 which is not always resolved
5	constant	constant	medium	
6	constant	constant	dark	
7	constant	constant	dark	
8	constant	constant	light	
9	constant	constant	light	
10	constant	slightly variable	very light	
11	constant	constant	dark	sometimes resolved into two bands (11a and 11b)
12	constant	constant	dark	

Table 2. Tentative identification of HIS species.

HIS Species	Probable Identification	Approximate Molecular Weight for Calf Thymus (in Daltons)
a	very lysine-rich f1	22,000
b	very lysine-rich f1	21,000
c	lysine-rich f2b and arginine-rich f3	14,000
d	lysine-rich f2a2	12,500
e	arginine-rich f2a1	11,000

the position marked x in Fig. 1. This protein species was judged to be an occasional contaminant from the rarity and randomness of its occurrence; therefore, it was not further considered.

HIS samples were not routinely reduced with 2-mercaptoethanol prior to electrophoresis. It has been determined for pea that histone f3 dimers exist as artifacts due to oxidation during extraction and that in vivo only the reduced monomer is present (Spiker and Chalkley, 1971). Presumably the inclusion of 2-mercaptoethanol during USP extraction reduced all histone f3 dimers to their natural monomeric form. In no HIS gel, was a band present at the appropriate position for a histone f3 dimer (approximately 28,000 daltons).

Analysis of USP and HIS Species Based on Comparative Fractionations

Figure 2 compares gel patterns obtained when the order of chromatin fractionation is changed. It can be concluded from these experiments that many nonhistones are soluble in acid, and direct acid extraction of chromatin results in highly contaminated histones. Measurement of the area under the alleged histone peaks of direct acid extraction gel scans resulted in the observation that 66-68% of all acid extracted proteins were presumed histones.

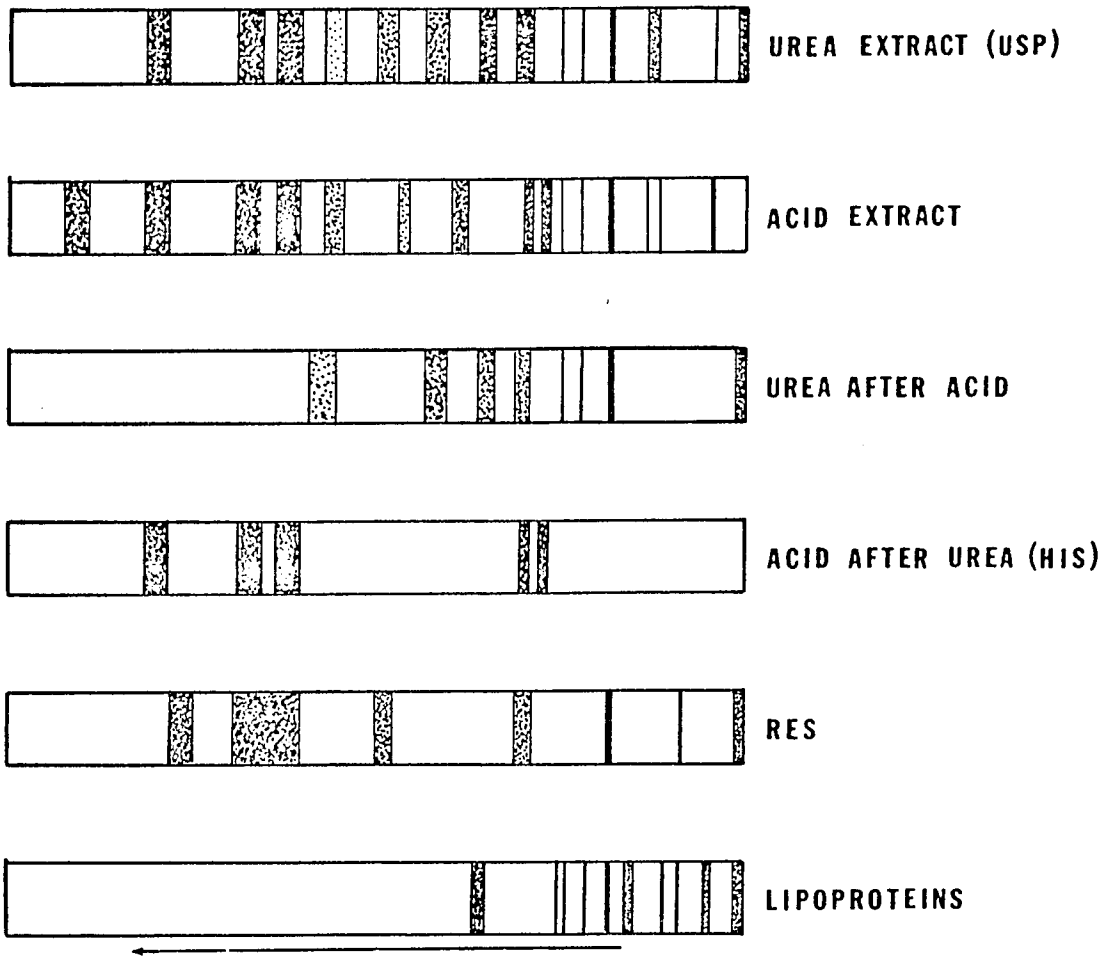

Figure 2. Gel patterns for proteins derived from altered fractionation order. -- USP are obtained from direct extraction of chromatin with urea buffer. Acid extract indicates proteins obtained when chromatin is directly extracted with acid. Urea after acid indicates proteins obtained when chromatin is extracted with urea buffer following removal of acid-soluble proteins and lipoproteins. HIS indicates proteins obtained when chromatin is extracted with acid following extraction with urea buffer. RES indicates proteins obtained from tris-SDS extraction following removal of USP and HIS. Lipoproteins indicates proteins obtained from extraction of chromatin with chloroform-methanol as described in Materials and Methods of Chapter 3. Arrow indicates direction of migration.

In the acid-soluble protein gels the bands did not occur at precisely the same position as in the USP gels. Although it is possible that these may represent different protein species, the change in position of urea-soluble protein bands following acid extraction demonstrates that a more likely explanation is that acid-treatment alters nonhistone protein species and these are in fact acid-soluble USP. This further demonstrates the desirability of direct extraction of nonhistones for any study attempting to examine their properties.

Although it was noted by Gronow and Griffiths (1971) that histone f2a1 was probably extracted in urea, a band corresponding to the expected position for histone f2a1 was also present in HIS gels. Bands were present in USP gels at positions equivalent to positions of alleged histones f2a1, f2a2 and f2b + f3 on HIS gels. Studies on dissociation of histones from chromatin in the presence of 5 M urea have shown that histone f2a2 dissociates at 0.15 M NaCl, and in the presence of 7 M urea, histones f2a1 and f3 dissociate at 0.15 M NaCl (Kleiman and Huang 1972). Since urea lowers the affinity of histones for DNA, it is possible that these USP species are actually histone contaminants. However the urea extraction buffer contained 0.05 M sodium phosphate, not 0.15 M NaCl, and another likely explanation is that the bands in question are simply

USP species of similar molecular weights to the histone species. No studies of histone affinity for DNA in the presence of 8 M urea are known.

Lipoproteins extracted prior to urea extraction of acid-extracted chromatin as described in Materials and Methods of this chapter, were also subjected to electrophoresis following aspiration of the chloroform-methanol solvent and solubilization in electrophoresis running buffer. The patterns obtained were highly suggestive of some USP bands, but with slightly different mobilities. It was concluded that either many USP species are lipoproteins, or no lipoproteins are extracted with urea. If the first case is true, then lipoproteins have also been examined within the context of USP. If the second case is true, then lipoproteins were not studies at all and may represent proteins tightly bound to DNA which were detectable in synthesis experiments (Chapter 5) when the final pellet was occasionally counted for radioactivity.

Comparison of USP and Nonhistones Obtained from Other Fractionation Procedures

Figure 3 illustrates gel patterns obtained when different methods of extraction of nonhistones were compared. Patterns obtained for PSNAP were strikingly similar to USP patterns. Again, variability of band position can probably be attributed, at least in part, to the harsh phenol

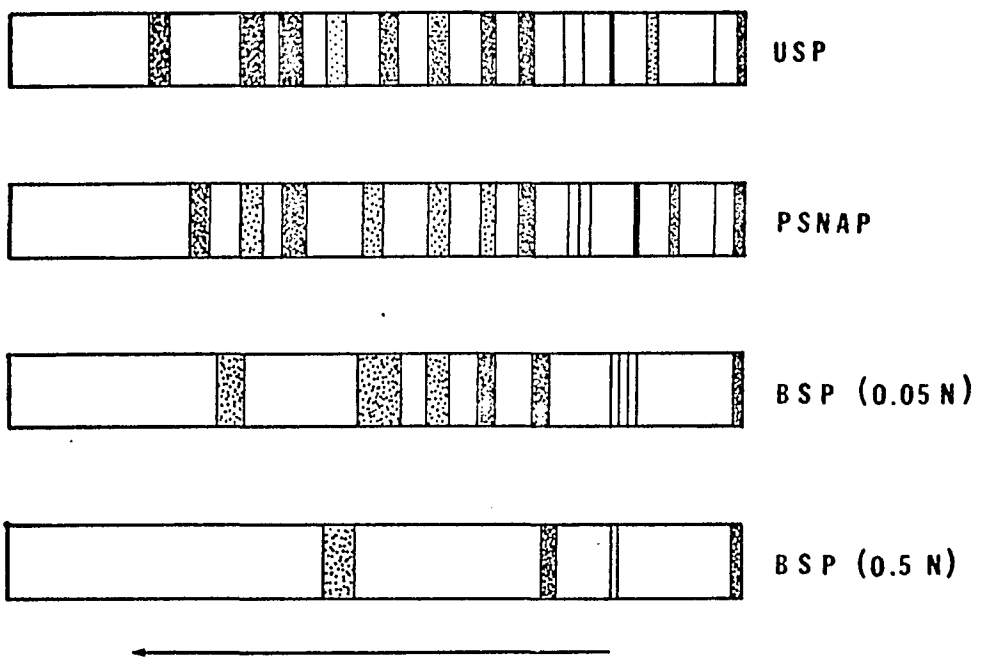

Figure 3. Gel patterns for proteins derived from different methods of extraction of nonhistones. -- USP are obtained from direct extraction of chromatin with urea buffer. PSNAP are obtained from extraction of chromatin with phenol following extraction of acid-soluble proteins and lipoproteins. BSP are obtained from extraction of chromatin with NaOH following extraction of acid-soluble proteins. Arrow indicates direction of migration.

treatment to which PSNAP were subjected. An interesting feature of the data presented here is that although PSNAP are extracted from chromatin which remains after acid and lipoprotein extraction, they appear more similar to USP than to urea extractions of chromatin which were treated similarly (urea after acid in Fig. 2). No explanations based on the data presented here can be offered for this phenomenon.

Base extraction following acid extraction resulted in fewer protein species on BSP gels than on either USP or PSNAP gels. However it may be noted that BSP patterns illustrated in Fig. 3 are similar to urea-soluble proteins following acid extraction (Fig. 2).

It was concluded from this series of experiments that direct urea extraction of chromatin, followed by acid extraction, represented the best way to fractionate non-histones from histones and was the most desirable method to obtain nonhistones (USP) for intensive study of their properties.

Identification and Variability of RES Bands

Figure 1 demonstrates a photograph of a RES gel with the bands numbered. There was much variability among RES gels and the band patterns were always similar to USP patterns as demonstrated in Figs. 1 and 2. It is possible

that RES are USP that were not completely extracted in urea and the numbering system employed in Fig. 1 reflects this interpretation. Data from synthesis experiments (Chapter 5) and responses to growth regulators (Chapter 6) support this interpretation. It is probable that USP are nonhistones tightly bound to DNA. Unlike the several bands on RES gels observed in this study, Gronow and Griffiths (1971) found only two low molecular weight species.

Comments on the Extraction and Fractionation Procedures

The charge has been leveled (Johns and Forrester 1969, Goodwin and Johns 1972) that a majority of studies on nonhistone chromatin proteins may actually be studies on cytoplasmic and nuclear sap proteins which were nonspecifically adsorbed to chromatin during washing with SSC, and the suggestion is made that washing chromatin with 0.2-0.35 M NaCl prior to fractionation results in the disappearance of all or nearly all so-called nonhistone chromosomal proteins. However, studies incorporating this additional chromatin wash have shown that very little protein is removed by this method. Stein and Baserga (1970) found a protein/DNA ratio (0.07) in the 0.35 M NaCl fraction as compared to 0.8 for histones and 0.91 for remaining nonhistones. The same study found low radioisotope incorporation (40 cpm during HeLa cell S-phase)

into the 0.35 NaCl fraction as compared to 940 cpm for histones and 1370 cpm for remaining nonhistones. Nonhistones in these studies were solubilized in 1% SDS following acid-extraction of chromatin. It has also been shown that nonhistone chromosomal proteins bind as efficiently to chromatin as they bind to DNA and approximately 12-15% of nonhistones cannot be outcompeted by any fraction of cytoplasmic proteins in binding to chromatin (Chaudhuri, Stein and Baserga 1972).

Since nearly all proteins of chromatin possess regions which are not bound to DNA (Simpson 1972) and it is known that the linkage of protein to DNA is a loose one (Mirsky, 1971), I contend that to use the criterion that any protein removable from chromatin by a method such as washing in 0.35 M NaCl indicates this protein is a contaminant, is not only a groundless criterion, but can lead to loss of information about proteins which may, in fact, be important regulatory molecules. It is for these reasons that my study was made on proteins <u>associated</u> with chromatin, and rigorous over-purification of chromatin was avoided.

Support for reasonable purity of the chromatin extracted for these studies can be derived from the observation that in order to get soluble histone sulfate, one must use very pure nucleoprotein because if an extraction is made from crude nucleoprotein only 10% of acid-soluble

protein is histone (Bonner et al. 1968a). It has been shown in my study that 66-68% of acid-soluble protein is alleged histone, and this may be taken as an indication of the purity of the chromatin, at least within the range acceptable to other workers in the field.

Standards have also been proposed specifying that a protein/DNA ratio of total isolated protein greater than approximately 2.5 indicates that the chromatin contains ribonuclear proteins or other contaminants (Bonner et al. 1968a). The majority of studies on chromatin cite figures well within the range of this standard for pure chromatin. It can be noted from data presented in Chapters 4 and 5 that most protein/DNA ratios cited in my study are far above the "desirable" range normally considered acceptable. These ratios are not believed due to contamination and the following arguments are given to support this contention.

The accepted ratio of histone/DNA has traditionally been about 1.0 but I believe this ratio is too low for the following reasons. Although early studies indicated that only about 50% of DNA is covered with protein of any sort (Clark and Felsenfeld 1971, also reviewed by Comings 1972), more recent work has concluded that 70-84% of DNA is protected by arginine-rich histones alone (Clark and Felsenfeld 1972) and histones bind to more than 80% of DNA (Billing and Bonner 1972). Most histone/DNA ratios cited

vary between 0.8 and 1.35, however the amino acid configuration of histones is such that a mass ratio of 1.35 is required for a stoichiometric complex of histone to DNA (Bonner et al. 1968b). Thus, if one accepts the evidence available, a histone ratio somewhat greater than 1 and possibly as large as 1.35 should be expected. In my study HIS/DNA ratios were slightly greater than 1.35 (usually about 1.4-2.0). My values should probably be revised downward due to the fact that the ratios are based on total HIS extracted/total DNA extracted from pellet only. Not only may there be unextracted DNA left in the pellet, but studies measuring DNA in every fraction have demonstrated that some DNA is lost at other points of the extraction procedure (e.g., see Shirey and Huang 1969). The loss of DNA from the final pellet would be expected to inflate all calculated protein/DNA ratios.

Surprising facets of this study were the observed USP/DNA and RES/DNA ratios (Chapters 4 and 5) which may be collectively considered nonhistone/DNA ratios. Most studies find nonhistone/DNA ratios to be considerably lower than histone/DNA ratios and the 1970 review by Paul states that ratios typically vary between 0.25 and 1.0. Studies such as those on Nicotiana (nonhistone/DNA = 8.3, Flamm and Birnstiel 1964), on Physarum (nonhistone/DNA = 9.1, Mohberg and Rusch 1970; PSNAP/DNA = 1.2, LeStourgeon and

Rusch 1971), and on HeLa cells (nonhistone/DNA = 1.635, Rovera and Baserga 1971), however, serve to throw doubt on the concept that the low nonhistone/DNA ratios are particularly "normal." Discrepancies such as these suggest that the nonhistone/DNA ratio is probably dependent upon the tissue used for extraction as well as upon the methodology employed. It is my contention that the majority of studies on nonhistones have seriously underestimated the amount present due (1) to loss of nonhistones into histone fractions and (2) to inefficiency of methods to extract nonhistones. In support of the first charge are observations by authorities such as Stein and Borun (1972) that histones extracted from whole chromatin, even 0.35 M NaCl-treated chromatin, are contaminated; and by Wilhelm et al. (1972) who find that 15% of nonhistones are extracted with the histone fraction. That many methods currently employed for nonhistone extraction are inefficient is suggested by an early study by Dounce and Hilgartner (1964) which showed that rat liver nuclei, from which lipoproteins and histones had been extracted, contained nonhistones that were highly insoluble in water, NaCl solutions of varying concentrations, and 8 M urea, among others. However, after treatment with sodium thioglycollate (a mercaptoacetate) the major portion of the protein was soluble in 8 M urea or in dilute alkali. This was thought due to the reduction

of disulfide bridges by the thioglycollate, resulting in "degelled" chromatin. If this action does indeed occur, the combination of mercaptoethanol and urea used by me to extract USP would be expected to have the same effect, resulting in extraction of nonhistones normally not dissociated from DNA.

Concerning their urea extraction procedure, Gronow and Griffiths (1971) claimed that 70.3 ± 5% of total nuclear proteins are extracted by the 8 M urea and further work on this procedure concludes only chromatin-associated proteins are extracted and "it is reasonable to assume that we are dealing with proteins which are truly chromosomal in origin" (Barrett and Gould, 1973).

Finally, although it is believed traditional nonhistone/DNA ratios are too low and values cited in my work reflect more accurate estimates of nonhistones actually associated with chromatin, it is possible that the values derived from my studies were high due to interference problems encountered in the Folin phenol test as noted in Chapter 2. It should be kept in mind that although the values here are comparable from one sample to another, little direct comparison of values can be made with other studies.

CHAPTER 4

TISSUE SPECIFICITY OF CHROMATIN-ASSOCIATED PROTEINS

Tissue and species specificity of some nonhistones is an expected characteristic if they truly include proteins which function as regulators of gene action. It has been hypothesized (Comings 1972) that two classes of nonhistones exist. The first class includes enzymatic and structural proteins such as polymerases, phosphokinases and proteases which would be expected to be similar if not identical in various tissues and species. The second class consists of regulatory proteins. These represent a small fraction of nonhistones which show greater variability among tissues and species. This idea is in accord with those of Teng et al. (1971) who have pointed out that since all cells synthesize certain RNA's in common, regulatory proteins concerned with the transcription of the more ubiquitous RNA's should be similar from cell to cell. Much evidence in the literature is found in support of these views.

Review of Literature

Recent reviews which comprehensively discuss tissue and species specificity of nuclear proteins include those

by Wilhelm et al. (1971), Spelsberg et al. (1972), Stellwagen and Cole (1969) and Elgin et al. (1971).

Protein/DNA Ratios

Many studies have reported protein/DNA ratios of various tissues and species (e.g., Elgin and Bonner 1970, Steggles et al. 1971), but few have reached any conclusions as to the significance of this measurement. Dingman and Sporn (1964) found that while HIS/DNA was constant for various chicken tissues, total protein/DNA ratios (which were positively correlated with RNA/DNA) generally declined with increasing age; while others (O'Meara and Herrmann 1972) found for mouse liver that total protein/DNA did not change related to age, although HIS/DNA increased with corresponding decreases in nonhistones/DNA and RNA/DNA. Bonner et al. (1968b) cite values for pea tissue showing that nonhistones varied inversely with histone content and template activity of chromatin. However, nonhistones are composed of a variety of different proteins, including structural species, species functioning to activate the genome, and repressor species; therefore, a correlation between tissue type and protein/DNA ratio would be unexpected if a majority of nonhistones were extracted.

Tissue and Species Specificity of Histones

Little specificity, on a gross basis, has been found for histones. Secondary modifications (phosphorylation, methylation and acetylation) have indicated far more heterogeneity for histones analysed by gel electrophoresis than probably exists on a level of primary structure. A well-documented tissue specific histone species, which appears to be an evolutionary decendant of histone f1, occurs in nucleated erythrocytes of birds, fish and frogs (reviewed by Comings 1972). This species may be involved in maintaining the highly repressed state of the erythrocyte chromatin (reviewed by Elgin et al. 1971).

Histone f1 is the most heterogeneous of all histone species varying, not only in amount, but in amino acid composition. Fambrough, Fujimura and Bonner (1968) found a low histone f1 content in the pea cotyledon paralleled by a low HIS/DNA ratio and high template activity, and the reverse situation in the pea bud.

Tissue Specificity of Nonhistones Analyzed by Gel Electrophoresis

The majority of investigators examining tissue (or species) specificity of nonhistones do so on SDS gel electrophoresis. Since this technique dissociates subunits into denatured polypeptide chains, it is possible that diversity in addition to that which has been found, exists

on the level of association of subunits in varying combinations (Platz, Kish and Kleinsmith 1970).

Tissue Specificity of Nonhistones Analyzed by Hybridization

A recent popular, and probably more informative, approach to tissue specificity of nuclear protein species has been to classify them on the basis of their effects on RNA synthesis rather than by their positions on gels. Techniques used for such analyses are comprehensively reviewed by Paul (1970).

Early experiments such as those by Paul and Gilmour (1968) used DNA/RNA hybridization to show that RNA's from various organs anneal to no more than 5-10% of DNA from the same organism, whereas RNA produced from DNA which had been stripped of its proteins annealed to 40-50% of DNA. Competitive hybridization was used to show that RNA produced from native chromatin transcribed *in vitro* behaves identically to RNA extracted from the corresponding tissue. This work implicated nonhistones as the molecules responsible for tissue specific transcription and suggested that while histones mask DNA, the effect is not specific.

In recent sophisticated experiments, chromatins from various tissues are dissociated and various combinations of histones, nonhistones and DNA from two different tissues are reconstituted. RNA's synthesized from an

in vitro system are isolated and competitive DNA/RNA hybridization is used to test which combinations of chromatin components lead to the synthesis of the same species of RNA's as the native chromatins transcribed *in vitro* or *in vivo*. Results from experiments such as those by Spelsberg and Hnilica (1970) and Spelsberg, Hnilica and Ansevin (1971) have clearly shown that it is the source of the nonhistones and not the source of the histones or DNA that determines tissue specific transcription.

Similar experiments measuring template activity of HeLa chromatin reconstituted with chromatin components from various points of the cell cycle have shown that it is nonhistones rather than histones which result in reduced template activity of mitotic chromatin (Stein and Farber 1972).

Experiments comparing different rat tissues as well as rat liver and Walker tumor, have shown that homologous nonhistones added to chromatin stimulate RNA synthesis to a greater extent than heterologous nonhistones and that homologous nonhistones stimulate transcription from DNA sequences in addition to those transcribed from native chromatin and different from those activated by heterologous nonhistones (Wang 1971, Kostraba and Wang 1972).

Other approaches have demonstrated differences in immunological properties of nonhistone-DNA complexes from

different tissues. Since 20% of the antigenic sites located on nonhistone-DNA complexes were accessible in the native chromatin and since antisera inhibited in vitro template activity, it has been suggested that nonhistones might be associated with transcribable DNA (Chytil and Spelsberg, 1971).

Nonhistones have been tested for species-specific binding to DNA and it has been shown that they include species which do not bind to DNA at low ionic strength, species with affinity for heterologous DNA and species binding only to homologous DNA (Teng, Teng and Allfrey 1970, 1971; van den Broek et al. 1973). It has been shown that the species specificity of DNA binding cannot be attributed to chromosomal RNA (Kleinsmith, Heidema and Carroll 1970) and it is concluded that the specificity must reside within the nonhistones themselves.

The examples presented here represent only a few of the many experiments which have demonstrated that nonhistones contain species which are both structurally and functionally tissue and species-specific as well as specific to tissues at different stages of development and different times of the cell cycle.

Materials and Methods

Growth and Harvest of Plant Tissue

Vicia faba seeds were surface-sterilized and germinated two days in running tap water as described previously in Chapter 3. Seed coats were then removed and naked seeds were planted to a uniform depth of approximately 3 cm in autoclaved Krum (perlite, Silbrico Corp.). Thirty-six seeds were planted in each of two 25 x 33 x 14 cm deep plastic dishpans. Initially, one liter of tap water was added to each pan with an additional 0.5 liters added on the third and sixth days after planting. The plants were kept in the dark at room temperature for a total of nine days after planting. During times that water was added, they were exposed briefly to green light. After the total 11 day growth period, etiolated seedlings were harvested and dissected into root meristems (2-mm apical ends of lateral roots), mature lateral root tissue (1-cm sections from the middle of lateral roots, at a minimum distance from the root apex of 1 cm), and stem tissue (2-cm sections of the stem at a distance of at least 2 cm from both the shoot apex and the point of cotyledonary attachment). Any plants visibly infected with fungus were discarded.

Cotyledons from 10 seeds were collected from batches of seeds grown as described in Chapter 3 in running tap

water for 48 or 120 hr. Cotyledons were inspected carefully for fungus infection before harvesting only uncontaminated tissue.

Embryos and seed coats were removed from dry seeds and the cotyledons were smashed with a hammer against a stone surface. The resulting pieces were ground to a coarse powder with a mortar and pestle. These are referred to as "0-hr cotyledons." Like other plant material, the 0-hr cotyledon powder was frozen with liquid nitrogen and stored at -15 C.

Excised epicotyl gels refer to gels generated from experiments discussed in Chapter 5.

At least two replications were performed for each tissue except 0-hr cotyledons. The procedure was repeated in its entirety for replications. That is, a new batch of tissue was grown and harvested for replicate experiments. As discussed in Chapter 3, little variation in gel banding pattern was noted from batch to batch.

Chromatin Extraction and Fractionation and Electrophoresis

Chromatin was isolated and fractionated as described in Chapter 2. Cotyledon chromatin was washed six times in 0.01 M tris, pH 8, rather than five times to facilitate removal of the excess starch occurring in that tissue.

Starch contamination was minimal in the final chromatin pellet.

Electrophoresis was performed as described in Chapter 2. In cases where large concentration differences made results difficult to interpret, additional electrophoresis of diluted control samples was performed.

Analysis of Data

Gels were analyzed for presence or absence of bands and for band position by a method designed to minimize the chance of making a Type I error. This process of gel analysis is fully discussed, with examples, in Appendix A.

Each tissue was compared as to protein/DNA ratio for each class of proteins and for these data, both the mean values and range of ratios are shown. Proportions of HIS subfractions were computed as described in Chapter 2.

Results and Discussion

Protein/DNA Ratios

Table 3 lists mean protein/DNA ratios calculated for each tissue examined and their ranges. Ratios for which no range is listed are based on one sample. In most such cases, the replicate values were not calculated due to protein concentrations too low to be measured. For each class of protein, ratios were compared with excised epicotyl and with one another. Ratios with overlapping

Table 3. Protein/DNA ratios for various tissues. -- Ranges of protein/DNA ratios are indicated below the mean ratio. Nonhistone/DNA = (USP + RES)/DNA. Total protein/DNA = (USP + RES + HIS)/DNA.

	Protein/DNA Ratio				
Tissue	USP/DNA	HIS/DNA	RES/DNA	Nonhistone/ DNA	Total Protein/ DNA
14-hr excised epicotyl	3.2 3.0-3.3	2.0 1.9-2.2	1.8 1.3-2.1	5.0 4.6-5.3	7.0 6.6-7.5
leaf	1.6 1.3-1.8	1.4 1.4	1.2 1.1-1.3	2.8 2.4-3.1	4.3 3.8-4.5
root meristem	2.4 1.5-3.4	1.5 1.0-2.0	2.0 1.6-2.3	4.4 3.1-5.7	5.9 4.1-7.7
mature lateral root	9.7 2.9-16.5	0.9	6.6 2.1-11.2	16.3 5.0-27.7	17.2 5.8-27.7
stem	0.7 0.3-1.0	1.5	2.5 1.0-4.0	3.2 1.3-5.0	4.7 2.8-5.0

Table 3. (Continued)

Tissue	Protein/DNA Ratio				
	USP/DNA	HIS/DNA	RES/DNA	Nonhistone/ DNA	Total Protein/ DNA
0-hr cotyledon	10.3	1.8	2.2	12.5	14.3
48-hr cotyledon	23.1	1.1	4.7	27.8	28.9
	9.0-37.3	1.0-1.3	2.8-6.5	11.8-43.8	13.1-44.8
120-hr cotyledon	4.5	1.1	1.8	6.3	7.4
	4.3-4.7	0.8-1.5	1.5-2.0	5.8-6.7	6.6-8.2

ranges were not considered significantly different from one another.

Excised epicotyl and root meristem demonstrated similar USP/DNA ratios, whereas leaf tissue had a USP/DNA value significantly different from excised epicotyl, but not from root meristem. Mature lateral root tissue did not appear to be different from excised epicotyl and root meristem. The mean protein/DNA ratios for mature lateral root tissue are inflated due to an abnormally low amount of DNA recovered for the second sample. The first sample (lower value of the range) was probably more indicative of the natural state in this tissue. Stem tissue demonstrated a USP/DNA ratio significantly lower than any other tissue examined. Cotyledons had significantly higher USP/DNA ratios which appeared to change during development.

The data derived from the studies with cotyledons, demonstrating different USP/DNA values at different times, indicated that USP/DNA may vary with age of tissue. The peak USP/DNA ratio in 48-hr cotyledons is coincident with V. faba cotyledon development stage II (Briarty, Coult and Boulter 1970) during which the number of mitochondria and free ribosomes in this tissue have increased. The USP/DNA ratio for cotyledons decreased at 120 hr, corresponding to development stage IV, a time of protein degredation.

Although, on the basis of the data presented here, no statistical difference has been proven, the consistently high HIS/DNA ratios of 14-hr excised epicotyls compared with the consistently lower ratios of some other tissues suggests that HIS/DNA may be different among tissues.

RES/DNA ratios are similar for every tissue except 48-hr cotyledons and for this tissue, the ratio is significantly higher than other ratio observed. If USP/DNA and RES/DNA are summed to reflect total nonhistone protein/DNA, the pattern observed is the same as for USP/DNA, except that the range for stem tissue is extended into that for excised epicotyl.

No trends such as inverse or positive correlations of nonhistone/DNA and HIS/DNA were apparent from these data. It can be concluded that although USP/DNA ratio is not related to any discernible feature of the tissues examined, the ratios do vary and different tissues exhibit characteristic USP/DNA ratios.

Proportions of HIS Species

Table 4 lists the mean proportions of HIS species for various tissues and their ranges. Bands c and d were pooled since they were not always completely resolved in electrophoresis.

The proportion of HIS band a and b are in line with literature values for histone f1 for pea (Fambrough et al.

Table 4. Proportions of HIS species for various tissues. -- Proportion expressed as percent of total HIS. Ranges are indicated below the mean value.

Tissue	HIS Species a+b (f1)	c+d (f2b+f3+f2a2)	e (f2a1)
leaf	17	47	36
	17	44-49	35-38
root meristem	18	40	42
	17-18	37-43	38-44
mature lateral root	13	49	38
	9-16	46-52	38-39
stem	8	41	50
	2-14	30-53	33-68
0-hr cotyledon	12	46	41
48-hr cotyledon	24	40	37
	20-27	30-50	23-50
120-hr cotyledon	14	46	39
	14-15	42-51	34-45

1968, f1 = 14-32%) for wheat (Spiker and Krishnaswamy 1973, f1 = 9-11%) and for V. faba (Stanley 1972, f1 = 14%). However the proportions of HIS c and d are significantly lower than expected for f2a2 + f2b + f3 (64-74% for pea, 68-74% for wheat and 67% for V. faba). This is consistent with problems encountered with quantitative densitometry using Coomassie blue stain, as described by Fishbein (1972). Thus, although proportions can probably be compared from one sample to another within this study, little direct comparison can be made with other studies of proportions of histone subfractions.

Leaf and root meristem have a somewhat greater proportion of HIS a and b (presumably histone f1) than mature lateral root, stem and most cotyledon tissues. An elevated proportion of lysine-rich histone in meristematic tissue appears to be in agreement with cytochemical studies in barley root by Yanagi and Kusanagi (1970), although the elevation is nowhere near that suggested by their work. Studies on tobacco by Srivastava (1971) comparing young and senescent leaves and pith suggest that the proportion of histone f1 is reduced in older tissues but experiments with different organs of pea plants (Fambrough et al. 1968) demonstrate no discernible tendency for the occurrence of a depressed proportion of histone f1 in nonmeristematic tissues such as stem. It is suspected that, although

ranges are not overlapping, the differences observed between the two types of tissue may be due to statistical error that would become more obvious if a greater number of samples were tested.

No significant changes are noted in the proportions of HIS c and d which is contrary to the findings of both Srivastava (1971) who found elevated histone f2a2 + f2b + f3 proportions in older tissues and those of Fambrough et al. (1968) who found higher proportions of f2a2 + f2b + f3 in cotyledon and bud than in mature seed and leaflet for pea. Fambrough et al. (1968) also found higher histone f2a1 proportions in mature seed and cotyledon than in leaflets of pea, and lower histone f2a1 proportions in pea bud. Other experiments with roots, shoots and leaves of winter and spring wheat under various conditions of development have demonstrated no changes in any HIS subfraction (Spiker and Krishnaswamy 1973).

The elevated proportion of HIS a and b in 48-hr cotyledons is believed to be significant. Fambrough et al. (1968) have observed that for pea cotyledons, the proportion of histone f1 changes during maturation from an initial depression of 6-8% increasing to 16% with increasing maturation. However this study was not carried into various ages of cotyledons during germination. Evidently, in V. faba, HIS a and b proportions continue to change during germination.

As noted by Fambrough et al. (1968) for pea cotyledon maturation, no significant changes in the proportion of other HIS species for V. faba cotyledon development were indicated.

Variability in USP Bands for Different Tissues

Figure 4 demonstrates USP banding patterns for various tissues of Vicia faba and Fig. 5 demonstrates the banding pattern with all invariant bands removed. Bands 0, 1, and 2 are not pictured, since variability in excised epicotyl tissue for these species did not present sufficient baseline information for decisions on significant variability in other tissues. No changes were discernible at the various stages in cotyledon development and only one pattern is shown for all cotyledons. Little variation in banding pattern occurs among USP from various sources, which is as expected since all tissues probably have many enzymes and structural proteins in common. One striking change is the development of a low molecular weight species (band 13) in 11-day old tissues, regardless of their state of differentiation. This band did not occur in any tissue collected at 120 hr or earlier.

Band 11b appears to be replaced by a species of slightly lower molecular weight in cotyledons. Bands 6 and 7 are replaced by one species of intermediate molecular

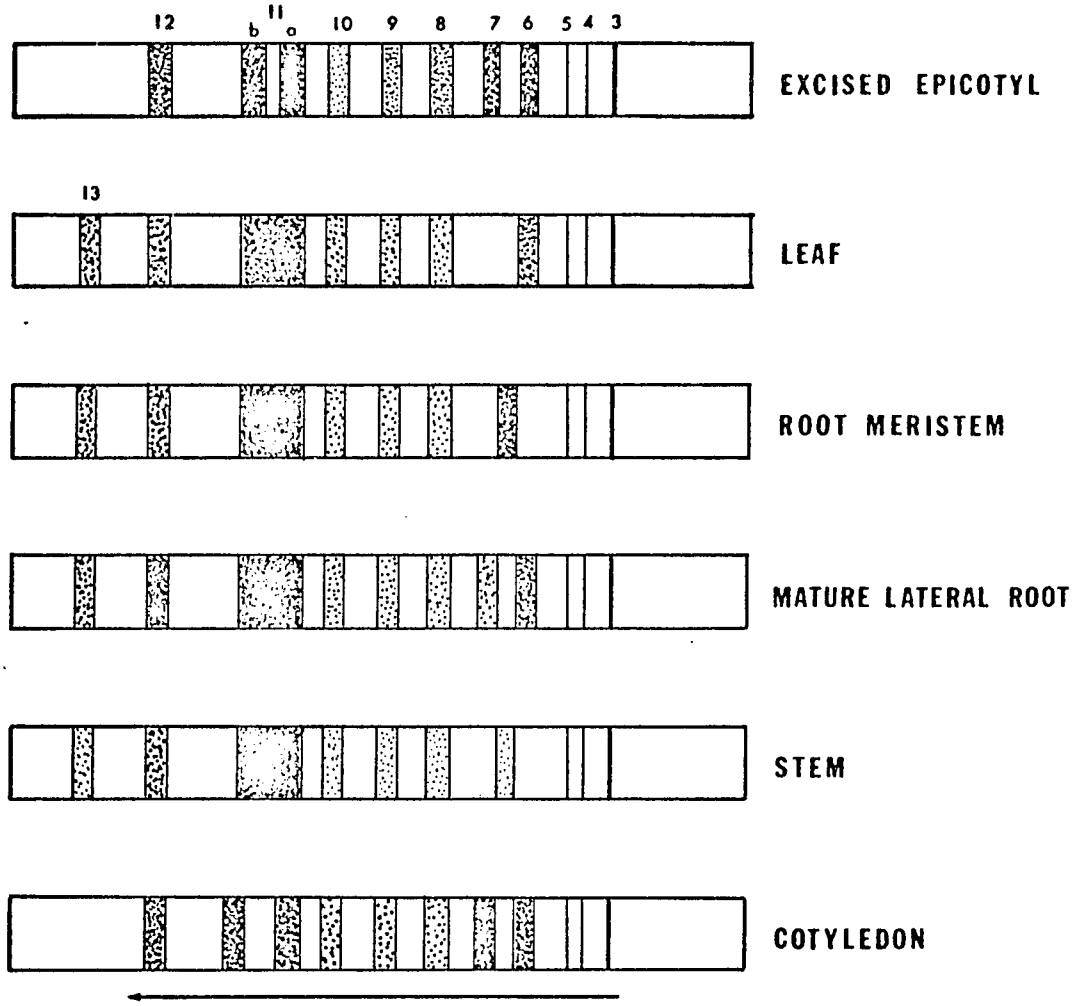

Figure 4. USP for various tissues of V. faba.

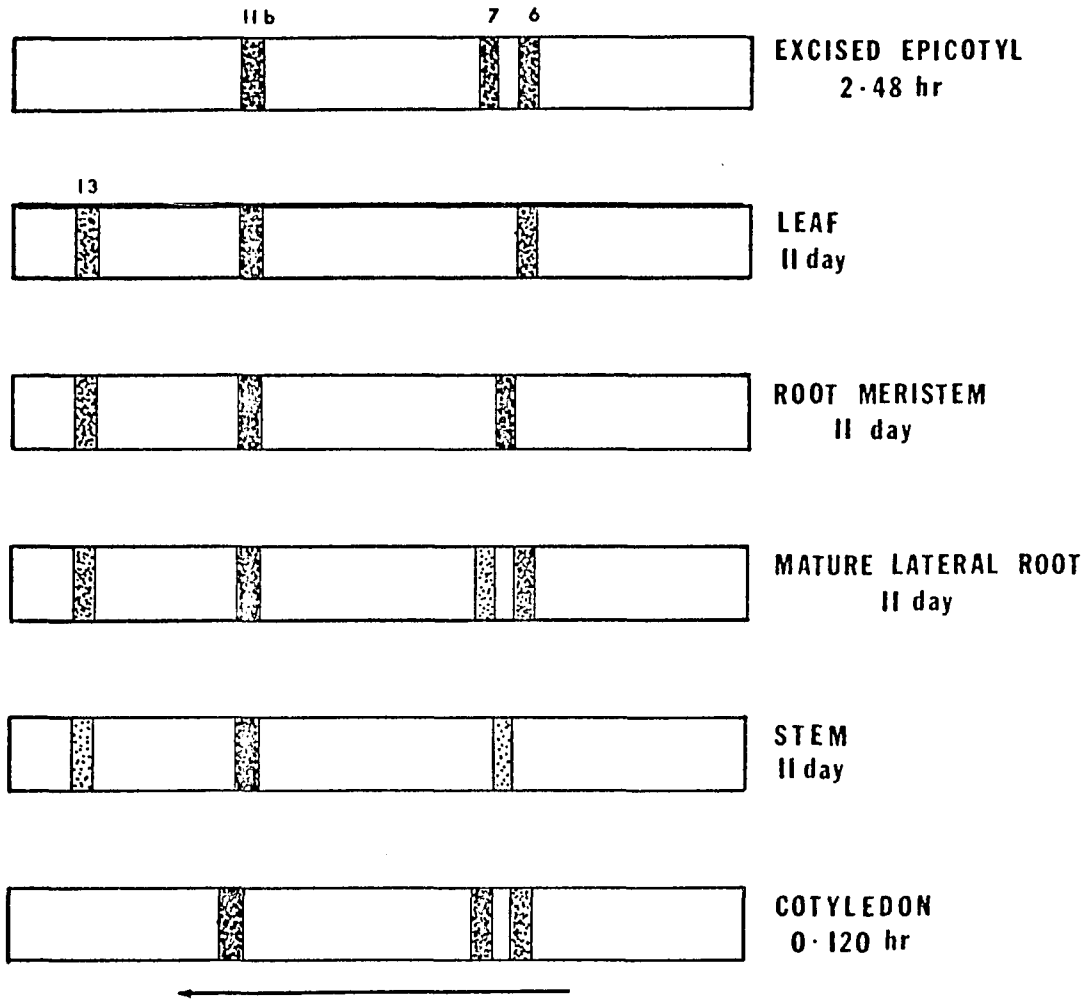

Figure 5. Tissue specific USP in V. faba.

weight for root meristem and stem and band 7 is lost entirely in leaf tissue. It is interesting that bands 6 and 11 are commonly extracted in RES samples, and if RES represent USP tightly bound to DNA, they may, in fact, be important regulatory molecules. For all tissues, bands 3, 4, 5, 8, 9, 10, and 12 were invariant.

The results of this experiment are typical of such experiments comparing electrophoretic banding patterns. Limited tissue specificity has been found for SDS-soluble nonhistones of rat and chicken tissues (Elgin and Bonner 1970, Wilhelm et al. 1972) and for acidic chromosomal and ribonuclear proteins of Phaseolus (Spelsberg and Sarkissian 1968, 1970b) among others. Tissue specificity has also been found for PSNAP of rat (Teng, et al. 1970, 1971) and extreme species and tissue specificity of USP has been observed from rat and chicken tissues using a two-dimensional electrophoretic system (Barrett and Gould 1973).

The results of experiments showing little or no tissue or species specificity of nonhistones (e.g., MacGillivray et al. 1972) can probably be attributed to failure to extract many nonhistones as evidenced by the low protein/DNA ratio and the failure to find high molecular weight species.

Due to high background staining on USP gels with Coomassie blue, no effort was made to calculate change in

proportions of various USP species. However, it is obvious that changes did occur as is demonstrated by Fig. A-7 in Appendix A.

Variability in HIS and RES Bands for Different Tissues

No variability in HIS banding patterns were detected in any tissues, as expected. RES banding patterns were not tested, as it is believed that these may be the same as unextracted USP species. It was evident by casual examination of the gels that there was great variability in RES gel banding patterns, both from tissue to tissue and from replication to replication.

CHAPTER 5

SYNTHESIS OF CHROMATIN-ASSOCIATED PROTEINS DURING EARLY STAGES OF GERMINATION

One of the features of chromatin-associated proteins which makes them likely candidates for regulatory proteins is their pattern of synthesis. Histones and nonhistones exhibit different characteristics in their biosynthesis; for detailed accounts of the work done in this field, the reader is referred to the comprehensive reviews by Stellwagen and Cole (1969), Elgin et al. (1971), Hnilica et al. (1971), McClure and Hnilica (1972), Spelsberg et al. (1972) and Comings (1972).

Review of Literature

Synthesis of Nonhistones

In dividing tissues given labelled amino acids, nuclear nonhistones have specific activities approximately three to six times higher than histone fractions (McClure and Hnilica 1972). For the most part, biochemical studies (e.g., Cross 1972, Sadgopal and Bonner 1969) have detected nonhistone synthesis throughout the cell cycle with labelled-amino acid incorporation highest in the G_1 phase. Agreement with this has been demonstrated on the cytochemical level

(Auer 1972). Nonhistone synthesis is typically stimulated at times of gene activation. For example, stimulation of haemoglobin synthesis by erythropoetin in mouse liver is associated with stimulation of nonhistone synthesis (Malpoix 1971), as is heterochromatization and genome repression in chicken erythrocyte (Sadgopal and Kabat 1969). The synthesis of nonhistone proteins observed in response to hormones is discussed in Chapter 6.

Control of Cell Division

The work of Stein and Baserga and their associates (comprehensively reviewed by Stein and Baserga 1971, and by Baserga and Stein 1971) has resulted in a hypothesis for the control of cell division in mammalian cells. This hypothesis states that in cells stimulated to proliferate by a variety of apparently unrelated stimuli such as change of medium (e.g., Stein, Chaudhuri and Baserga 1972) or SV 40 infection (Rovera, Baserga and Defendi 1972) a series of complex and interdependent biochemical events precedes the onset of DNA and mitosis. Abundant evidence is presented that gene activation is involved in this process and it is suggested that nonhistones are involved in the control of cell proliferation. It is thought that a cytoplasmic inhibitor is lost following the stimulus for cell proliferation, and the loss of this inhibitor results in translation of a pre-existing RNA template for a nonhistone protein.

The newly-synthesized nonhistone is transferred to the nucleus where it binds in the major groove of the DNA and activates a portion of the genome, resulting in rounds of RNA and protein synthesis that eventually lead to the onset of DNA synthesis and cell division (Baserga and Stein 1971). It appears that the synthesis of nonhistone proteins is closely related to cell division, at least in HeLa cells.

Synthesis of Histones

Synthesis of histones is apparently not as important for genetic regulation as are secondary modifications such as methylation, acetylation and phosphorylation (Benjamin 1971). Histone synthesis occurs, for the most part, in the S-phase of the cell cycle and turnover is extremely slow. Early studies on the biosynthesis of individual histone fractions showed decreased labelling with increasing lysine content of the fraction. However later studies have not borne this out. Biosynthetic rates of individual histone fractions remain one of the most controversial aspects of histone biology (reviewed by Hnilica et al. 1971). Much disagreement is voiced on whether DNA synthesis is dependent on histone synthesis, histone synthesis is dependent on DNA synthesis or whether the syntheses of histones and DNA are independent. On the basis of circumstantial evidence, histones have recently been proposed as

chain elongation proteins in the synthesis of DNA (Weintraub 1972). In most cases studied, DNA and histone syntheses do appear to be closely associated; therefore when DNA synthesis is stimulated, stimulated histone synthesis is also observed.

Materials and Methods

Germination and Radioisotope Labelling of Excised Epicotyls

Epicotyls were dissected from dry V. faba seeds. Batches of 100 epicotyls (approximately 200 mg dry tissue) were weighed and placed into 10-ml dry, sterile glass vials with polyethylene snap caps. These were stored at room temperature for varying periods up to two months.

In vitro excised epicotyl development, hereafter referred to as germination, was initiated by the addition of 5.0 ml water (cold, sterile distilled) containing 1585 units/ml of Potassium Penicillin G (Calbiochem, B grade). Epicotyls were germinated in the dark at room temperature for 14, 24, 36 or 48 hr. During the last 12 hr of each germination period, the water was pipetted out and 0.5 ml fresh water (with penicillin as above) containing 2.0 µc ^{14}C-leucine or 8.75 µc ^{3}H-leucine, was added. The addition of radioisotope was performed in semi-darkness with only incident light from neighboring rooms. After 12 hr of

incubation with radioisotope, the epicotyls were washed at least three times in distilled water, frozen, and stored as usual.

Three series of replicates were germinated, two with ^{14}C-leucine as a label and one with ^{3}H-leucine as a label.

Chromatin Extraction and Fractionation and Electrophoresis

Methods used were exactly as described in Chapter 2.

Scintillation Counting and Analysis of Gels

Ratioisotope incorporation into each class of chromatin-associated proteins was measured for 100 µliter aliquots. In addition, 100 µliter aliquots of the initial cell homogenate from each sample were counted in order to estimate the relative intracellular concentration of the radioisotope at each stage of germination.

In order to determine the synthesis of the specific protein species at each stage of germination, representative USP, RES and HIS gels were sliced, digested and counted as described in Chapter 2. For all HIS and for 12-24 hr USP and RES, one ^{14}C-labelled gel and one ^{3}H-labelled gel were sliced and counted. For USP at each of the other stages of germination, one ^{14}C-labelled gel was examined.

Scintillation counter settings for detection of radioisotopes were 8.5% gain for ^{14}C or 100% gain for ^{3}H. For either radioisotope, a nearly completely open window of 50-1000 was used. At these settings, efficiency of ^{14}C detection was approximately 63% and efficiency of ^{3}H detection was approximately 36% when 0.1 μc ^{14}C-leucine or 1.0 μc ^{3}H-leucine was counted with no protein present.

Results and Discussion

Synthesis of USP, HIS and RES

In Fig. 6 are shown the mean specific activities of USP and RES labelled and extracted at various stages of germination for the two series labelled with ^{14}C-leucine. Superimposed on these diagrams are the specific activities of the ^{3}H-leucine series. The data used to generate these diagrams are listed in Appendix B, Table B-1. Total incorporation into each fraction is listed in Table B-2. The highest specific activity (and the highest total incorporation) for both USP and RES is for samples labelled during 12-24 hr germination. Specific activities of 2-14 hr samples are 76% (USP) and 67% (RES) as large as those labelled during 12-24 hr germination. Although the trend is similar for HIS, shown in Fig. 7, with peak specific activity (and highest total radioisotope incorporation) occurring from 12-24 hr germination, the specific activity

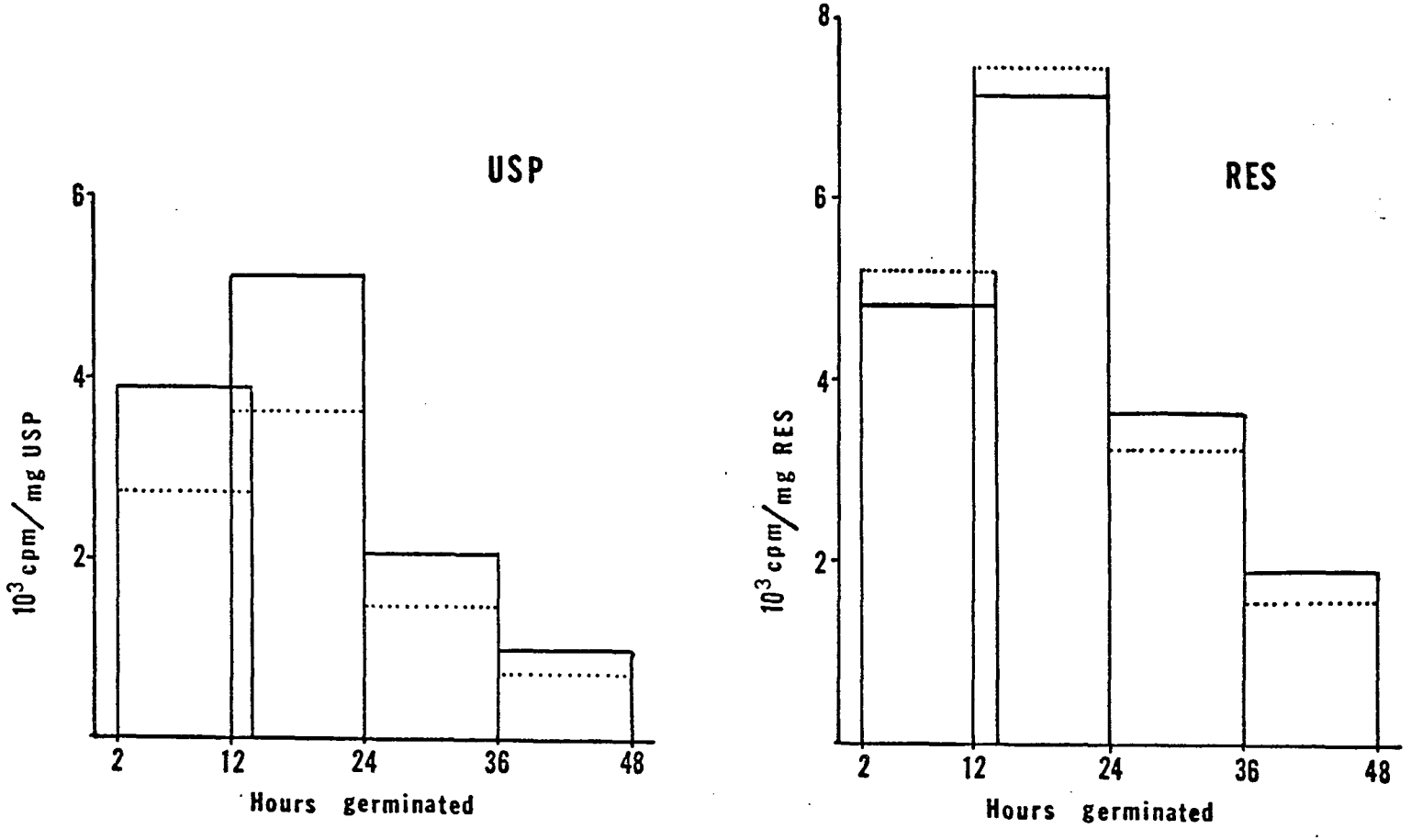

Figure 6. Specific activities of USP and RES during various stages of germination. -- Solid line indicates mean specific activity for two ^{14}C-leucine-labelled samples; dotted line indicates specific activity for one ^{3}H-leucine-labelled sample. Samples were labelled from 2-14 hr, from 12-24 hr, from 24-36 hr or from 36-48 hr.

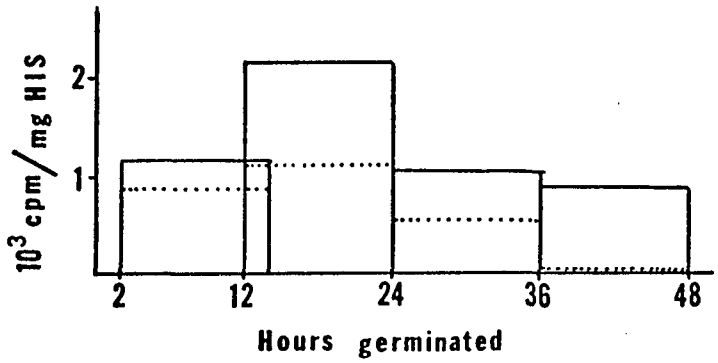

Figure 7. Specific activities of HIS during various stages of germination. -- Solid line indicates mean specific activity for two ^{14}C-leucine-labelled samples; dotted line indicates specific activity for one ^{3}H-leucine-labelled sample. Samples were labelled from 2-14 hr, from 12-24 hr, from 24-36 hr or from 36-48 hr.

of samples from 2-14 hr germination is only 41% that for samples labelled during 12-24 hr germination.

The differences observed in specific activity are an indication of synthesis of the chromatin-associated proteins although the experiments were not designed to rule out changes in leucine pools. The differences cannot be attributed to differential uptake of labelled-leucine at various times during germination. The intracellular concentration of labelled-leucine was approximately the same for all samples as shown by the data displayed in Appendix B, Table B-3.

The data for HIS may be compared to that of Stanley (1972) who found net histone synthesis during V. faba excised epicotyl development to peak during 2-14 hr germination, falling to a low level from 12-24 hr and increasing again at 24-36 and 36-48 hr. His data were corrected to cpm/200 mg initial dry weight of epicotyls. However, when data from my experiments are corrected to dry weight, the general pattern of protein synthesis is not different from that in Figs. 6 and 7. It is thought that the results achieved by Stanley (1972) were due in part to contamination by selectively-extracted acid-soluble non-histones and in part to light conditions during germination at variance from those used in these experiments. An indication that light conditions of germination may be an

important factor was demonstrated in further experiments not reported here.

Mean chromatin-associated protein/DNA ratios and their ranges are listed in Table 5. It is apparent that for USP there is an accumulation of protein by 36-48 hr germination which may be reflected in RES as well, although the significance of the RES values are in question.

The protein/DNA ratio of HIS at any stage was the same, as expected. In Table 6 the mean proportions of various HIS species at the different stages of germination are shown. At 36-48 hr germination, HIS a+b (presumably histone f1) appears to have decreased in amount and HIS e (presumably histone f2a1) appears to have increased in amount. However, the differences at these times of germination are not thought to be large enough to constitute a significant difference.

Data presented for these experiments are in agreement with observations of all other investigators who have examined synthesis of nuclear proteins, in that if total nonhistones are extracted, their specific activity is higher than that for histones, and the nonhistones which are tightly bound to DNA exhibit the highest specific activities (reviewed by McClure and Hnilica 1972).

It has been reported for HeLa cells that histones are synthesized mainly during the S-phase of the cell cycle

Table 5. Protein/DNA ratios during various stages of growth. -- Ranges of protein/DNA ratios indicated below the mean ratio.

Stage of Germination	Protein/DNA Ratio		
	USP	HIS	RES
2-14 hr	3.2	2.0	1.8
	3.0-3.3	1.9-2.2	1.3-2.1
12-24 hr	4.3	1.7	2.6
	2.6-6.8	1.2-2.0	1.8-3.9
24-36 hr	4.0	1.9	2.6
	2.8-5.3	1.7-2.0	2.6-2.7
36-48 hr	7.7	2.0	3.7
	6.4-8.5	1.7-2.7	3.4-4.1

Table 6. Proportions of HIS species at various stages of growth. -- Proportions expressed as percent of total HIS. Ranges are indicated below the mean value.

Stage of Germination	HIS Species		
	a+b (f1)	c+d (f2b+f3+f2a2)	e (f2a1)
2-14 hr	16	50	35
	14-18	49-50	33-36
12-24 hr	18	49	33
	15-22	46-51	32-34
24-36 hr	14	51	35
	13-15	49-53	32-38
36-48 hr	11	38	51
	10-12	24-52	36-67

(e.g., Sadgopal and Bonner 1969, Stein and Borun 1972) and that nonhistones are synthesized during the G_1 phase of the cell cycle (Stein and Borun 1972, Borun and Stein 1972). Data from the work of Stanley (1972) has demonstrated that ^{32}P incorporated into DNA of excised V. faba epicotyls occurs at all stages of germination measured, increasing at 24-36 hr and peaking at 36-48 hr. This is consistent with reports that the first major round of DNA synthesis in V. faba roots occurs at about 40 hr (Davidson 1966, Jakob 1972). Thus the results of the experiments reported here demonstrating that histone and nonhistone syntheses peak mainly at 12-24 hr germination appear inconsistent with other work done. It must be remembered, however, that the biological system tested in these experiments consisted of a population of tissues that were not specifically synchronized and that labelling was done over very long periods of time compared to some other studies done. In spite of the disadvantages of this system for synthesis experiments, it was believed worthwhile to examine the synthesis of the various chromatin-associated protein species on a band-by-band basis.

Synthesis of Individual USP and RES Species

Results for fractionation of replicate ^{3}H-leucine and ^{14}C-leucine USP gels are shown in Fig. 8 and for RES

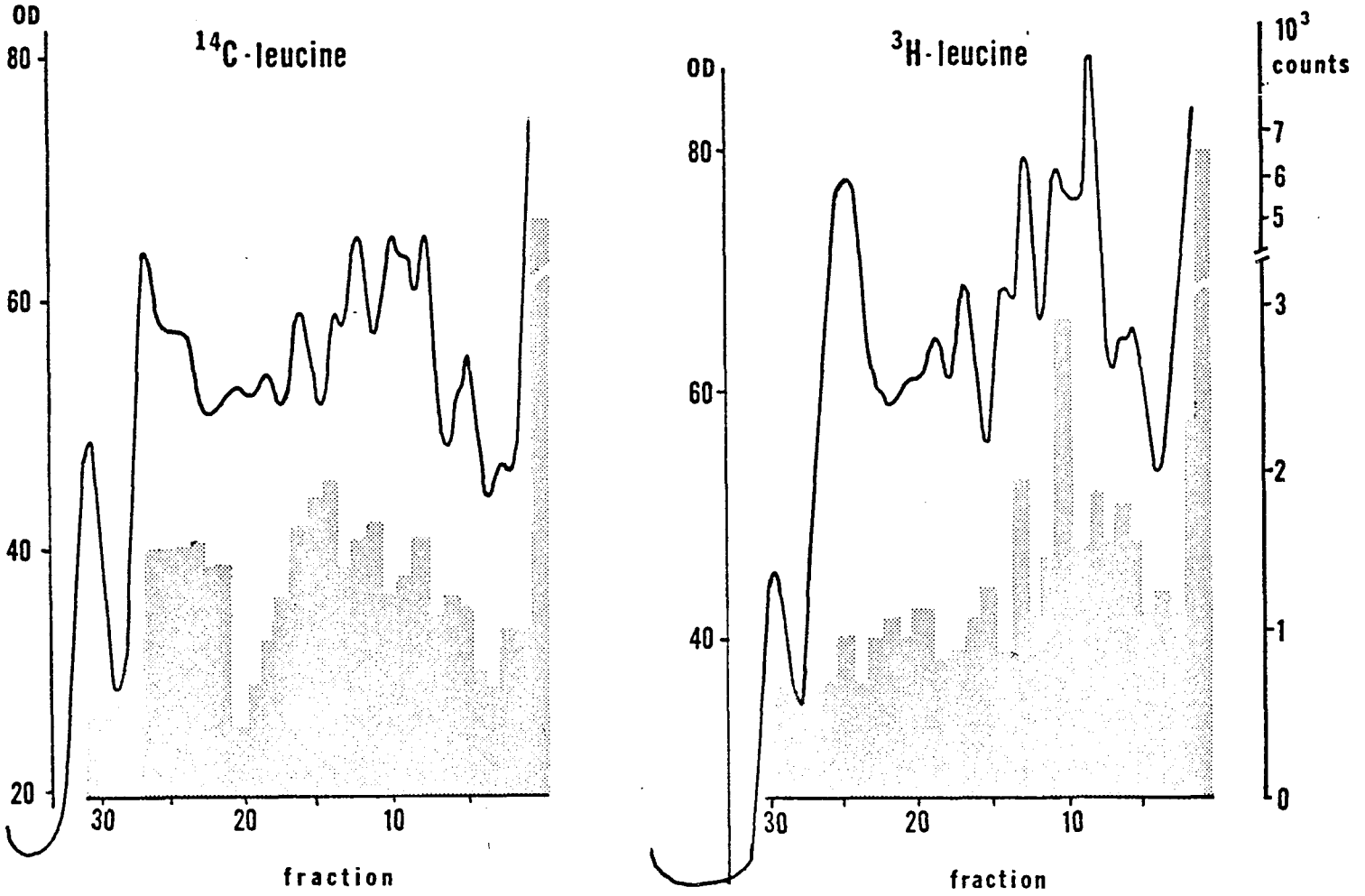

Figure 8. Fractionation of replicate gels of USP labelled from 12-24 hr germination. -- Gels were sliced, beginning at origin with fraction 1, and slices were digested and counted. Solid line indicates gel scan; shading indicates counts in 50 min/slice. OD is percent full-scale absorbance. Applied to the ^{14}C gel was 169 µg protein (30200 counts) and to the ^{3}H gel, 175 µg protein (29400 counts).

gels in Fig. 9. It can be seen from these illustrations that, while not identical, labelling patterns were similar for replicate gels. Due to the low detection of label in sliced and digested gels, long counting periods were required. Therefore, only one ^{14}C-leucine gel was counted for USP at times other than 12-24 hr. Figures 10 and 11 demonstrate the results obtained with USP for the four stages of germination. When these data are examined on a band-by-band basis, it is apparent that each USP species has a characteristic pattern of synthesis and three basic patterns are observed. The mean counts in 50 min/slice for each of the three basic groups of USP species, classified by their synthetic patterns, is shown in Fig. 12. Group A, demonstrating high labelling at 12-24 hr and 36-48 hr germination includes species 0, 1, 5, 6, 7 and 8. Group B, demonstrating high labelling at 12-24 hr germination only includes species 2, 3, and 4 and group C with the highest labelling at 24-36 hr germination includes species 9, 10, 11 and 12. Such stage specific differences in the rate of labelling of different bands has also been noted for HeLa nonhistones (Stein and Borun 1972). It is an interesting facet of these data, that species of similar molecular weight, for the most part, demonstrate similar labelling patterns.

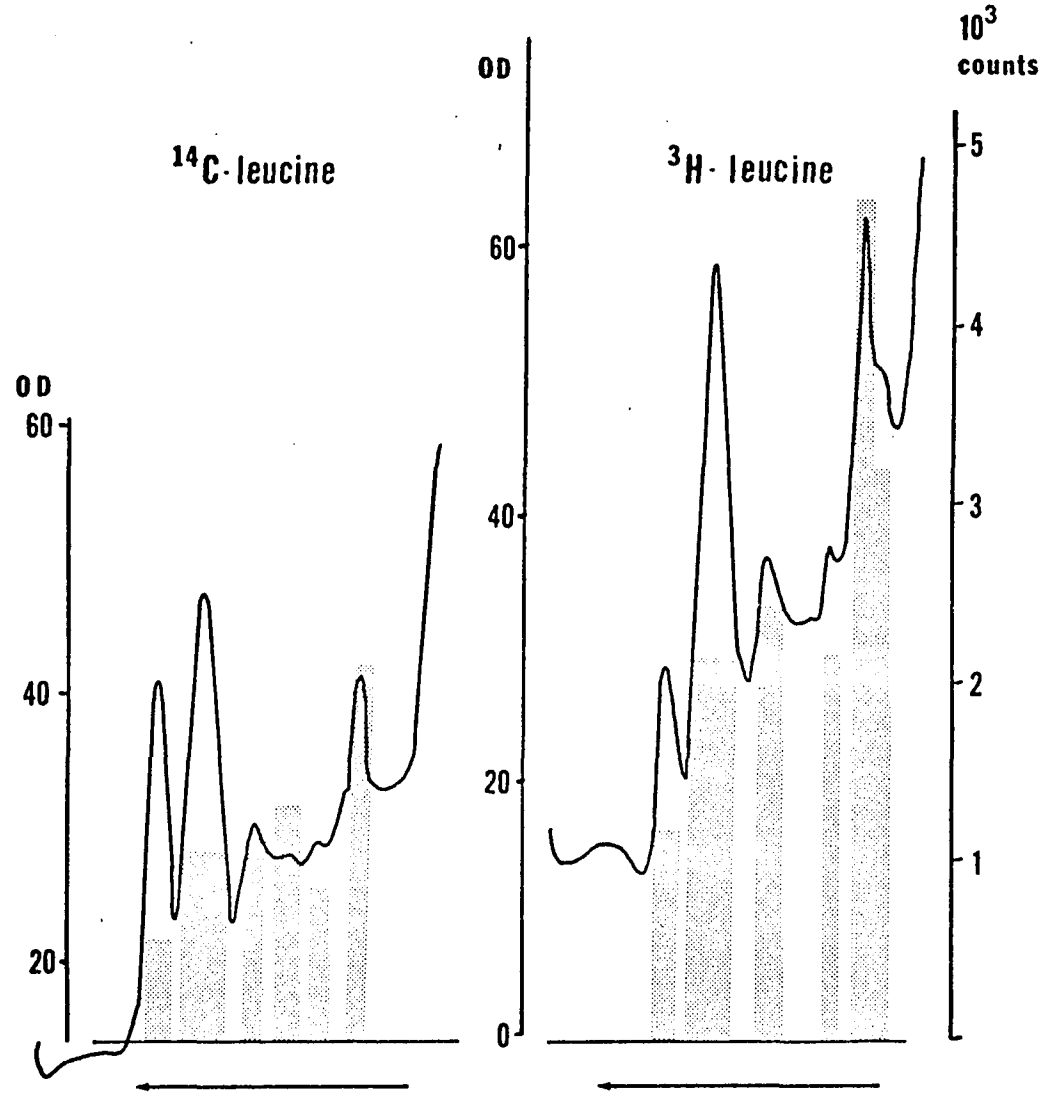

Figure 9. Fractionation of replicate gels of RES labelled from 12-24 hr germination. -- Individual bands were sliced from gels, digested and counted. Solid line indicates gel scan; shading indicates counts in 50 min/slice. OD is percent full-scale absorbance. Applied to the ^{14}C gel was 123 µg protein (40300 counts) and to the ^{3}H gel, 141 µg protein (52400 counts). Arrow indicates direction of migration.

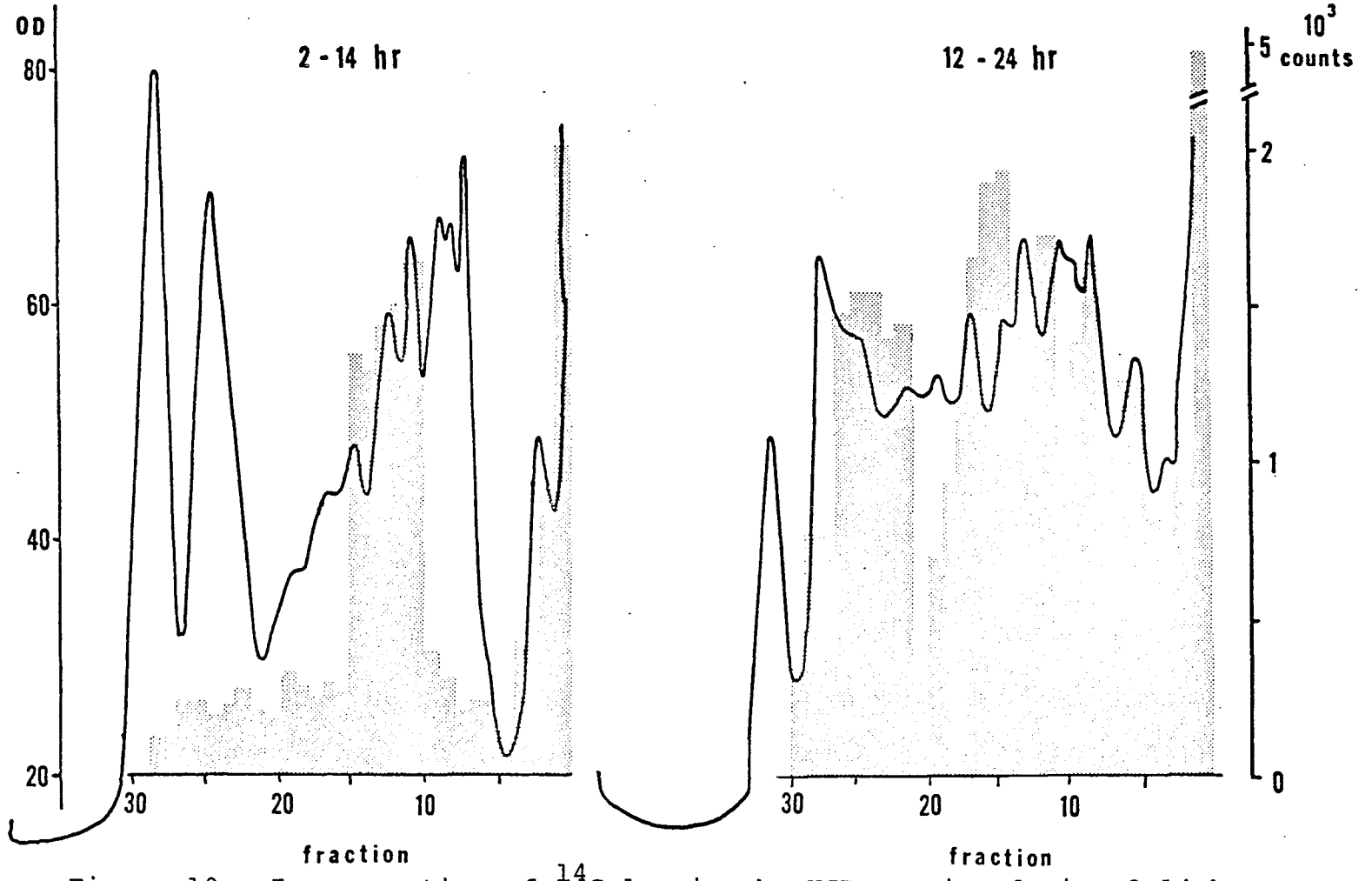

Figure 10. Incorporation of ^{14}C-leucine by USP species during 2-14 hr and 12-24 hr germination. -- Gels were sliced, beginning at origin with fraction 1, and slices were digested and counted. Solid line indicates gel scan; shading indicates counts in 50 min/slice. OD is percent full-scale absorbance. Applied to the 2-14 hr gel was 85 µg protein (13100 counts) and to the 12-24 hr gel, 169 µg protein (30200 counts).

Figure 11. Incorporation of ^{14}C-leucine by USP species during 24-36 hr and 36-48 hr germination. -- Gels were sliced, beginning at origin with fraction 1, and slices were digested and counted. Solid line indicates gel scan; shading indicates counts in 50 min/slice. OD is percent full-scale absorbance. Applied to the 24-36 hr gel was 139 µg protein (9000 counts) and to the 36-48 hr gel, 193 µg protein (7200 counts).

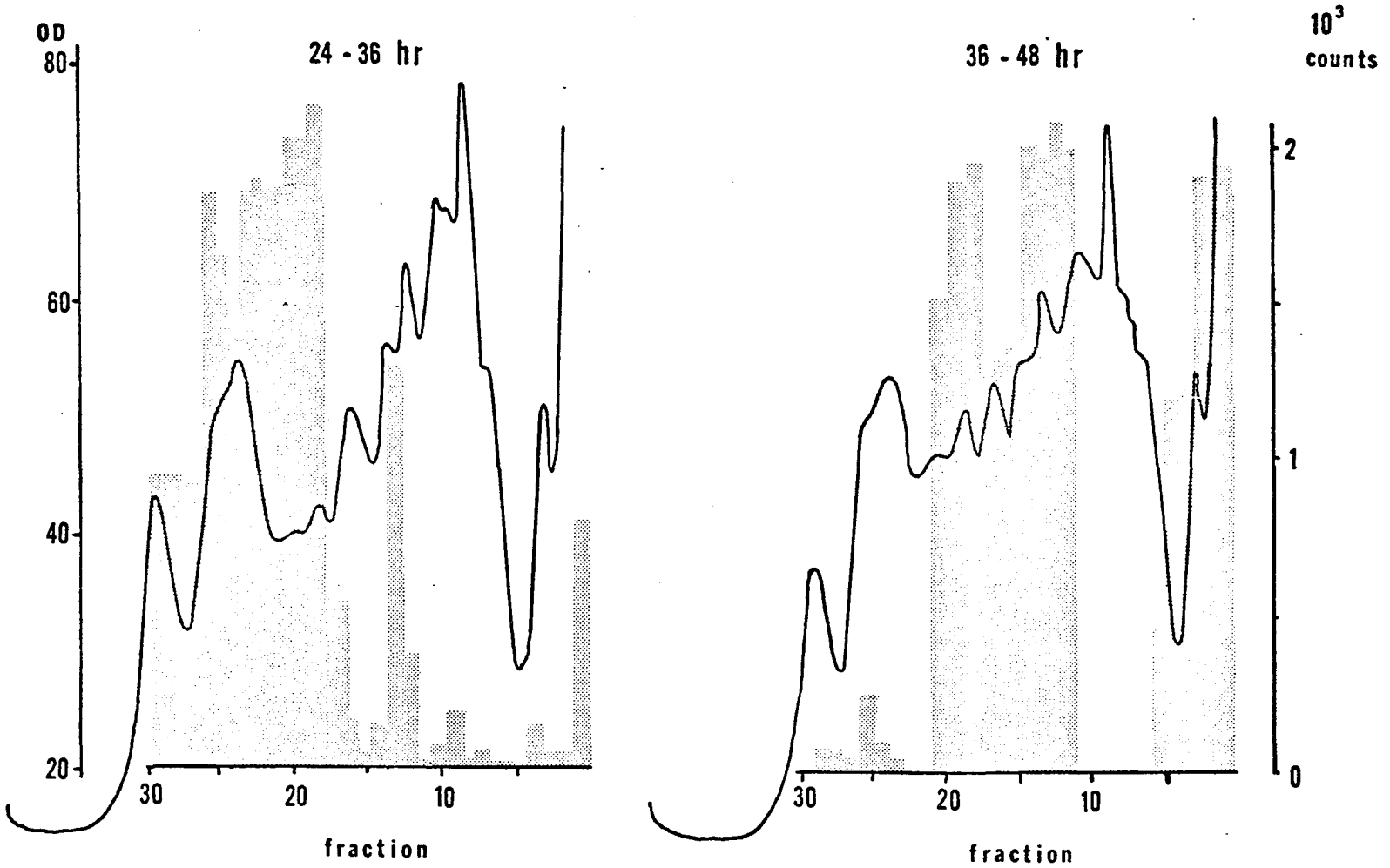

Figure 11. Incorporation of ^{14}C-leucine by USP species during 24-36 hr and 36-48 hr germination.

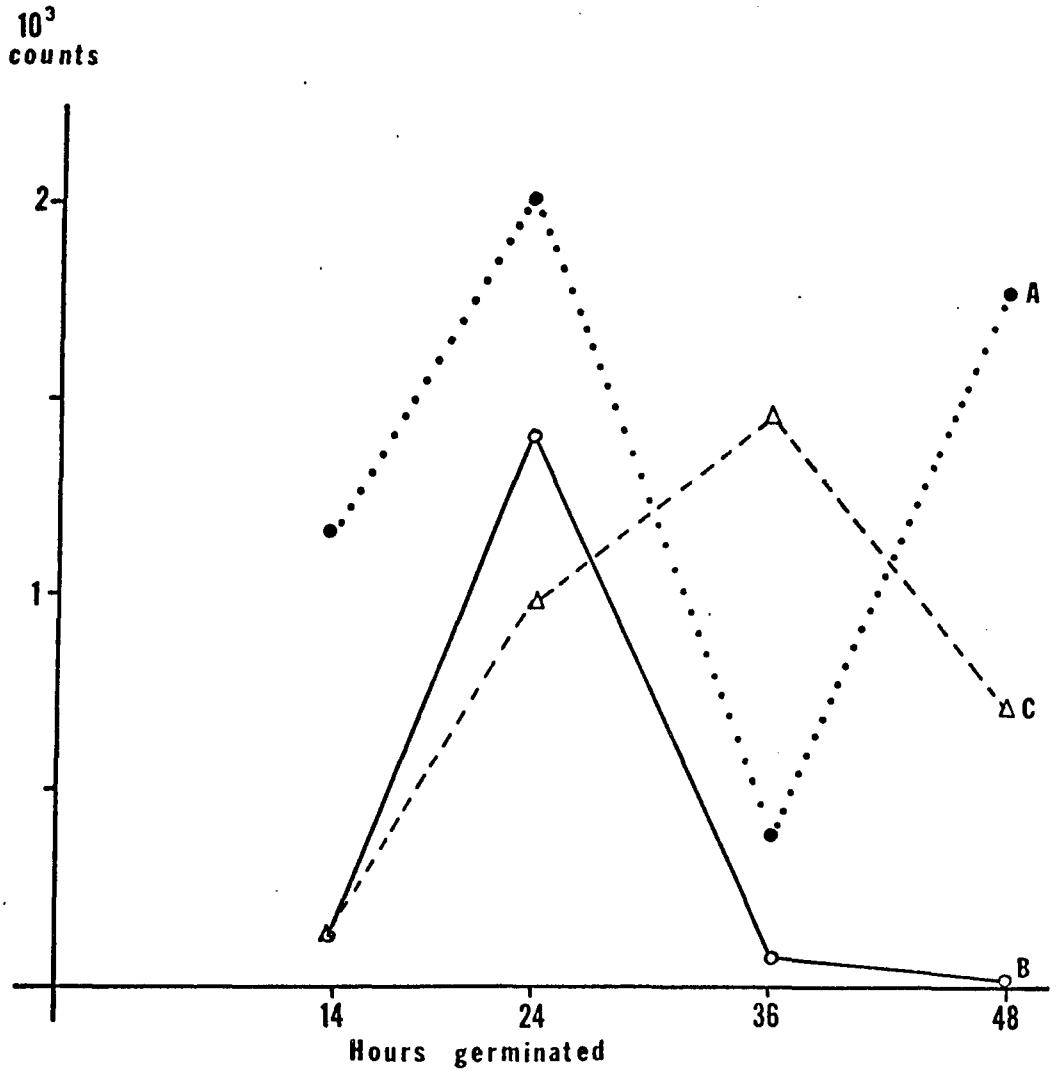

Figure 12. Labelling of USP species during various stages of germination. -- Mean labelling for each group of USP species having similar high or low ^{14}C-leucine incorporation during each stage of germination was calculated from data displayed in Figs. 10 and 11. Group A (dotted line) includes species 0, 1, 5, 6, 7, and 8. Group B (solid line) includes species 2, 3, and 4, and group C (dashed line) includes species 9, 10, 11, and 12. Average of counts observed are graphed vs. the endpoint of the labelling period.

RES species pictured in Fig. 9 migrate roughly to the same positions as USP species 2, 4, 7, 9, 10, 11 and 12. However the different distribution of counts in the RES gels as compared to USP gels would suggest that not all RES species are identical to USP species.

Synthesis of Individual HIS Species

Good agreement was obtained between ^{14}C-labelled and ^{3}H-labelled HIS replicates tested from 12-24 hr germination, as demonstrated in Fig. 13. However due to the extremely low specific activities in HIS species during other stages of germination, no agreement was seen between replicate gels at those times.

Table 7 compares the counts observed for each HIS species corrected to the area under each curve, for replicate gels at 12-24 hr germination. It has been hypothesized by some investigators, from work with other plant and animal systems, that two classes of histones exist. The first class, including histones f3 and f2a1 demonstrate turnover with or without DNA synthesis. The synthesis of the second class, which includes histones f1 f2a2 and f2b is thought to be dependent on DNA synthesis (Chalkley and Maurer 1965, Sadgopal and Bonner 1969). If this is true, then the high specific activity observed for HIS band a, presumably histone f1, implies that DNA synthesis in

Figure 13. Fractionation of replicate gels of HIS labelled from 12-24 hr germination. -- Individual bands were sliced from gels, digested and counted. Solid line indicates gel scan; shading indicates counts in 50 min/slice. Applied to the ^{14}C gel was 62 µg protein (6200 counts) and to the ^{3}H gel, 99 µg protein (8000 counts). Arrow indicates direction of migration.

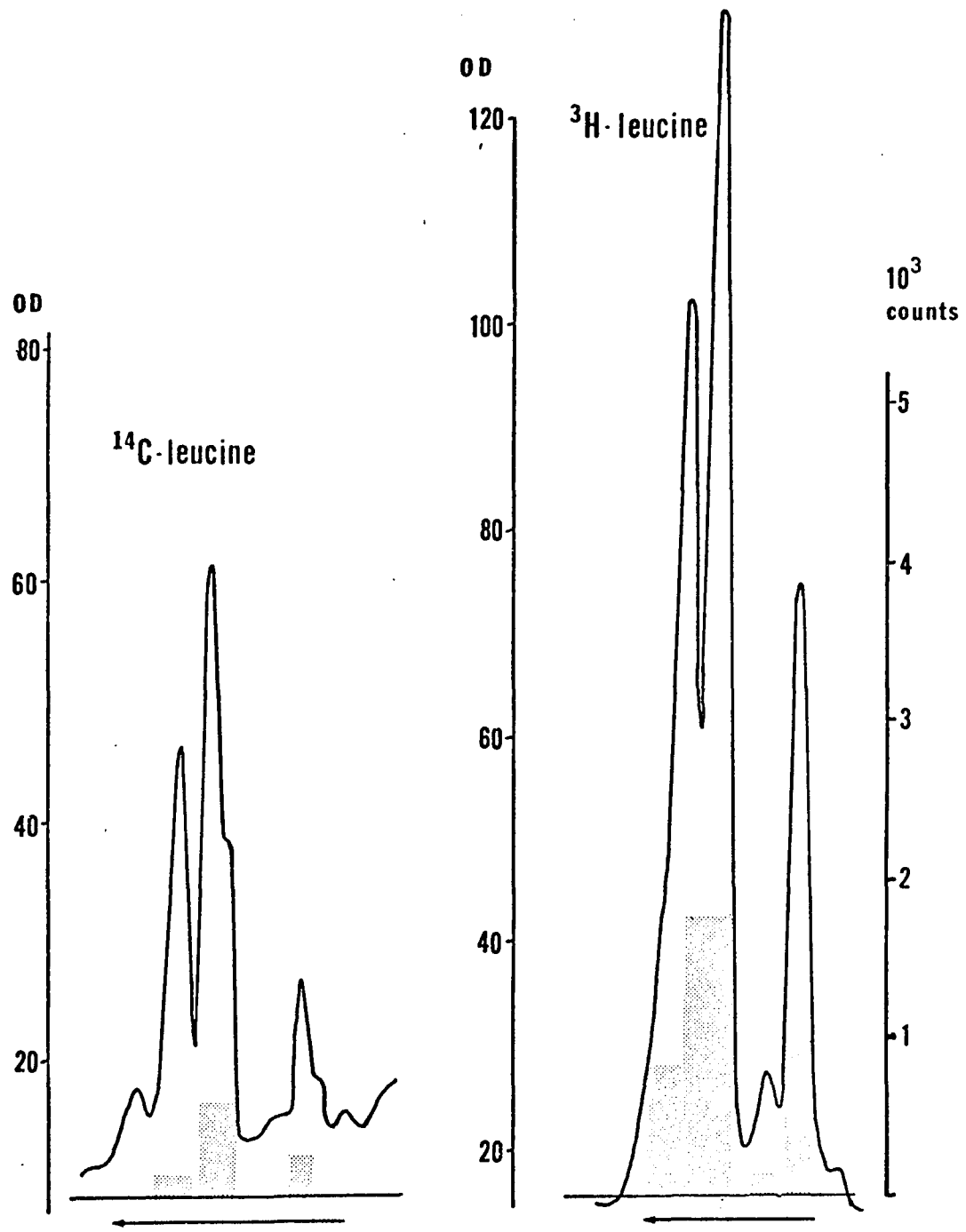

Figure 13. Fractionation of replicate gels of HIS labelled from 12-24 hr germination.

Table 7. Specific activities of HIS species during 12-24 hr germination. -- Bands were cut from gels, digested and counted and net counts were corrected to area under peak for each species.

	HIS Species		
Replicate	a+b (f1)	c+d (f2b+f3+f2a2)	e (f2a1)
^{3}H-leucine gel	301	262	172
^{14}C-leucine gel	471	302	83

excised epicotyls of V. faba does occur during the 12-24 hr germination stage. However, lack of synchrony in the tissue used does not permit further conclusions to be drawn.

It can be concluded from these experiments that, as expected, nonhistones have a higher turnover rate that histones, the radioisotope incorporation of each class of chromatin-associated protein shows a characteristic pattern with that of RES similar to that of USP but of higher specific activity, and finally that species of proteins within the various classes of chromatin-associated proteins each have their own characteristic synthesis pattern.

CHAPTER 6

RESPONSES OF CHROMATIN-ASSOCIATED PROTEINS TO GROWTH REGULATORS

For more than a half century, a central problem of plant physiology has been to discover the effects of plant growth regulators and the mechanisms by which they cause these effects. Despite the literally millions of man-hours devoted to this task, few answers have been found. Plant hormones appear to have ubiquitous effects and plant responses differ, often in diametrically opposite directions, with hormone concentration and with plant tissue and age. Plant hormones not only interact with one another, but some appear to demonstrate positive feedback on their own endogenous levels (reviewed by Scott 1972). Much research on hormone mechanisms has been performed with questionable methodology and it is not the exception to find even well-designed experiments, purporting to test the same mechanism, achieving very different results. The vast scope of plant growth regulator research is much too extensive to be thoroughly treated in this literature review and only the most pertinent studies have been selected for inclusion.

Review of Literature

The development of an organism is programmed, not only by the information contained within the genome, but by the features of its environment which in turn act upon the genome specifying which information is transmitted and when. Among such features which appear to act as controlling devices for higher plant genomes are the plant growth regulators: auxins, cytokinins and gibberellins. It has been suggested that the cytokinins and gibberellins dominate the early phases of plant development and auxins become dominant in later phases. Many investigators agree that the primary site of action of plant hormones is close to the gene (reviewed by van Overbeek 1966).

Auxins

The classical action of auxins on target cells is to cause cell elongation (reviewed by Ray 1969 and Cleland 1972). The initial action of auxin is thought to be on the mechanical properties of the cell wall but auxin-influenced metabolism, especially of nucleic acids, has further effects in plant development (reviewed by Scott 1972). In Vicia roots, auxin appears to stimulate reactivation and reorganization of a new meristem (Grant and Fuller 1971, Scott 1972). A popular hypothesis is that auxins act on growth by inducing specific protein synthesis via the promotion of transcription (reviewed by Key 1969 and Trewavas

1968b). A recent mechanism proposed for auxins is that the interaction of auxin with the plasma membrane may release a cytoplasmic factor that interacts with a specific RNA polymerase resulting in an altered transcriptional pattern (Hardin et al. 1972).

Cytokinins

Named for their promotion of cell division, cytokinins appear to have roles in such diverse processes as germination, flowering, organ formation, senesence, apical dominance and transport (reviewed by Helgeson 1968). They appear to normalize and stabilize growth and have a striking capacity to modify the action of other hormones (reviewed by van Overbeek 1966). Cytokinins are frequent constituents of tRNA, having a functional significance in transcription, although it is not known if free cytokinins serve as precursors to cytokinin ribonucleosides in tRNA (reviewed by Skoog and Armstrong 1970 and Key 1969). Like auxins, cytokinins appear to affect metabolism of both RNA and DNA (reviewed by Trewavas 1968b and Key 1969).

Gibberellins

Gibberellins are particularly known for their ability to overcome seed dormancy (reviewed by Wareing and Saunders 1971). Of all the plant hormones, they have perhaps the most defined mechanism. Many experiments have

led to the acceptance of the theory that gibberellins secreted by the plant embryo in certain cereals, activate aleurone cells to induce de novo synthesis and secretion of certain hydrolytic enzymes (reviewed by van Overbeek 1966, Trewavas 1968b and Key 1969). However, recent arguments have been advanced against the primary action of gibberellins as initiators of transcription, suggesting they function instead at a post-transcriptional level (Carlson 1972).

Responses of Nucleoproteins to Animal Hormones

Much progress has been recently realized on the effects of mammalian hormones on nucleoproteins. Stimulation of RNA synthesis in liver cells, followed by an increase in some liver enzymes suggested that hydrocortisone resulted in increased availability of genetic material for transcription (Dahmus and Bonner 1965). Hydrocortisone induction was associated with increased content of diffuse (active) chromatin in liver nuclei and corresponding decreased content of compact (repressed) chromatin (Arbuzova et al. 1968). Not only was increased incorporation of labelled amino acid into total nonhistone chromosomal proteins observed (Buck and Schauder 1970) but the specific synthesis of one PSNAP species was found to be a response to hydrocortisone (Shelton and Allfrey 1970). Most

experiments investigating the effects of hydrocortisone on histones have no responses (Hanoune and Feigelson 1969, Shelton and Allfrey 1970, Tsai and Hnilica 1971).

Other mammalian hormones have been found to induce similar effects in the synthesis of nucleoproteins: insulin has been shown to increase general nonhistone synthesis with no effect on histones (Buck and Schauder 1970); testosterone has been shown to induce both nonhistone and histone synthesis (Chung and Coffey 1971); and estradiol has been shown to stimulate label incorporation into one fraction of uterine (but not liver) nonhistones (Teng and Hamilton 1970) and into an acid-soluble nonhistone isolated from a complex with histone f3 (Barker 1971).

This phenomenon of hormone induction of nonhistone synthesis appears to be wide-spread throughout the biological spectrum as shown by the induced formation of a phenol-SDS-soluble nonhistone species (molecular weight 42,000) as a response to ecdysone (Helmsing and Berendes 1971).

Cyclic AMP

A widespread element in biochemical control mechanisms is cyclic AMP (reviewed by Jost and Rickenberg 1971). This molecule has been implicated as a mediator of hormone action for a variety of animal hormones and as a result of the massive research in this area, the second messenger hypothesis has been developed (reviewed by Rasmussen 1970

and Pastan and Perlman 1971). This hypothesis proposes that the hormone (the first messenger) is an external stimulus specific for a particular cell type, binding with a specific receptor present in the plasma membrane of target cells to activate a membrane-bound adenyl cyclase. The activated cyclase causes the generation of cyclic AMP (cAMP) on the inner surface of the membrane and the newly-synthesized cAMP diffuses through the cell to bring about responses. One known response to cAMP is kinase activation and a model for control of transcription via activation of a specific histone kinase has been developed by Langan (1971). Kinase activity has been found associated with some nonhistone chromosomal protein fractions (reviewed by Elgin et al. 1971).

Gibberellin and Cyclic AMP

Sufficient evidence has been accumulated to implicate cAMP as a second messenger for only one plant hormone, gibberellin. Lettuce hypocotyl elongation caused by cell-wall loosening has been demonstrated as a response to both gibberellin and cAMP (Kamisaka et al. 1972). Cyclic AMP can substitute as a trigger for gibberellin in barley to stimulate increased secretion of soluble sugar and amylase synthesis (Duffus and Duffus 1969, Pollard and Venere 1970, Pollard 1970 and Pollard 1971). Both cAMP and gibberellin induce similar total amounts and rates of release

of protease and acid phosphatase (Nickells, Schaefer and Galsky 1971). Caffeine and theophylline (cAMP phosphodiesterase inhibitors) promote the level of acid phosphatase attainable with gibberellin alone, and the induction of acid phosphatase by either cAMP or gibberellin is repressed by the same inhibitors. Since gibberellin is not needed during the lag period for induction, it is suggested that it possesses only the initial action of cAMP induction (Gilbert and Galsky 1972). A soluble adenyl cyclase has been identified in barley seeds which catalyzes the conversion of ATP to a cAMP analogue and the synthesis of this enzyme appears to be regulated by gibberellin and cAMP (Alveraz 1971). Gibberellin appears both to stimulate cAMP synthesis (Pollard 1970) and to repress cAMP degredation (Pollard 1971) and the promotion of ATPase release appears to follow a similar time course for both gibberellin and cAMP (Earle and Galsky 1971).

Because amylase induction by cAMP and gibberellin is inhibited by inhibitors of gibberellin biosynthesis, Kessler and Kaplan (1972) have proposed cAMP as a first messenger and gibberellin as the second messenger.

Synergism of cAMP and gibberellin (Galsky and Lippincott 1969) and different levels of enzyme production attainable (Pollard 1971) have suggested that gibberellin has other activities for which cAMP does not act as a second messenger.

Materials and Methods

Growth and Harvest of Plant Tissue

Excised epicotyls were used as described in Chapter 5. Batches of 200 epicotyls were germinated in sterile distilled water with penicillin for 12 hr at which time the water was replaced with either a 0.5 ml solution of growth regulator plus 30 μc ^{3}H-leucine or a blank solution plus 3 μc of ^{14}C-leucine. Each blank solution consisted of distilled water and ethanol at a concentration equivalent to that present in the corresponding growth-regulator sample.

For the second series of experiments, growth regulator solutions contained 3 μc ^{14}C-leucine and the blank solution contained 30 μc ^{3}H-leucine. For the control series, 3 μc ^{14}C-leucine in sterile distilled water was given to one batch of epicotyls and 30 μc ^{3}H-leucine in sterile distilled water was given to another batch. No growth regulators were applied to either control sample.

After 12 hr labelling, each sample was rinsed at least three times in distilled water, frozen and stored separately.

Chromatin Extraction and Fractionation and Electrophoresis

Prior to initial homogenization, the epicotyls from each set of growth regulator-plus-labelled-leucine and the

corresponding blank-plus-labelled-leucine were mixed. The extraction and fractionation of chromatin was performed exactly as described in Chapter 2 on the set of pooled epicotyls. Figure 14 illustrates the labelling and pooling of the set of samples for the first series of experiments.

Electrophoresis was performed as described in Chapter 2.

Growth Regulators

The following plant growth regulators were applied in the final concentrations listed: auxin (5×10^{-6}M indoleacetic acid, Calbiochem), kinetin (5×10^{-6}M 6-furfurylaminopurine, Calbiochem A grade), gibberellic acid (10^{-4}M gibberellin X, K salt, Calbiochem), cyclic AMP (5×10^{-5}M adenosine- 3', 5' cyclic phosphate, Schwartz Bioresearch). Auxin (IAA) and kinetin were initially dissolved in a small amount of 100% ethanol, and serially diluted to the final concentration which contained 1% (v/v) ethanol or less. Gibberellic acid (GA) and cyclic AMP (cAMP) were soluble in water.

Scintillation Counting and Analysis

Radioisotope incorporation into each class of chromatin-associated protein was measured for 200 µliter aliquots. In addition, 100 µliter aliquots of each initial cell homogenate were counted. For the second series of

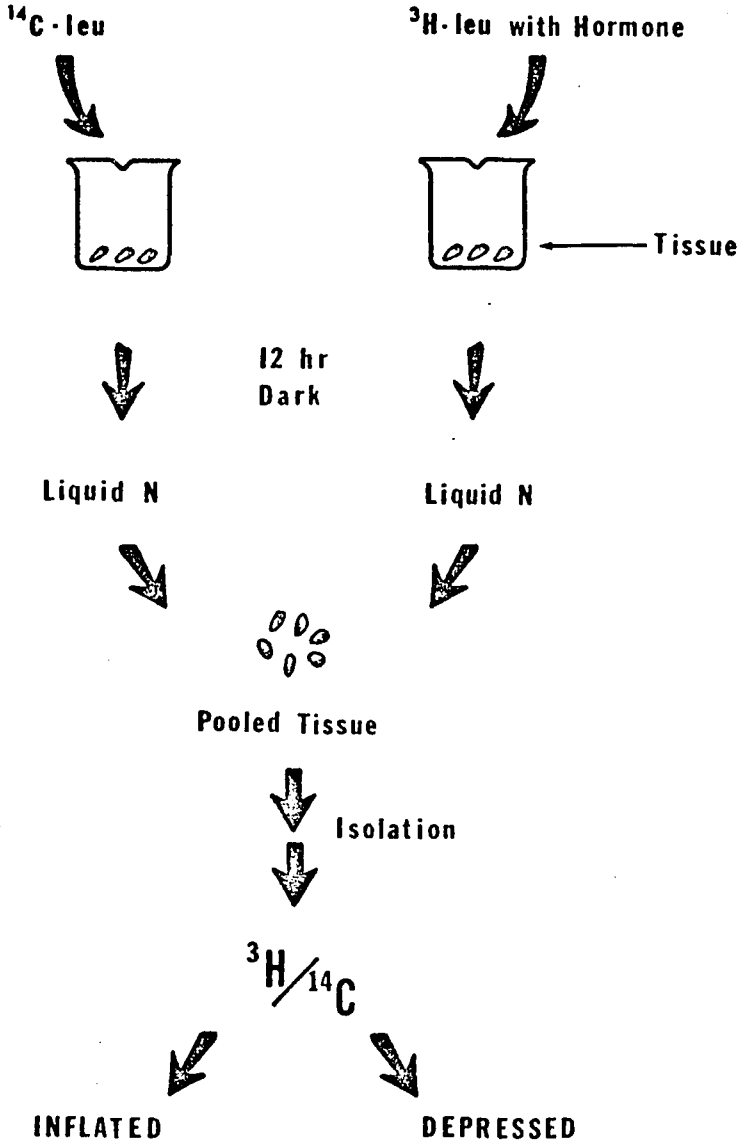

Figure 14. Experimental design to test for responses to plant hormones. -- Inflated ratio indicates stimulation of ^{3}H-leucine incorporation; Depressed ratio indicates repression of ^{3}H-leucine incorporation. For reciprocal labelling, interpretation of ^{3}H/^{14}C is opposite.

experiments, an aliquot of the supernatant recovered from the first centrifugation was precipitated with cold 10% (w/v) TCA, washed at least twice with cold 10% TCA and once with 95% ethanol, and counted in order to estimate radioisotope incorporation into soluble proteins.

Gels were sliced and digested for counting as described in Chapter 2.

Two channels of the Packard Tricarb liquid scintillation spectrophotometer were used simultaneously to detect the two radioisotopes. The ^{3}H channel was set for 100% gain with a 50-450 window and the ^{14}C channel was set for 8.5% gain with a 200-1000 window. At these settings, the efficiency of ^{3}H detection was approximately 18% and the efficiency of ^{14}C was approximately 39% when 1.0 μc ^{3}H-leucine plus 0.1 μc ^{14}C-leucine were counted simultaneously in the absence of protein. The cross-talk factor for ^{14}C in the ^{3}H channel ranged from 10-15%. There was less than 1% cross-talk of ^{3}H into the ^{14}C channel and this was considered to be zero. Tritium counts were determined as net counts minus ^{14}C counts in the tritium channel and ^{14}C counts were determined as net counts in the ^{14}C channel plus ^{14}C counts in the tritium channel. Both ^{3}H and ^{14}C counts were corrected to 200 mg dry weight of epicotyls before calculation of $^{3}H/^{14}C$ ratios for total proteins. This correction to dry weight was not done for gel analysis.

For all experiments $^{3}H/^{14}C$ ratios were observed, not $^{14}C/^{3}H$ ratios.

With the addition of protein to the vials, many problems with the analysis of the double-labelled samples became apparent. These problems and their resolution are discussed in Appendix C.

Results and Discussion

Responses of USP, RES and HIS to Growth Regulators

The values of the $^{3}H/^{14}C$ ratios calculated for each class of chromatin-associated protein in response to the various growth regulators are listed in Table 8. For the first series of experiments (1) ^{3}H-leucine was added with the growth regulator and for the second series (2) ^{14}C-leucine was added with the growth regulator. The S-values (discussed in Appendix C) are listed in this table for each experiment. Significance, defined for these experiments in the following paragraph, is not intended in the usual statistical sense. The S-values which appear to be significantly greater than 1.0 (indicating stimulation of radioisotope incorporation as a response to the growth regulator) or significantly less than 1.0 (indicating repression of radioisotope incorporation) are marked with an asterisk. Those S-values not significantly different from 1.0 indicate no response to the growth regulator.

Table 8. Responses of chromatin-associated proteins to growth regulators. -- $^{3}H/^{14}C$ ratios: (1) - ^{3}H-leucine added with growth regulator. (2) - ^{14}C-leucine added with growth regulator. For computation of S-values see Appendix C. S-values appearing significantly different from 1.0 are indicated by *.

Growth Regulator	Chromatin-associated Protein		
	USP	HIS	RES
IAA			
(1)	3.789	1.900	3.029
(2)	2.524	1.924	1.456
S-value	1.288*	0.996	1.470*
Kinetin			
(1)	2.808	2.256	1.609
(2)	2.700	1.782	1.679
S-value	1.036	1.192*	1.075
GA			
(1)	2.711	1.656	2.058
(2)	2.810	3.358	2.219
S-value	0.997	0.711*	0.978
cAMP			
(1)	2.020	1.713	1.484
(2)	2.558	2.621	1.942
S-value	0.949	0.810*	0.948
none (control)			
$^{3}H/^{14}C$	3.283	2.030	2.550

Although the experiments reported here were not designed to distinguish between pool effects, nuclear membrane permeability effects, and synthesis, radioisotope incorporation is presumably a measure of synthesis and the two terms will be used more or less interchangeably, with the thought in mind that this one-to-one correspondence has not been proven.

Figure 15 shows the distribution of S-values around 1.0 for the data recorded in Table 8. The majority of S-values are clustered around 1.0 (between 0.9 and 1.1). The mean of the clustered S-values is 0.997 and the range of this mean ± two standard deviations for the clustered values is indicated on Figure 15. S-values falling outside of this range are deemed to be significantly different from 1.0 and have been set off by asterisks in Table 8.

Synthesis of USP appears to be stimulated by IAA and is unaffected by other growth regulators. HIS synthesis appears to be repressed by both GA and cAMP and stimulated by kinetin but unaffected by IAA. If RES represent USP that are tightly bound to DNA, as suggested in previous chapters, it is expected that RES and USP would show similar responses to growth regulators. This appears to be the case as RES synthesis appears stimulated by IAA and unaffected by the other growth regulators.

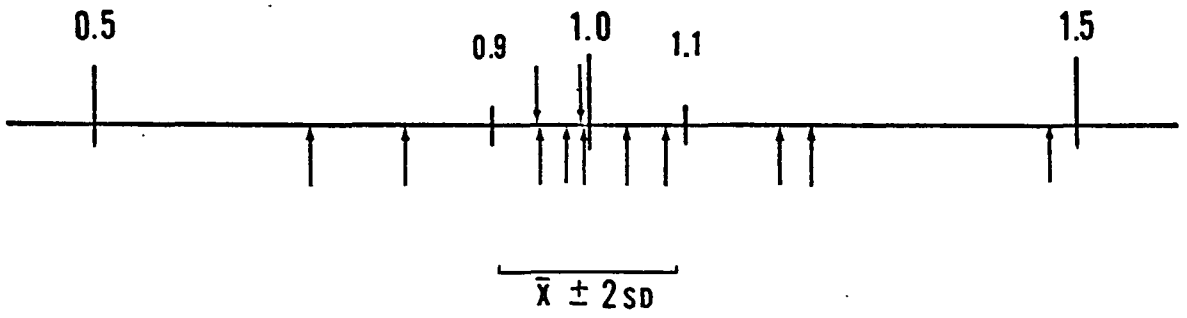

Figure 15. Distribution of S-values around 1.0. -- S-values from Table 8 are indicated by arrows showing position of value with respect to 1.0. Mean of S-values clustered between 0.9 and 1.1 is 0.997; range of this mean ± two standard deviations of clustered S-values is enclosed by bracket below line. S-values outside of the bracketed area are deemed significantly different from 1.0.

In order to show that the effect of growth regulators was not an effect of cell permeability to leucine, incorporation of labelled-leucine into the total cell homogenate was measured. These results are presented in Table 9. The S-values outside of the significance range in Figure 15 are marked with an asterisk in this table. The interpretation is that kinetin and IAA have no effect on the permeability of the cell to leucine, but both GA and cAMP appear to repress leucine permeability slightly. This lowered permeability, in part, may be responsible for the decreased leucine incorporation into HIS in response to GA and cAMP. However, the degree to which leucine permeability is repressed is less than the degree to which leucine incorporation is repressed. Also, there appears to be no effect on incorporation of leucine into USP or RES in response to GA or cAMP.

Effects of Growth Regulators on Soluble Protein Synthesis

Data regarding the effect of growth regulators on leucine incorporation into total soluble proteins is presented in Table 10. Since this was measured only for the second series of experiments in which ^{14}C-leucine was added with the growth regulator, no S-values can be calculated. However, the high $^{3}H/^{14}C$ ratio observed for the cAMP-treated set is suggestive of repression of total protein

Table 9. The effect of growth regulators on cell permeability to leucine. -- $^{3}H/^{14}C$ ratios: (1) ^{3}H-leucine added with growth regulator; (2) ^{14}C-leucine added with growth regulator. For computation of S-values see Appendix C. S-values significantly different from 1.0 are indicated by *.

Growth Regulator	Cell Homogenate
IAA	
(1)	0.741
(2)	0.927
S-value	0.905
Kinetin	
(1)	0.784
(2)	0.927
S-value	0.927
GA	
(1)	0.709
(2)	1.019
S-value	0.842*
cAMP	
(1)	0.742
(2)	1.017
S-value	0.860*
none (control)	
$^{3}H/^{14}C$	0.969

Table 10. The effect of growth regulators on soluble protein synthesis. -- $^{3}H/^{14}C$ ratio was calculated only for series in which ^{14}C-leucine was added with growth regulator.

Growth Regulator	$^{3}H/^{14}C$
IAA	1.973
Kinetin	1.395
GA	1.747
cAMP	2.531
none (control)	1.442

synthesis. This phenomenon is very interesting in light of the reports that exogenous cAMP inhibits growth of human fibroblasts in culture (Froehlich and Rachmeler 1972) and that low endogenous cAMP levels are characteristic of transformed cell cultures (Sheppard 1972). The data from this experiment suggest that in higher plants, as well as in mammals, cAMP may function, in part, as a regulator of cell growth. It is interesting that in this experiment GA does not appear to have the same effect as cAMP.

Responses of Specific USP and RES to IAA

In Figs. 16 and 17 are shown the results obtained from counting USP and RES gels of IAA-treated samples. RES bands appear to have approximately the same molecular weight as USP species 2, 9, 11, and 12 on the basis of mobility. A skewed scale is used to graph $^{3}H/^{14}C$ ratios since the degree of $^{3}H/^{14}C$ elevation for experiments in which ^{3}H was added with the growth regulator is always greater than the degree of $^{3}H/^{14}C$ repression for reciprocal experiments. Since the device used for slicing gels did not always give exactly even fractions, the $^{3}H/^{14}C$ ratios for reciprocally labelled gels may not line up with one another at all points. From these data, it appears that IAA stimulates nearly all USP species. Species 6, 10 and possibly 12 are disproportionately stimulated as indicated by disproportionately high $^{3}H/^{14}C$ ratios for experiments in which ^{3}H-leucine was added with the growth regulator, and low $^{3}H/^{14}C$ ratios for reciprocal experiments.

USP species 8, and 9 appear to have repressed synthesis from this diagram. As discussed in Chapter 5, all USP species except 9, 11 and 12 have relatively high rates of synthesis during 12-24 hours germination. Species 9 is of particular interest since this species appears to respond to IAA with repressed synthesis when considered with USP and with stimulated synthesis when considered with RES.

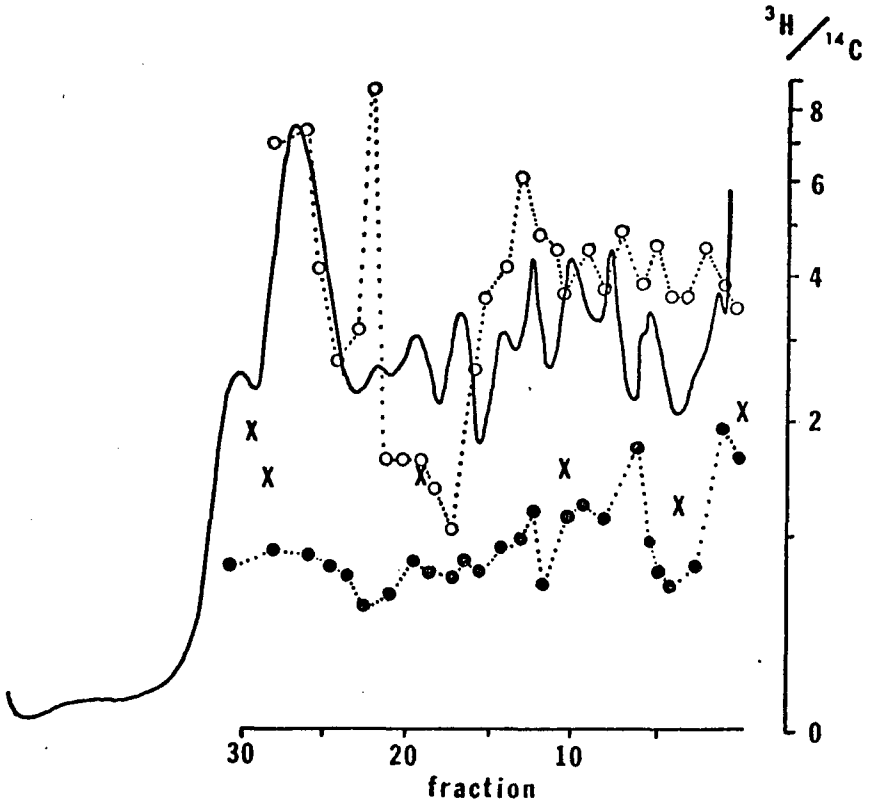

Figure 16. Responses of USP species to IAA. -- Gels were sliced, beginning at origin with fraction 1, and slices were digested and counted. Solid line indicates gel scan. Upper dotted line (open circles) represents $^3H/^{14}C$ for experiment in which ^3H-leucine was added with growth regulator (1). Lower dotted line (closed circles) represents $^3H/^{14}C$ for experiment in which ^{14}C-leucine was added with growth regulator. X indicates $^3H/^{14}C$ from control gel for which no growth regulator was added.

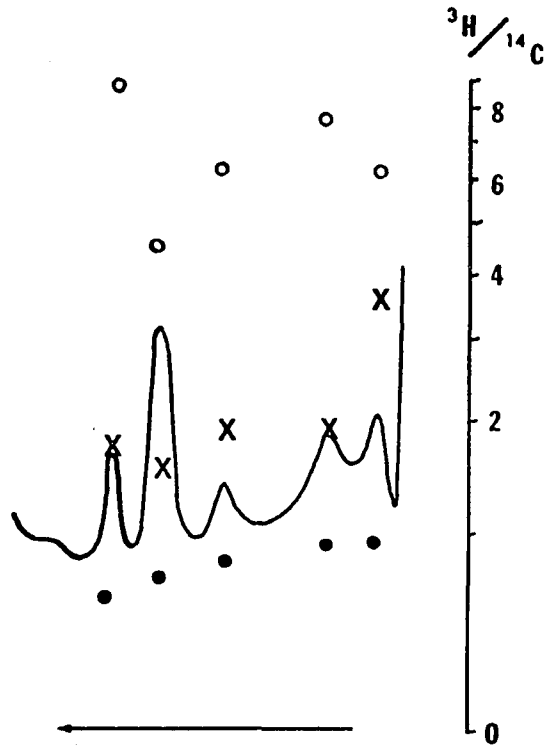

Figure 17. Responses of RES species to IAA. -- Individual bands were sliced from gels, digested and counted. Solid line indicates gel scan. Open circles represent $^3H/^{14}C$ for experiment in which ^3H-leucine was added with growth regulator (1). Closed circles represent $^3H/^{14}C$ for experiment in which ^{14}C-leucine was added with growth regulator (2). X indicates $^3H/^{14}C$ from control gel for which no growth regulator was added. Arrow indicates direction of migration.

This may be further evidence that the USP and RES species banding at the same position on gels are different proteins of similar molecular weight, or it may be an artifact due to uneven gel slicing of USP. The responses of USP species 6 and 10 are the same sort of responses that have been found for rat PSNAP to cortisol and glucagon. The molecular weight of the PSNAP demonstrating stimulated synthesis in response to cortisol has been estimated at 41,000 (Shelton and Allfrey 1970) and the molecular weights of PSNAP species demonstrating stimulated synthesis in response to glucagon have been estimated at 60,000 and 8,000 daltons (Enea and Allfrey 1973). The molecular weight of USP species 6 is believed to be somewhat less than 67,000 daltons since bovine serum albumin of that molecular weight bands at approximately the same position as USP species 4.

The responses of individual HIS species to growth regulators were not discernible since the counts were too low to give consistent replication, even with extended counting periods.

Conclusions Regarding the Mechanism of Action of Growth Regulators

Although auxin, kinetin and gibberellin appear to share the effect of stimulating DNA and RNA synthesis (reviewed by Trewavas 1968b, Key 1969), it is evident that they have different effects on nucleoprotein metabolism. The

data presented in the experiments reported here are not meant to infer relationships between effects of growth regulators on nucleoproteins and the alteration of template activity; rather, they are used to establish whether growth regulators have separate and measurable effects on nucleoprotein metabolism comparable to the effects demonstrated with mammalian hormones. This has been demonstrated.

Contrary to other reports (Trewavas 1968a, Kuraishi, Uematsu and Yamaki 1967), IAA did not appear to increase general protein synthesis. This is in agreement with Datta and Sen (1967). Neither did GA increase total protein synthesis, although it is known to induce synthesis of some enzymes (reviewed by Key 1969 and van Overbeek 1966). Although cAMP may indeed function as a second messenger for some GA effects, it evidently has effects of its own on cell growth separate from those of GA as indicated by the suggested repression of total protein synthesis. Kinetin has been implicated in the inhibition of proteolysis in excised tissue rather than in the promotion of general protein synthesis (Shibaoka and Thimann 1970) and data presented here is not contradictory to this hypothesis.

Inferences that alteration of template activity is due to a difference in the chromatin of auxin-treated (O'Brien et al. 1968) and GA-treated (Fukasawa and Konno 1972) plants have been supported by these experiments.

Datta and Sen (1965) and Patterson and Trewavas (1967) noted changes in labelled-amino acid incorporation into cellular fractions believed to include nuclear material in response to IAA. Sarkissian and Spelsberg (1967) noted induction of a base-soluble nonhistone band on electrophoretic gels in response to IAA treatment of Phaseolus hook. The careful methodology applied in my experiments shows these effects to be real; IAA does indeed increase radioisotope incorporation specifically into nonhistone proteins and certain nonhistone species are specifically affected to a greater degree than others. Fellenberg (1969) suggests that by binding, IAA alters nonhistones causing them to act as histone antagonists. Nonhistones are known to reverse histone induced inhibition of RNA synthesis (Marushige, Brutlag and Bonner 1968, Wang 1969, Spelsberg and Hnilica 1969). The synthesis of nonhistones induced by IAA may serve simply to increase the concentration of non-specific derepressors or it may serve to induce the synthesis of specific regulatory nonhistones.

Kinetin has been found to alter the electrophoretic mobility of one nonhistone species from Phaseolus hypocotyl and to reduce the level of nonhistones in both hook and hypocotyl. In Phaseolus hypocotyl, histone levels were apparently reduced (Spelsberg and Sarkissian 1970a). Datta and Sen (1965) observed stimulation of labelled-amino acid

incorporation into total nuclear protein with kinetin treatment. Cytochemical studies on onion root (Piesco and Alvarez 1972) have demonstrated an increased arg/lys ratio in kinetin-stimulated cells and increased Feulgen binding suggested to these workers that kinetin may alter DNA-protein relationships. Experiments presented in this chapter suggest increased histone synthesis is a response to kinetin; however, this may be a result of increased DNA synthesis since cytokinins are known for their effects on the synthesis of DNA.

Stimulation of labelled amino-acid incorporation by gibberellin has been observed for total nuclear protein, but nuclear proteins were not fractionated (Datta and Sen 1965).

Gibberellin-treated dwarf pea plants do not demonstrate altered histone/DNA ratios and it has been suggested that it is unlikely that gibberellin modifies histone synthesis since the large effect of gibberellins on RNA metabolism would lead one to expect more than a subtle change of histone quantity (Spiker and Chalkley 1971). However, the data presented here suggest that GA does repress histone synthesis, but a causal relationship between this suppression and resultant increases in RNA synthesis has not been established.

cAMP has been implicated as a second messenger for IAA (Salomon and Mascarenhas 1971, 1972; Wood and Braun

1973) and as a third messenger for cytokinin, which is believed to induce cytokinesin as a second messenger (Wood, Lin and Braun 1972, Wood and Braun 1973). It has also been suggested that the stimulatory and inhibitory effects of cytokinin on the growth of human lymphocytes may involve cAMP metabolism (Gallo et al. 1972). No evidence was found in my studies suggesting cAMP could serve as a second messenger for IAA or kinetin effects on the metabolism of chromatin-associated proteins.

In agreement with the overwhelming evidence implicating cAMP as a second messenger for some GA effects, as discussed in the literature review for this chapter, the interpretation of the data presented here is that HIS synthesis is repressed in response to cAMP and in response to GA. As found in other experiments comparing the effects of GA and cAMP (Kamisaka et al. 1972, Pollard 1971, Duffus and Duffus 1969), the response to cAMP was not to the same degree as the response to GA, even though cAMP was present at a higher concentration than GA. Repression of HIS synthesis was less with cAMP than with GA.

Finally it can be concluded that at least one primary action of the growth regulators examined here appears to be at the level of nucleoprotein metabolism.

CHAPTER 7

CONCLUSIONS

This dissertation has defined a method of fractionating chromatin-associated proteins into histones and nonhistones for higher plants. Nonhistones are fractionated into two classes whose relationship is not clear. The two classes may represent different protein species of similar molecular weights or they may represent two populations of identical proteins, binding to DNA with different tenacities. Evidence is presented which favors both interpretations.

The fractionation method developed for this work is believed to be superior to methods formerly used because it has the following advantages:

1. The method avoids treatment of nonhistones with acid or other harsh reagents prior to their extraction.

2. The method extracts a greater proportion of chromatin-associated proteins than former methods.

3. There is no cross-contamination of nonhistones into histone fractions.

4. With the exception of one nonhistone fraction, results are highly reproducible.

5. The method is rapid and simple to perform.

Data presented in this dissertation implicates chromatin-associated proteins, particularly nonhistones, as regulators of gene activity during development. The inference is based on the following evidence:

1. Although nonhistone/DNA ratios are not correlated with any one feature of various tissues, some types of tissue appear to have characteristic nonhistone/DNA ratios. HIS/DNA ratios appear constant for every tissue but an elevated proportion of the species presumed to be histone f1 is observed in root meristem and leaf tissues, compared with lateral root and stem tissues; and in cotyledons at 48 hr germination, compared with the same tissue examined at other stages.

2. Histones are qualitatively identical in every tissue examined as judged by SDS gel electrophoresis banding patterns. However some USP species vary in different tissues whereas some are invariant. The development of one USP species (no. 13) may be age-related. RES species are highly variable, not only from tissue-to-tissue and age-to-age, but from extraction-to-extraction.

3. Nonhistones have significantly higher specific activities than histones and nonhistones more tightly bound to DNA exhibit the highest specific activities. In excised epicotyls, peak synthesis for all chromatin-associated proteins occurred during the period of 12-24 hr

germination. Accumulation of nonhistones was evident by 36-48 hr germination. Individual species of chromatin-associated proteins exhibited characteristic patterns of synthesis.

4. Chromatin-associated proteins appear to exhibit separate and distinctive responses to various plant growth regulators. In response to auxin, stimulation of labelled-leucine into USP and RES is observed. Stimulated leucine incorporation is a response of HIS to kinetin and repressed leucine incorporation is a response of HIS to gibberellic acid and cAMP. In addition, gibberellin and cAMP appear to repress cell permeability to leucine although only cAMP appears to repress incorporation of leucine into total cellular proteins. It is inferred that cAMP may be a second messenger for gibberellin effects on histone synthesis.

5. Not all USP species respond identically to auxin. Individual species exhibit distinctive degrees of increased or repressed labelled-leucine incorporation.

APPENDIX A

ANALYSIS OF GELS FOR TISSUE SPECIFICITY

The null hypothesis was that all bands would be present at the same position for every tissue. A Type I Error, which for this analysis would result in finding tissue specificity when in fact none existed, is considered more undesirable than a Type II Error, or overlooking tissue specificity which indeed exists. Gel analysis was performed by a method designed to minimize Type I Errors.

The "best" (cleanest) USP gel scan, usually from the gel with the greatest amount of protein applied was chosen for each tissue and traced onto tracing paper. This was compared to other scans from the same tissue and any peaks indicating additional protein species which were present on another scan and either not present, or present only as a shoulder on the best scan, were added to the best scan. The resulting scan was considered the best composite gel scan (BCT) for that tissue. An example of the derivation of the BCT for the root meristem is presented in Fig. A-1.

The BCT was then overlaid on scans of excised epicotyl gels from synthesis experiments until a scan of the same length was found. The same-length excised epicotyl

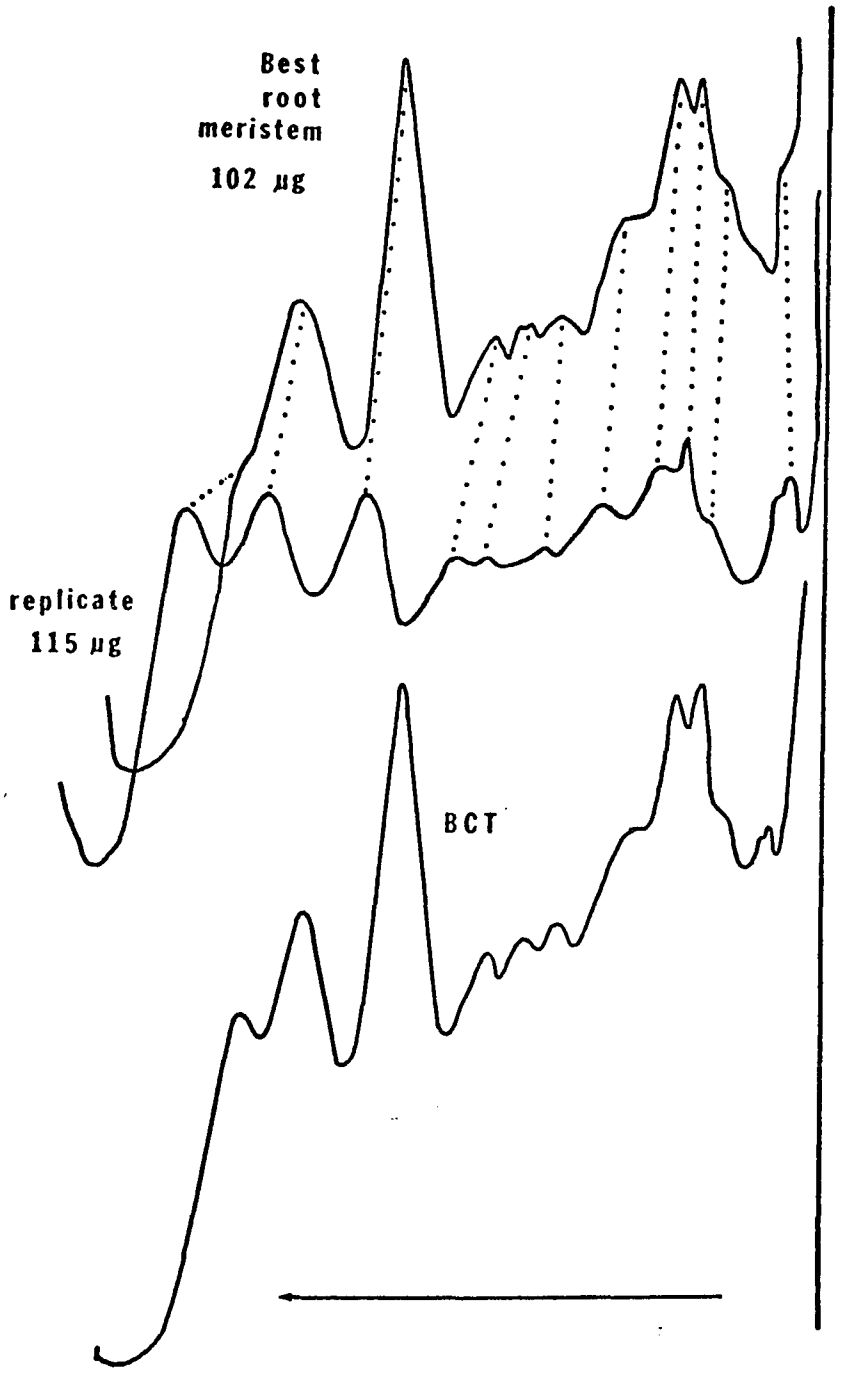

Figure A-1. Derivation of BCT for root meristem USP. -- Dotted lines connect peaks for same USP species on replicate gels. Arrow indicates direction of migration.

gel scan was traced over the BCT on the same piece of tracing paper so that band positions could be compared. The excised epicotyl gel scan most nearly matching the BCT with respect to amount of protein applied to the gel was used as a guide to adjust the height and shape of peaks of the same-length epicotyl gel scan if necessary to demonstrate the similarities between the two scans. Next, the excised epicotyl gel scan most similar in appearance to the BCT regardless of length or amount of protein applied, was used to further adjust the peak height and shape of the excised epicotyl gel scan in order to emphasize its similarity to the BCT. The excised epicotyl gel scans used during this process for root meristem are illustrated in Fig. A-2. The resultant comparison of the BCT and the best composite excised epicotyl gel scan (BCE) is shown in Fig. A-3. The final graph was used to compare the scans on a peak-by-peak basis to determine whether or not all bands were present and at the same position on each. A representation of the gels (pictured below the graph in Fig. A-3) was drawn indicating the position of each band. Although the area under each peak (amount of each species) was not generally taken into account for this analysis, the darkness of the band is roughly indicated on the drawings of the gels. The final illustrations of the gels were then compared both to photographs of gels and to the

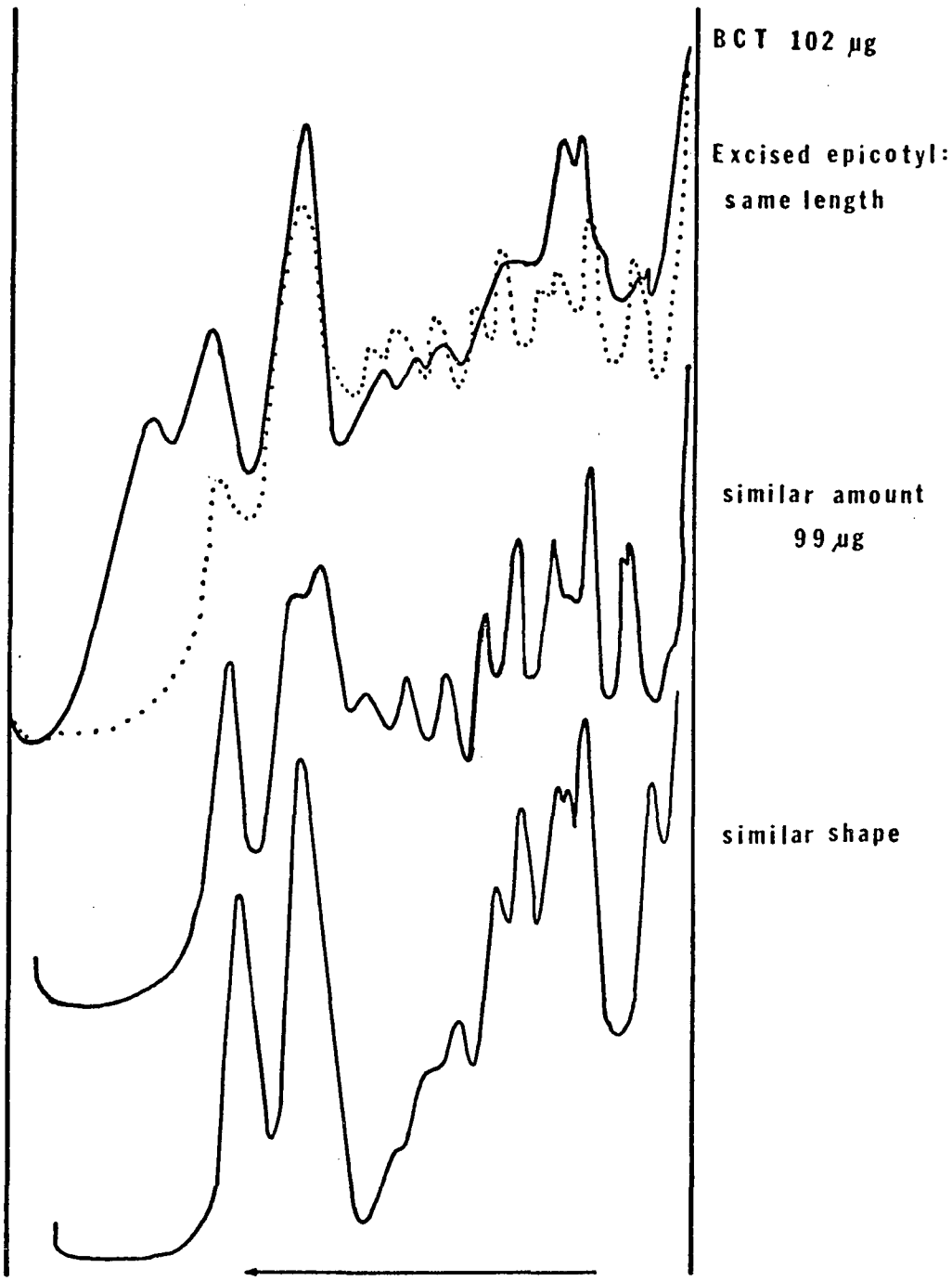

Figure A-2. Excised epicotyl gel scans for derivation of BCE to compare with root meristem BCT. -- Dotted line indicates excised epicotyl gel scan of same length as BCT. Arrow indicates direction of migration.

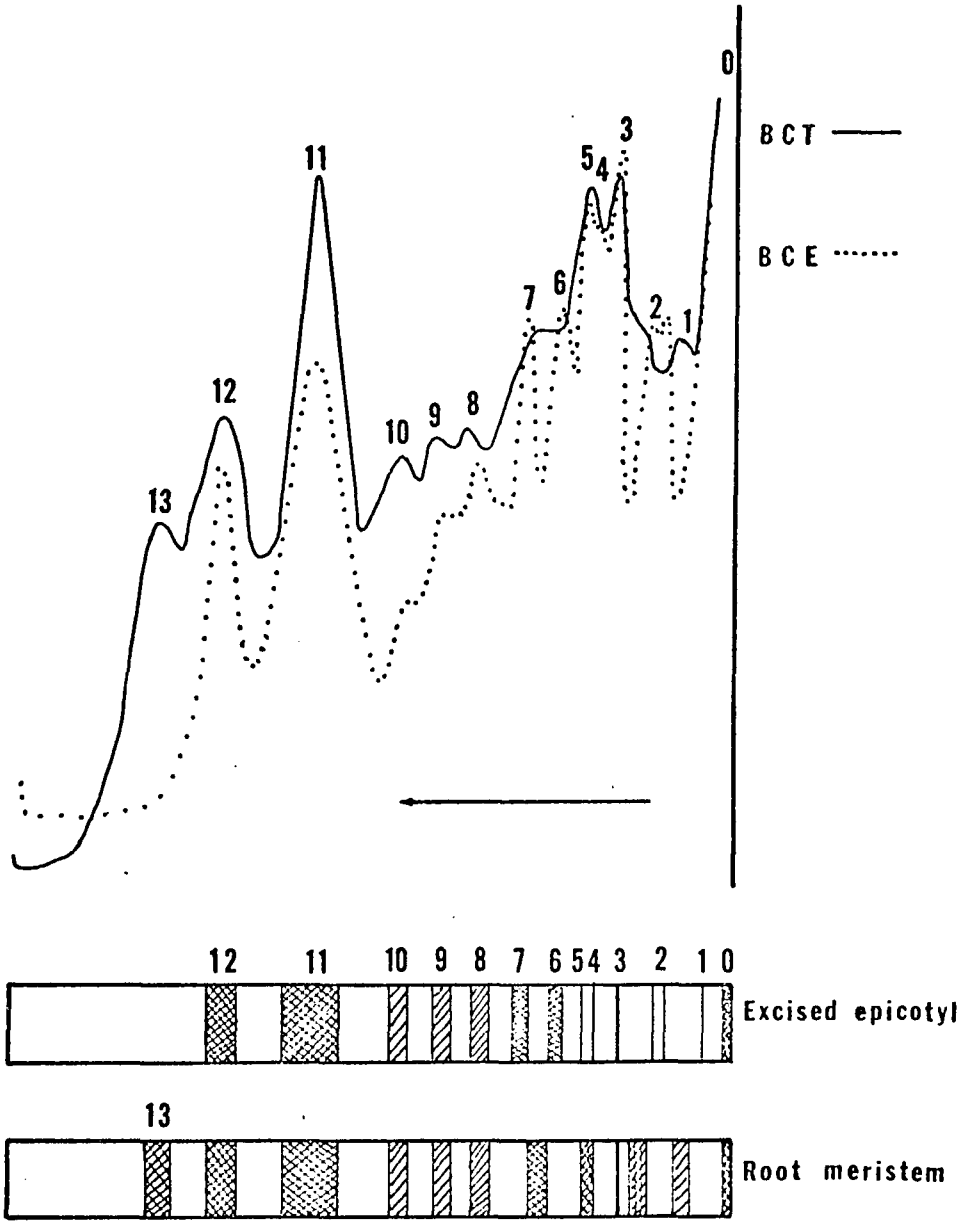

Figure A-3. Comparison of BCT and BCE for root meristem USP. -- Species numbered in gel drawings correspond to species numbered in gel scans. Arrow indicates direction of migration.

gels themselves to determine if the drawing was a correct representation of the gel. For every tissue examined, this was the case.

Figures A-4 through A-7 demonstrate the final composite gel scans BCT and BCE from which drawings shown in Chapter 4 were derived for leaf, mature root tissue, stem and cotyledons.

Figures A-8 and A-9 illustrate comparative gel scans from which drawings shown in Chapter 3 were derived comparing USP and acid-extracted proteins and comparing acid-extracted proteins and acid-extract after urea (HIS). Figure A-10 illustrates comparative gel scans for RES vs. USP.

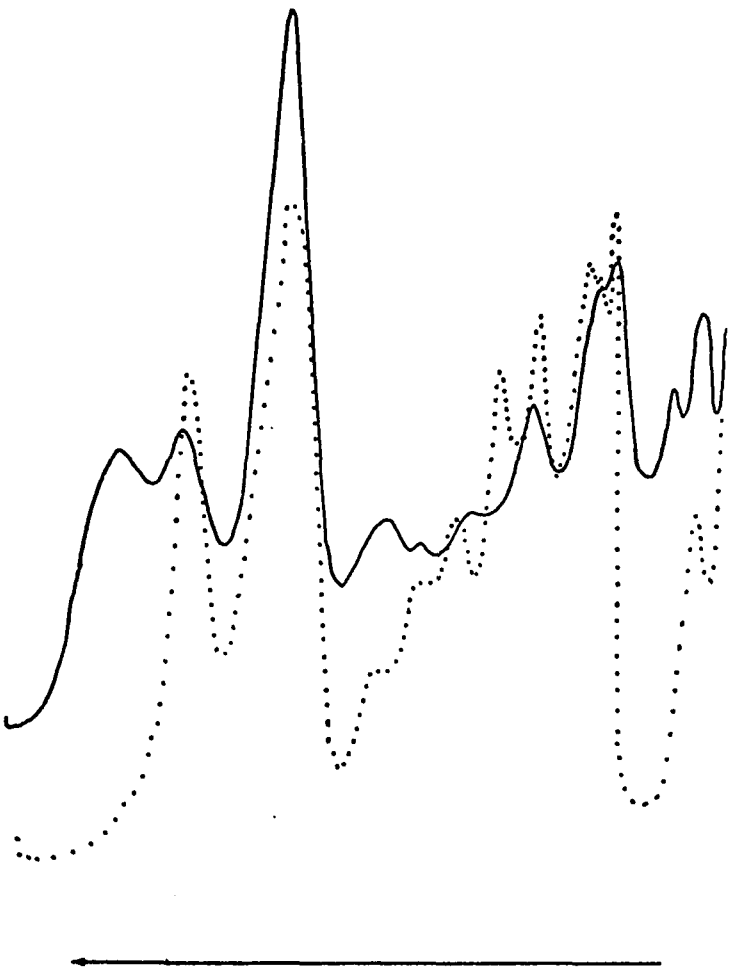

Figure A-4. Comparison of BCT and BCE for leaf USP. -- Solid line indicates BCT; dotted line indicates BCE. Arrow indicates direction of migration.

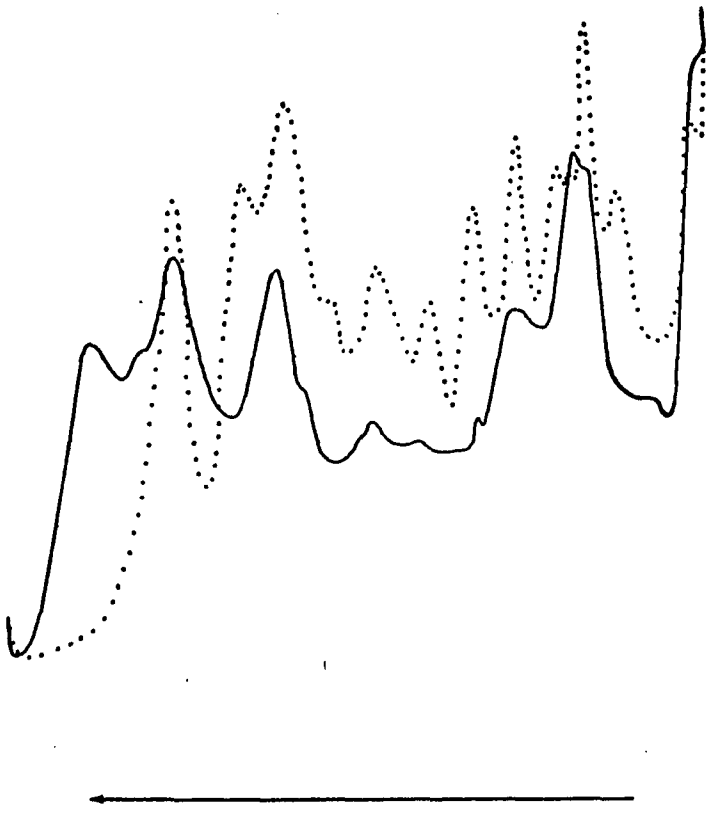

Figure A-5. Comparison of BCT and BCE for mature lateral root USP. -- Solid line indicates BCT; dotted line indicates BCE. Arrow indicates direction of migration.

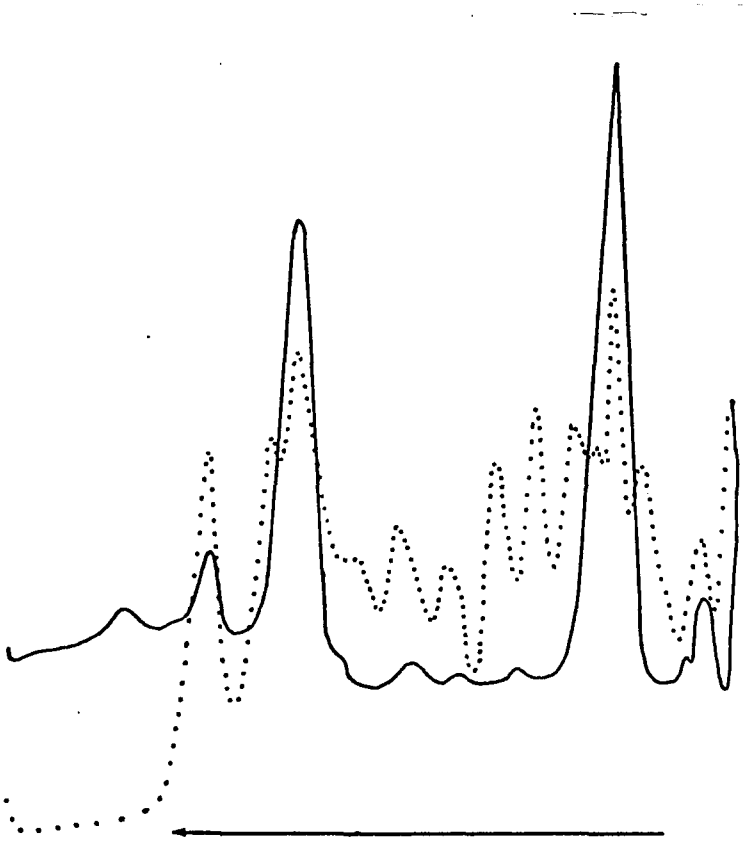

Figure A-6. Comparison of BCT and BCE for stem USP. -- Solid line indicates BCT; dotted line indicates BCE. Arrow indicates direction of migration.

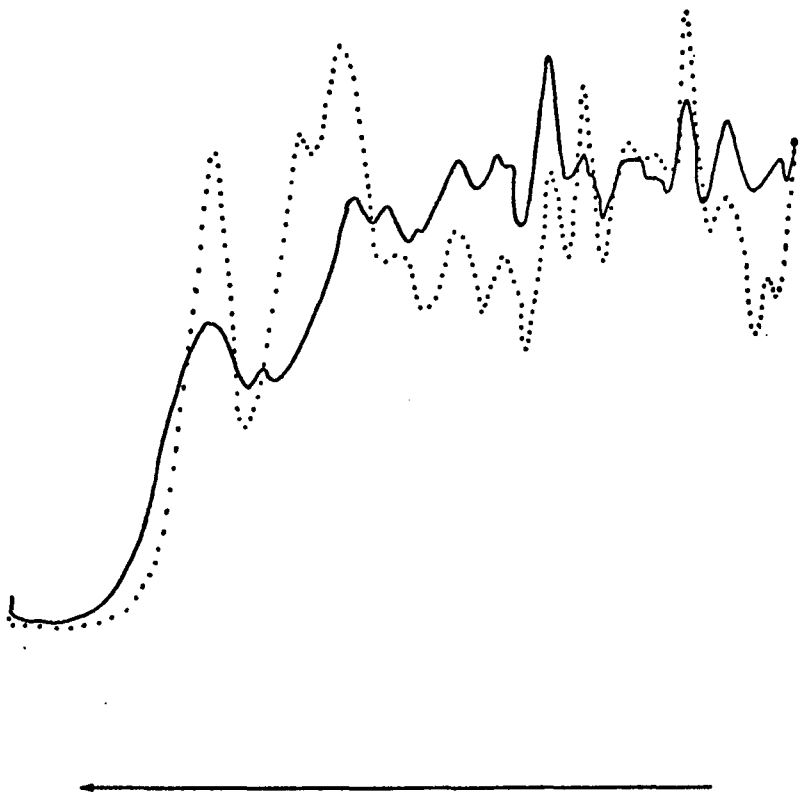

Figure A-7. Comparison of BCT and BCE for cotyledon USP. -- Solid line indicates BCT; dotted line indicates BCE. Arrow indicates direction of migration.

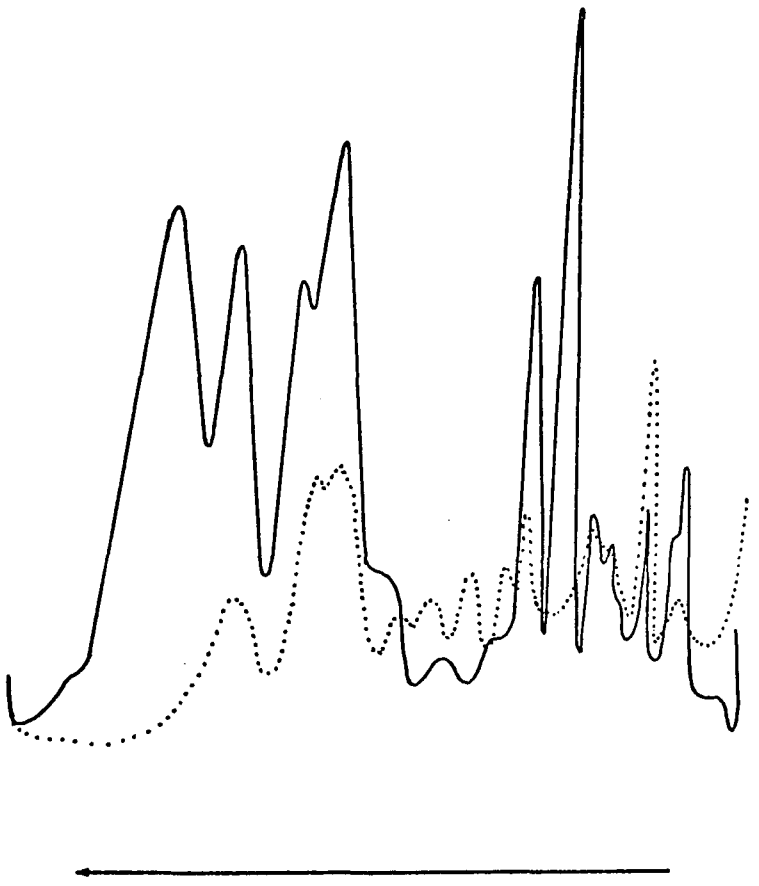

Figure A-8. Comparison of proteins from direct acid extract and USP. -- Solid line indicates species extracted directly from chromatin in acid; dotted line indicates species extracted directly from chromatin in urea buffer (USP). Arrow indicates direction of migration.

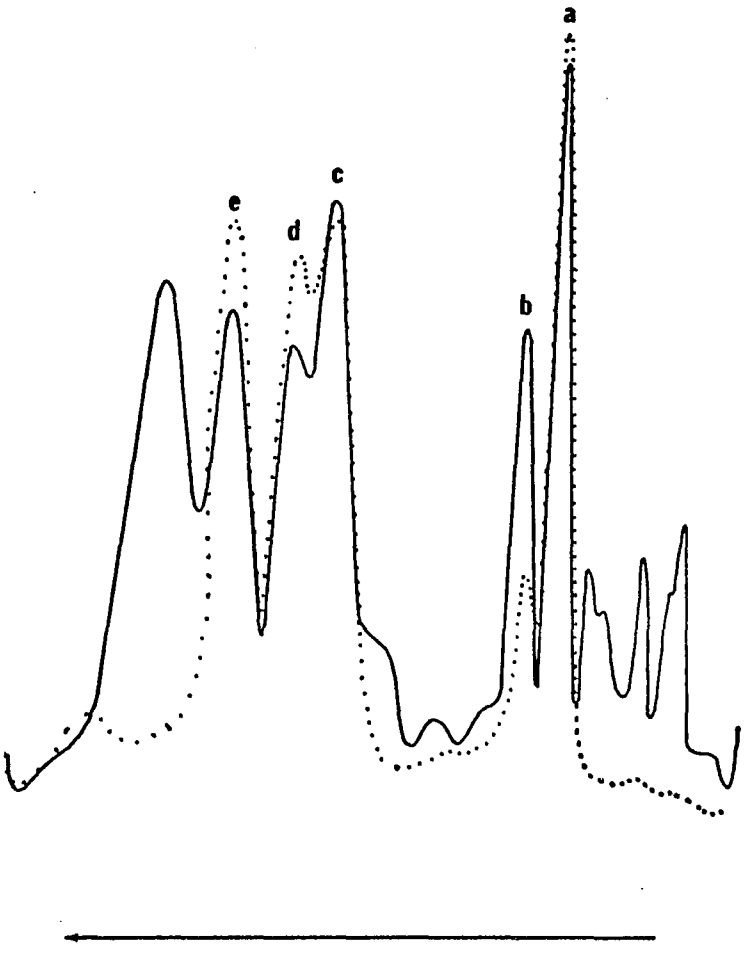

Figure A-9. Comparison of proteins from direct acid extract and HIS. -- Solid line indicates species extracted directly from chromatin in acid; dotted line indicates species extracted in acid after chromatin is extracted with urea buffer (HIS). Letters indicate HIS species. Arrow indicates direction of migration.

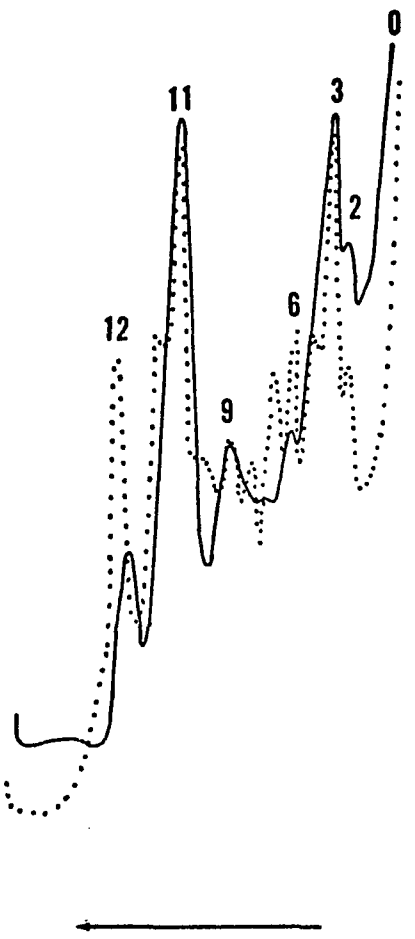

Figure A-10. Comparison of RES and USP. -- Solid line indicates RES species; dotted line indicates USP species. Numbers indicate USP species with mobility similar to or identical with RES species. Arrow indicates direction of migration.

APPENDIX B

SPECIFIC ACTIVITIES OF CHROMATIN-ASSOCIATED PROTEINS AND LEUCINE INCORPORATION

Table B-1. Specific activities of chromatin-associated proteins at various stages of germination. -- Data are expressed as net cpm/mg protein. For experiments 1 and 2, ^{14}C-leucine was used; for experiment 3, ^{3}H-leucine was used. Mean is average of experiments 1 and 2.

Chromatin-associated Protein	Stage of Germination (hr)			
	2-14	12-24	24-36	36-48
USP				
Experiment 1	2550	3000	1050	650
Experiment 2	5150	7150	2990	1360
Mean	3850	5080	2020	1000
Experiment 3	2770	3360	1650	730
HIS				
Experiment 1	930	1990	470	440
Experiment 2	860	1920	660	540
Mean	900	2200	560	490
Experiment 3	930	1230	770	0
RES				
Experiment 1	3240	6560	2960	1590
Experiment 2	6380	7850	4380	2170
Mean	4810	7200	3670	1880
Experiment 3	5520	7440	3580	1600

Table B-2. Incorporation of labelled-leucine into chromatin-associated proteins at various stages of germination. -- Data are expressed as net cpm. For experiments 1 and 2, ^{14}C-leucine was used; for experiment 3, ^{3}H-leucine was used. Mean is average of experiments 1 and 2.

Chromatin-associated Protein	Stage of Germination (hr)			
	2-14	12-24	24-36	36-48
USP				
Experiment 1	131	302	90	72
Experiment 2	269	896	366	139
Mean	400	599	228	211
Experiment 3	137	294	169	73
HIS				
Experiment 1	28	62	20	12
Experiment 2	39	103	45	22
Mean	34	83	33	17
Experiment 3	34	66	36	0
RES				
Experiment 1	67	403	202	83
Experiment 2	278	560	374	134
Mean	173	482	288	109
Experiment 3	207	524	221	65

Table B-3. Incorporation of labelled-leucine into cellular homogenates. -- Data are expressed as net cpm/200 mg dry tissue. For experiments 1 and 2, ^{14}C-leucine was used; for experiment 3, ^{3}H-leucine was used.

Experiment	Stage of Germination (hr)			
	2-14	12-24	24-36	36-48
1	2639	2769	2637	2460
2	2321	2460	2562	2257
mean	2480	2545	2600	2358
3	1192	1550	1082	888

APPENDIX C

DOUBLE-LABEL COUNTING PROBLEMS AND DATA ANALYSIS

Originally, a comparison of sample $^{3}H/^{14}C$ ratios to a standard base ratio of $^{3}H/^{14}C$ of a simple mixture of the two radioisotopes at the appropriate concentrations was proposed. It was expected that, if the response of the nuclear protein to the growth regulator under scrutiny was synthesis (increased radioisotope incorporation) then the ratio for the series in which ^{3}H-leucine was added with the growth regulator would be larger than the standard base ratio and the reciprocal series would demonstrate a $^{3}H/^{14}C$ ratio smaller than the standard base ratio. If no response occurred, each series was expected to have the same value as the base. However, it was found that regardless of which radioisotope was added with the growth regulator, the value of the $^{3}H/^{14}C$ ratio was always greater than the standard base ratio.

A series of control experiments on counting conditions demonstrated that this effect was due to the presence of protein in the sample. For these double-label counting control experiments, a stock of ^{3}H-leucine plus ^{14}C-leucine was made with the proportions 10 μc: 1 μc respectively. Aliquots of this stock solution were placed into

scintillation vials under various conditions, including mixture with various unlabelled nuclear proteins. Examples of the results obtained are shown in Table C-1. Replicates demonstrated that the variance in efficiency and in $^{3}H/^{14}C$ ratio was not attributable to pipetting error but rather to the presence of the other materials, including the class of protein present in the scintillation vial.

For this reason, control experiments incorporating either ^{3}H-leucine or ^{14}C-leucine with no growth regulators present were devised. The ratio obtained for each chromatin-associated protein from the control experiment was used as a standard base ratio against which the ratio of a chromatin-associated protein from a hormone-treated sample could be compared.

The method devised (W. T. Starmer, personal communication) for the analysis of radioisotope incorporation as a response to an exogenous growth regulator was based on the following equations, where:

H = ^{3}H-leucine incorporated in control (no growth regulator) sample

C = ^{14}C-leucine incorporated in control (no growth regulator) sample

H_r = ^{3}H-leucine incorporated in sample to which ^{3}H-leucine was added with growth regulator

C_r = ^{14}C-leucine incorporated in sample to which ^{14}C-leucine was added with growth regulator

R_h = $^{3}H/^{14}C$ ratio of sample to which ^{3}H-leucine was added with growth regulator

Table C-1. Results obtained from double-label counting control experiments. -- Each sample contained 0.5 μc ^{3}H-leucine plus 0.05 μc ^{14}C-leucine.

Conditions Under Which Radioisotope Mixture was Added to Scintillation Vial	Unlabelled Protein Added (100 μliter)	$\frac{1}{10^5}$ ^{3}H Counts	^{3}H/^{14}C
directly in vial	none	2.8	1.7
on paper filter	none	1.3	0.8
on fiberglass filter	none	7.3	2.1
directly in vial	cell homogenate	0.1	0.7
on paper filter	cell homogenate	1.2	1.7
on fiberglass filter	cell homogenate	3.0	2.7
directly in vial	USP	3.9	2.5
on paper filter	USP	0.8	0.7
directly in vial	HIS	7.9	2.8
on fiberglass filter	HIS	8.4	2.4

R_c = $^3H/^{14}C$ ratio of sample to which ^{14}C-leucine was added <u>with</u> growth regulator

N = $^3H/^{14}C$ ratio of control sample to which <u>no</u> growth regulator was added

$$H_r/H = H_r/C \times C/H = R_h/N$$

$$C/C_r = H/C_r \times C/H = R_c/N$$

$$\tfrac{1}{2}(H_r/H + C_r/C) = S$$

where S represents the value of the response of the chromatin-associated protein with respect to radioisotope incorporation (presumably a measure of synthesis).

Therefore, if the S-value equals 1, the protein exhibited no response to the growth regulator. If the S-value is greater than 1, then radioisotope incorporation was stimulated in the presence of the growth regulator and if the S-value is less than 1, then radioisotope incorporation was repressed in the presence of the growth regulator.

Since for analysis of sliced gels, each gel represented a self-contained system with a straight line as its own standard, the method discussed above was not applied to gel analysis.

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