BASIC NUCLEAR PROTEIN DEVELOPMENT IN COTYLEDONS
OF GERMINATING PUMPKIN (CUCURBITA PEPO L.)

by

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Changes in the nucleic acid content and histone composition were monitored in cotyledons of pumpkin (*Cucurbita pepo* L., cv, Small Sugar) during the first four days of development. Seedlings were grown in vermiculite at $37 \pm 1^\circ C$ under a 12-hr photoperiod.

The DNA content of cotyledons increased 2.5-fold between the first and third days and the RNA content increased 4.5-fold between the first and fourth days. Major increases in both DNA and RNA occurred between the second and third days.

Histones were extracted using various techniques and were identified by their migration rate with respect to bovine serum albumin and to calf thymus histone. The slightly lysine-rich histones ($f2a2 + f2b$, nomenclature of Johns) increased in their proportion of the total histone composition from about 39% to about 52% between the second and fourth days. The arginine-rich histones ($f2a1$ and $f3$) and the lysine-rich histone ($f1$) did not change significantly during the period tested.

It was concluded that there was no quantitative change in the arginine-rich histones corresponding to a change in DNA replicating rate or in template activity. The precise effect of the change in slightly lysine-rich histones was not determined.
In higher organisms, the genetic material, deoxyribonucleic acid (DNA), is not present in the free state but is present in close association with basic proteins, nonbasic proteins, and a small, but detectable, amount of ribonucleic acid (RNA). Of these associated components, the basic proteins (histones) are consistently present in amounts nearly equaling that of DNA (23). Because of their close association with DNA and their possible role as regulators of genetic activity, histones have been the subject of a vast amount of research in recent years.

Early investigations into the nature of histones indicated a high degree of heterogeneity in the histone composition. This prompted Stedman and Stedman (55) to propose that histones are responsible for the change in genetic expression during differentiation. An implied corollary to this hypothesis is that different tissues of the same adult organism would have a different complement of histones, and that there would be detectable changes in the histone composition as template activity of chromatin changes during embryogenesis.

The use of more reliable isolation techniques has determined that there are only five major histone fractions found in most organisms, but quantitative changes in the relative composition of these five different fractions have been detected in chromatin from different
tissues of the calf (40). Changes in the composition of histones have also been detected during spermatogenesis and also in the male pronucleus following fertilization in snails (10), and during development of sea urchin embryos (9, 56, 60, 61, 62).

The objective of this study was to determine if there would be a quantitative change in the histone complement of a rapidly developing plant tissue, and if this change would correlate to any change in DNA or RNA synthesis rates. To test this proposition, it is desirable to have a system in which changes in genetic activity are readily observable. Pegelow (43) has shown that the DNA and RNA content of germinating pumpkin seedlings change dramatically within five days of germinations. When grown at 37 ±1°C, the DNA content per cotyledon tripled between the second and third day and thereafter remains constant, while the RNA content per cotyledon increased approximately 6-fold between the second and fourth days and thereafter decreased. Therefore, if changes in gene activity can be correlated to changes in the relative histone composition, these changes should be observed within a four-day period following germination of a plant that is readily available.
CHAPTER 2

LITERATURE REVIEW

The original hypothesis of Stedman and Stedman (55) was based on the assumption that there could be a specific type of histone for each gene. However, as techniques for isolation and characterization of histones improved, it became evident that there were a limited number of histones. The number of classifications obtained depended on the technique of isolation. Calf thymus histone can be separated by electrophoretic fractionalization into 3 fractions; arginine-rich, lysine-rich, and slightly lysine-rich.

Starch gel electrophoresis as employed by Hnilica (26) split the histones into 4 fractions; lysine-rich f1, slightly lysine-rich f2a and f2b, and arginine-rich f3. However, the f2b reported by Hnilica did not have the same characteristics ascribed to the currently accepted f2b. It exhibited evolutionary stability and it had a molecular weight of 22,000 as compared to the 13,774 reported by DeLange and Smith (22). The selective extraction technique of Johns (28) also divided histones into 4 groups.

The polyacrylamide gel electrophoresis technique employed by Panyim and Chalkley (39) and others (11, 28) provides a rapid qualitative and quantitative analytical technique for characterizing histones into fractions. Calf thymus histone can be separated into five bands.
using this technique. Panyim and Chalkley also introduced their own nomenclature system for identifying these bands, adding to a field already encumbered by many nomenclature systems. Table I correlates some of the nomenclature systems used by the more widely published authors. The nomenclature system of Johns (28) was used in this report.

The amino acid sequence for 3 of the 5 calf thymus histones is known. The complete sequence of f2a1 and partial sequences of f3 and f1 are given in a review by DeLange and Smith (22), the sequence of f2a2 is given by Yeoman et al. (64), and that of f2b by Iwai (in 27). The amino acid sequence for some histones isolated from peas and other sources (22) is also known. There is a remarkable degree of similarity between the arginine-rich fractions of the various sources; for example, of the 102 amino acids present in f2a1, only 1 residue is different when calf thymus f2a1 is compared to pea f2a1 (21). Panyim et al. (41) have proven the arginine-rich f3 to have identical electrophoretic mobilities in several species. This degree of evolutionary stability may suggest the function of arginine-rich histone is so related to its structure that only very conservative mutations are not lethal.

The gel electrophoresis technique also characterizes a number of subfractions for each fraction other than f2b. These are believed to be caused by enzymatically induced side chain modifications occurring after the entire protein has been synthesized (22,38,40,58). Each fraction except f2b has modifications which are either acetylations, methylations or phosphorylations of one or more of the amino acid residues.
Table I. A correlation of nomenclature systems used by various authors and the general class of the histone fraction.

<table>
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<th>General class</th>
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<tr>
<td>Lysine-rich</td>
<td>f1</td>
</tr>
<tr>
<td>Slightly lysine-rich</td>
<td>f2a2</td>
</tr>
<tr>
<td></td>
<td>fab</td>
</tr>
<tr>
<td>Arginine-rich</td>
<td>f3</td>
</tr>
<tr>
<td></td>
<td>f2a1</td>
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a. Johns (28).
b. Bonner et al. (12).
c. Panyim and Chalkley (40).
d. Srivastava (54).
e. Yeoman et al. (64).
To understand the significance of the various histone fractions and the possible importance of side chain modifications of histones, it is desirable to understand something of the interactions among histones and DNA and other chromosomal constituents.

The Relationship Existing Among Histones and Other Components of Chromatin

The DNA found in native chromatin has been found to be less available to interaction with dyes (1,3) or with DNase (35) in its environment than is isolated DNA. The template activity of chromatin is less than that of an equal amount of purified DNA although Bonner at al. (12) indicated that chromatin and purified DNA are capable of binding the same number of polymerase enzyme molecules. Also, the addition of histones to de-histonized chromatin shows a nonspecific decrease in template activity that is proportional to the amount of histone added (12).

Bekhor, Kung, and Bonner (8), Spelsberg and Hnilica (50), and Paul and Gilmour (42) have conducted the following informative series of studies which clearly demonstrated the general role of the various chromatin constituents. Native chromatin is capable of transcription in vitro, but the transcription ability is far less than that of purified DNA. They found that selective removal of only histones resulted in template activity that approached that of purified DNA. When the histones were reassociated with the de-histonized chromatin, the template activity reverted to that of native chromatin. They found that chromatin could be totally dissociated and reconstituted from its
components, but the template activity of the reconstituted chromatin was dependent on the order of addition of the components. The DNA in the fully dissociated state has a template activity equal to that of purified DNA. If histones alone were added to the DNA, a high degree of inhibition resulted and the template activity was much lower than that of native chromatin. The addition of acid nuclear protein (ANP) after the histone-DNA complex had formed did not result in de-repression of the template activity. However, if ANP were allowed to complex with either DNA of the histones prior to the formation of the complex, the degree of inhibition was lower than that found with only histone DNA complexes and the amount of de-repression was proportional to the amount of ANP added. If the amount of ANP added was equal to that in native chromatin, the reconstituted chromatin had a synthesis rate equal to that of native chromatin.

Bekhor, Kung, and Bonner (8) indicated that if the chromatin were dissociated in such a way that the chromosomal RNA (C-RNA) was destroyed, the RNA synthesized by the reconstituted chromatin will not compete successfully with labeled RNA produced in vitro using native chromatin. If, however, the C-RNA were not destroyed, then the RNA will compete with that synthesized by native chromatin. Wang (63) reported that addition of excessive exogenous ANP will de-repress histone inhibition of transcription beyond that of native chromatin. Acid nuclear protein from any of the sources tested was effective, but the degree was greatest when homogenous systems were added, for example, when spleen ANP was added to a spleen system.
The combined data of these and other experiments clearly demonstrated that the histones are the entity in chromatin that is responsible for inhibition of transcription. Some of the ANP may be responsible for modifying histone inhibition of transcription and the C-RNA may add the required specificity for regulation of genetic activity.

Selectively Removal of Histones and In Vitro Activity of Chromatin

A number of experiments were conducted to determine the effect of specific histone fractions on the template activity of chromatin. Georgiev (in 24) found that treating chromatin with 0.6 M NaCl led to removal of 15% of the histones and a 50% increase in template activity. Only lysine-rich f1 was removed at this concentration. A further increase in NaCl concentration led to the removal of more protein and further increased template activity but the percent increase in template activity for percent protein removed was far lower than it had been for f1. From these data it was concluded that f1 had a major role in controlling transcription. Bartly and Chalkley (6) have since reported that f1 is removed by a 0.6 M NaCl solution, while f2a2 and f2b are removed by salt solutions between 0.8 and 1.4 M NaCl.

The validity of conclusions drawn from this type of experiment was questioned. Clark and Felsenfeld (19) reported that the use of 0.6 M NaCl led to inherent errors in calculating template activity. Partially de-histonized chromatin containing unlabeled proteins and DNA was incubated in a 0.6 M NaCl solution containing labeled DNA. The solution was treated with DNase and the protected DNA was checked for
the presence of label. It was discovered that the protein associated with approximately 41% of the binding sites was capable of migrating to the labeled DNA.

The effect of selective removal of histones on the template activity was tested by a different technique by Smart and Bonner (47) using sodium deoxycholate solutions varying from 0 to 0.15 M. Slightly lysine-rich histones were removed by solutions 0.01 to 0.04 M sodium deoxycholate, arginine-rich histones by solutions 0.025 to 0.05 M sodium deoxycholate and lysine-rich histones by 0.03 to 0.08 M sodium deoxycholate. At these concentrations of sodium ion, it is doubtful that any proteins in chromatin shifted their position on the DNA strand. They found that the percent increase in template activity and the percent protein removed varied only slightly from a 1:1 ratio and that the effect of removal of f1 was only very slightly greater than that for the other fractions. As a result of these sodium deoxycholate studies, it was concluded that each histone fraction may play a role in the regulation of template activity.

The Nature of Histone-DNA Associations

Bonner et al. (12) reported that the ratio of histone:DNA in chromatin varies when cells of different tissues are compared, ranging from 0.75 to 1.35, but remaining constant within a particular cell at various stages of development. Clark and Felsenfeld (19) found that approximately one-half of the DNA is bound to protein, as determined by titration with polylysine and by digestion with DNase. They also determined that the total histone was not bound to regions rich in either
G+C or A+T, since the base pair ratio of protected DNA is 58% A+T in calf thymus, which is the same base pair ratio as in the entire strand. The total histone is distributed along the DNA strand in such a manner that there are no detectable long, continuous protein-free stretches of DNA, as indicated by the melting profile data presented by Tuan and Bonner (56) and by Smart and Bonner (46).

Arginine-rich histones are located in regions that are rich in G+C, as indicated by the analyses of DNA that was protected from DNase activity by chromatin that had been previously stripped of all but arginine-rich histones (20). The association of arginine-rich histones to G+C rich DNA was calculated on a theoretical basis by Smythes (in 49), using a helical wheel to arrange the known sequence of f2al into the most likely conformation and computing the nucleotide sequence that would give the best interaction.

The location of the slightly lysine-rich histones on the strand has been postulated with less assurance since it has not been possible to isolate strands of DNA containing only slightly lysine-rich histones. However, slightly lysine-rich histones probably do not cover long continuous strands of DNA as determined by the lack of a change in the melting profile caused by the removal of slightly lysine-rich histones using sodium deoxycholate as reported by Smart and Bonner (46).

The results of selective extraction experiments (6,13,24,37,44) demonstrate that the lysine-rich f1 dissociates in lower concentrations of most ionic solvents than do the other histone fractions despite the fact that f1 has the greatest net positive charge. It has
also been shown by Smart and Bonner (45) that f1 is the histone most subject to proteolytic activity and that f1 contributes more to the hydrophobic nature of chromatin than does any other histone fraction. These results indicate that f1 is the fraction most subject to interaction with the environment and possibly surrounds the complex like a sheath.

The combined results of these and other experiments suggest a model of the histone-DNA complex. The arginine-rich and slightly lysine-rich histones appear to be tightly complexed to DNA, possibly in one of the grooves (22). The arginine-rich fractions are localized and possibly clustered in G+C rich regions. The slightly lysine-rich and the lysine-rich fractions are aligned in such a fashion as to prevent long protein-free strands. Lysine-rich f1 or at least part of the molecule is free to wrap around the outside of the complex.

**Effects of Side Chain Modifications of Histone Fractions on the In Vitro Template Activity**

Although lysine-rich f1 has been shown to be capable of protecting DNA from DNase activity, Mirsky (in 35) reports that phosphorylated f1 was not capable of blocking DNase activity. Since only a few amino acid residues are phosphorylated [4 in f1 as reported by Balhorn et al. (5)], this indicates that minor alterations can effect profound changes in histone-DNA interactions and possibly in the template activity of chromatin. An enzyme system capable of regulating the degree of phosphorylation in histones has been isolated from the human lymphocyte system by Murray (in 36). The system consists of a
phosphatase and a cyclic-AMP stimulated protein kinase which, while not specific for histones, had a higher activity with arginine-rich and lysine-rich histones than with any other protein tested, including whole histone. This would imply that the activity with slightly lysine-rich histones was low.

The results of these and many other experiments cited indicate that there are two mechanisms by which the DNA in chromatin can be allowed to interact to a greater extent with its nuclear environment and, in doing so, increase the template activity; histones can be removed from chromatin, or the structure of the histone molecule may be chemically altered by enzymatic activity.

**Alterations in Composition of Histones in Developing Tissues**

If changes in the relative proportions of the histone fractions were correlated to modifications in genetic expression or template activity, different proportions of the specific subfractions should be observed in chromatin isolated from different tissues in the same organism. This difference was reported by Panyim and Chalkley (40) for various tissues of the calf. By the use of very long acrylamide gels they demonstrated that histones isolated from different tissues contained different proportions of the subfractions and that the subfractions were modified to different degrees.

Changes in the proportions of the histone fractions have been demonstrated during embryogenesis of the sea urchin (2,4,9,57,59). The histone composition in the early blastula was similar to that of the
late blastula but varies significantly from that in the gastrula (61). Benttininen and Comb (9) reported three significant changes during early embryogenesis in the sea urchin. At the 32-cell stage the histone fractions were present in the proportion f2b > f1 > f3 > f2a2 > f2a1 in the blastula the proportion was f1 > f2b > f2a2 > f2a1 > f3; and in the gastrula f3 > f1 > f2a2 > f2b > f2a1. The predominant change noted as the embryo entered a stage of high template activity was in increase in arginine-rich f3 and a decrease in slightly lysine-rich f2b.

Prior to gastrulation in sea urchins, the embryo synthesized no new RNA. Thaler (in 57) reported that the sea urchin egg contained long lived mRNA for arginine rich histones since that fraction was synthesized, even in the presence of Actinomycin D. Lysine-rich and slightly lysine-rich histones were not synthesized until after synthesis of new mRNA has occurred.

Asao (2) reported both quantitative and qualitative changes in the histone complement of the developing Japanese newt. Embryos had no protein that was analogous to adult histone. In agreement with results for sea urchin embryos there was an increase in the arginine-rich histone complement as gastrulation occurred in the newt embryo followed by a late increase in slightly lysine-rich histones.

These data and that of many others show the existence of changes in relative amounts of histones occurring during periods of changes in template activity and show the proportion of arginine-rich histones is higher during periods of high template activity. Evidence is also
available that indicates side chain modification can alter template activity. If this is true, a side chain modification should be noted in the histone fractions of active systems.

Gutierrez-Cernosek and Hnilica (25) correlated the time sequence of DNA synthesis to histone synthesis and histone phosphorylation in regenerating rat liver. Histone synthesis occurred prior to or concurrently with DNA synthesis. The various histone fractions were synthesized simultaneously and the rate of $^{14}$C lysine incorporation into each fraction was constant for the first 30 to 36 hr following partial hepatectomy. However, the ratio of the uptakes of $^{32}p: ^{14}$C was constant only for f3 and the ratio for the other fractions reached a peak at 6 to 12 hr and then dropped to a steady level for the remainder of the study. It was reported that the peak was highest for f2b, but it should be recalled that f2b as reported by Hnilica is not the same protein as the f2b reported by Johns (28) or DeLange and Smith (22), as mentioned earlier in the Literature Review. The synthesis of DNA occurred between 12 and 24 hr. This demonstrated that phosphorylation of the histones did precede an increase in genetic activity.

Studies on plant systems relating the composition of the histone complement to genetic activity are less numerous and the total picture of histone metabolism in plants is further from being complete than that discussed for animal systems. Srivastava (54) reported that older tobacco leaves have a higher template activity and the chromatin contains proportionally more arginine-rich histones than younger leaves. These data are in good agreement with most data from animal tissues.
Conversely, Spiker and Krishnaswany (52) compared the histones isolated from chromatin of spring and winter wheat samples grown under different conditions and harvested at different ages and concluded that there was no significant change in the quantitative or qualitative composition of the histones. Working with dwarf peas, Spiker and Chalkley (51) demonstrated that there was no difference observed in the histone composition of chromatin isolated from the epicotyl of 10-day-old control plants and that isolated from the epicotyl of plants that had been stimulated with gibberellic acid at an age of 8 days and harvested at 10 days. The results of Spiker's experiments imply that changes in the histone composition of some plant chromatin in relation to changes in template activity may not be detected.

Clearly there is a need for more information relating proportional changes in histone composition to changes in nucleic acid metabolism in plant tissues.

Nucleic Acid Metabolism in Cotyledons of Developing Seedlings

In pumpkin (43) as well as in peanuts (17), synthesis of both DNA and RNA has been demonstrated to occur in the cotyledons during germination. In pumpkins, the RNA content increased approximately 6-fold between the second and fourth days and then decreased while the DNA content increased approximately 3-fold between the second and the third days and remained constant for the remainder of the first week. While these changes clearly demonstrated template activity, the total protein content per cotyledon decreased dramatically, implying that there are
other factors involved in protein metabolism in cotyledons of germination seedlings.

**Protein Metabolism in Cotyledons of Developing Seedlings**

Cotyledons serve as a storage area for nitrogen and other compounds required by the embryonic axis. The principal nitrogen reserves of some plants are globulins stored in the cotyledons in subcellular protein bodies (7). As germination progresses the total protein content of the cotyledon decreases (7,15,16,18,31,43,53), while the protein content of the embryonic axis increases, and the total nitrogen content of the entire seedling remains constant (7). Chou and Splittstoesser (18) reported a decrease in total protein per cotyledon. By the ninth day 80% of the reserve protein had been utilized and 50% of the reserve nitrogen had been transferred out of the cotyledon. They also reported that the carbons in the arginine of the storage protein were given off as CO₂ indicating a high level of proteolytic activity in the cotyledon.
CHAPTER 3

MATERIALS AND METHODS

Seeds of *Curbita pepo* L. variety, Small Sugar, were purchased from the Dessert Seed Co., El Centro, California. These were planted as described by Pegelow (43) in 29 cm by 20 cm by 6.4 cm plastic trays containing 2 liters of vermiculite. They were then covered with 1 liter of vermiculite, watered with 120 ml of tap water, and incubated in a 37±1°C growth chamber under an 8:00 A.M. to 8:00 P.M. photoperiod of about 800 foot candles light intensity at plant height. Planting and harvesting were done between 9:30 A.M. and noon and trays were watered daily at noon with 600 ml of tap water for 1- and 2-day-old seedlings and 400 ml of modified Hoagland's solution (43) for 3-day and older seedlings. Six trays containing about 75 seeds each were planted and harvested for each experiment. Eight cotyledons from each harvest were stored in a -60°C freezer for nucleic acid analysis and the remainder were chilled for 30 min at -20°C and extracted for their histone content.

**Extraction of Histones**

Histones were extracted from purified chromatin by a modification of the technique of Bonner et al. (11). The grinding solution (0.25 M STKM) which contained 0.25 M sucrose, 0.005 M MgCl₂, 0.025 M KCl, 0.05 M tris, 0.05 M NaHSO₃; pH 7.6, was modified from the...
solutions used by Dr. R. McDaniel (personal communication, Department of Agronomy and Plant Genetics, The University of Arizona) by the addition of NaHSO₃ which is believed to limit proteinase activity (39). Unless otherwise stated, all reagents and equipment were kept between 0 and 4 °C during histone extraction. Centrifugations were conducted using a Sorvall SS-34 head in a Sorvall RC-2B centrifuge and G forces noted are maximum values.

In early experiments, the cotyledons were ground using a pre-chilled mortar and pestle in 1.5 ml of 0.25 M STKM per gram fresh weight. In later experiments the cotyledons were ground in 180 ml of 0.25 M STKM using a Waring Blender at top speed for 90 sec. The blender method was employed to further reduce the possibility of protease activity by dispersing the material immediately into as large a volume as possible. The homogenate was then filtered through two layers of cheesecloth and two layers of Miracloth (Cal Biochem). The cheesecloth and the Miracloth were hand wrung, using gloves in order to discard as little of the homogenate as possible. The nuclei were pelleted by centrifugation at 3000 × g for 8 min and the residual lipids were removed from the walls of the tube using "Kimwipes" (Kimberly Clark). The nuclei were further purified by successively washing with 50 ml of 0.25 M STKM made to 2% in Triton X-100 (Rohm and Haas), followed by centrifugation at 1000 × g for 5 min. The final nuclear pellet was essentially free of chlorophyll coloration.

Chromatin was extracted from the purified nuclei by first washing three times with 100 ml of 0.01 M tris pH 8.0. After each washing
the suspensions were centrifuged at 10,000 x g for 10 min and the pellet was retained in all cases. The final pellet was suspended in 40 ml of 0.01 M tris, pH 8.0, and layered in 5-ml aliquots over a semidiscontinuous sucrose gradient. The gradient was prepared by rapidly pipetting 10 ml of buffer I (0.8 M sucrose, 0.025 M KCl, 0.005 M MgCl₂, 0.002 M CaCl₂, 0.04 M tris-HCl, pH 7.5) over 10 ml of 2.3 M STKM (as 0.25 M STKM except 2.3 M in sucrose) in a 50-ml centrifuge tube. The rapid pipetting resulted in a rough gradient at the interphase.

Chromatin passed through the gradient and was pelleted on centrifugation at 40,000 x g for 45 min, and materials adhering to the tube were removed using Kimwipes. This process was repeated for further purification of the chromatin from 1- and 2-day-old cotyledons. The chromatin was resuspended in a total of 40 ml of 0.01 M tris, pH 8.0, combined into two tubes and pelleted by centrifugation at 10,000 x g for 10 min. The pellet was next washed three times with 25 ml of 1 x SSC (SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) and was collected after each wash by centrifugation at 3,000 x g for 5 min.

In most cases, histones were extracted from the final chromatin pellet after the washes with 1 x SSC, but in some cases attempts were made to remove nonhistone basic proteins before extraction for histones using 1 M NaCl (32, 33, 34, 53). This was done by homogenizing the chromatin pellet after the final 1 x SSC wash in 30 ml of 1 M NaCl, then centrifuging at 3,000 x g for 5 min and saving the supernatant fraction which contains dissolved chromatin.
Histones were extracted from the chromatin by homogenizing the pellet in 0.3 to 1.0 ml of buffer I made to 0.4 N in H$_2$SO$_4$. The combined supernatant fractions were either stored at -20°C or separated by gel electrophoresis immediately.

**Characterization and Quantification of Basic Proteins Using Gel Electrophoresis**

Basic proteins were characterized by a modification of the gel electrophoresis technique of Panyim and Chalkley (39). The gels were prepared as described by McDaniel (personal communication, Dr. R. G. McDaniel, Department of Agronomy and Plant Genetics, The University of Arizona), by dissolving 5.7 g of urea in 8 ml of solution C and 6 ml of solution A. Solution C was 30.0 g of acrylamide and 0.40 g of bisacrylamide made to 100 ml with water and adjusted to pH 2.9 with HCl; solution A was 71 ml of glacial acetic acid and 1.55 ml of N,N,N',N'-tetramethylethylenediamine (TEMED) made to 100 ml with water and adjusted to pH 2.9 with KOH. When the urea was completely dissolved, 2 ml of 1.4% ammonium persulfate in water was added; the solution was evacuated to remove air bubbles and transferred to 8 cm by 0.6 cm (inside diameter) plastic tubes using a Pasteur pipette. A 1-cm layer of water was carefully layered over the acrylamide solution and the tube was then placed three inches from a fluorescent light source to effect polymerization. The gels were prerun prior to sample addition, using 0.9 N acetic acid as tray buffer (39), at 4 mA per gel for 3 hr or until a methylene blue marker had traveled the length of the gel. The electrophoretic apparatus was as described by Bonner et al. (11). Current source was a
Heathkit IP17 constant amperage power supply unit. The tray buffer was replaced with a prechilled fresh preparation, and 10 μl of an 8-g bovine serum albumin per 10 μl buffer I solution was layered on each gel; a current of 2 mA per gel was applied for 10 min. Subsequently, 90 to 120 μl of the appropriate protein extract was layered on the gels and electrophoresis was conducted, at 2 mA per gel, for 4 hr. After electrophoresis, the gels which adhered to the tube were removed by first moving a long, thin needle around the perimeter of the gel while the tube was submerged under water, and air pressure from a "Propipette" was then applied to expel the gel. The gels were stained for 3 to 4 hr in a 1% amido black solution in 7% acetic acid and 20% ethanol, stored overnight in 7% HAC, 20% ethanol, and detained electrophoretically in a horizontal rapid destaining apparatus. The gels were transferred and stored in tubes containing destaining solution.

The gels were scanned in a Gilford 240 spectrophotometer with a model 2400 linear transport at 620 nm and the areas under the curves were determined by weighing cutout sections of the photocopies of the graphs.

Although all histone proteins do not bind the same quantity of dye, it has been shown that for any given protein the amount of dye absorbed is a reflection of the quantity of that protein (29). Therefore changes in the ratio of the areas under the curve on the graph were considered to represent changes in the composition of total histone.
Determination of Nucleic Acids in Cotyledons

The nucleic acid per cotyledon was determined by a modification of the technique of Smillie and Krotkov (48) described by Cherry (14). Eight cotyledons, stored at -60°C, were weighed and ground using an ice cold mortar and pestle in 10 ml of cold 0.05 N formic acid in methanol. The mixture was quantitatively transferred to a centrifuged tube using an additional 5 ml of formic acid-methanol wash. The combined homogenate was mixed for 5 min, then centrifuged at 4500 x g for 5 min. The pellet was then washed successively with 10 ml of 0.05 formic acid in methanol; twice with 5 ml of cold 0.2 N perchloric acid (PCA); once with 10 ml of cold 95% ethanol; once with 10 ml of a 2:2:1 (v/v/v) solution of ethanol, ether, and chloroform; and finally with 10 ml of ether. The homogenates in the last two washes were heated in a water bath at 50°C for 60 sec before centrifugation. All washes were centrifuged at 1500 x g for 5 min and only the pellets were retained. The defatted and depigmented powder was dried, then suspended in 3 ml of 0.5 PCA and extracted in a hot water bath at 85°C for 15 min. The supernatant obtained after centrifugation at 7700 x g for 5 min was transferred into a vial which had been previously calibrated to contain 5 ml. The pellet was next treated with 2 ml of 0.5 M PCA, the supernatant following centrifugation was added to the vial, and the combined volume was then made to 5 ml with 0.5 M PCA.

Total nucleic acid concentration was determined by measuring the $A_{260} - A_{220}$ of nucleotides suitably diluted with 0.5 M PCA. The
difference was multiplied by 57 to determine the total nucleic acid content (21).

The DNA contents were determined using the Burton procedure as modified by Kupila (in 30), using deoxadenosine as a standard. The RNA values were determined as the difference in value of total nucleic acid minus DNA.
CHAPTER 4

RESULTS

Nucleic Acid Composition of Developing Pumpkin Cotyledons

Pegelow (43) reported that when pumpkin seeds were grown at 37 ± 1°C under 800 foot candles light intensity the cotyledons emerged on the third day and the nucleic acid content underwent characteristic changes. The DNA per cotyledon was found to increase 2- to 3-fold between the second and third days and the RNA content per cotyledon increased 5- to 6-fold between the second and fourth days. Since these dramatic changes in DNA template activity and replicating ability was the basis for conducting studies on histone metabolism in pumpkin cotyledons, a representative sample of eight cotyledons from each harvest was set aside and analyzed for nucleic acid content. The results of these analyses are shown on Table II and Figure 1.

These results varied somewhat from those reported by Pegelow (43), but general trends found for the metabolism of the nucleic acids are the same. In this study the DNA content per cotyledon increased approximately 2.5-fold between the first and the third day while the RNA content per cotyledon increased approximately 5-fold between the first and third day. The greatest change in the content of DNA and RNA per cotyledon occurred between the second and third days. No significant change in the DNA content between days 1 and 2, or between days 3
Table II. The nucleic acid content of cotyledons from pumpkin seedlings of various ages.

<table>
<thead>
<tr>
<th>Age of seedlings</th>
<th>DNA (μg)</th>
<th>Na</th>
<th>RNA (μg)</th>
<th>Nb</th>
<th>TNA (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>15.32</td>
<td>1</td>
<td>139</td>
<td>7</td>
<td>167 ± 44.6</td>
</tr>
<tr>
<td>2 day</td>
<td>19.61 ± 2.21</td>
<td>3</td>
<td>312 ± 18</td>
<td>7</td>
<td>321 ± 39.2</td>
</tr>
<tr>
<td>2½ day</td>
<td>23.92 ± 1.32</td>
<td>2</td>
<td>427 ± 31</td>
<td>2</td>
<td>451 ± 27.6</td>
</tr>
<tr>
<td>3 day</td>
<td>32.33 ± 7.41</td>
<td>4</td>
<td>619 ± 127</td>
<td>10</td>
<td>653 ± 79.2</td>
</tr>
<tr>
<td>4 day</td>
<td>36.25 ± 4.15</td>
<td>4</td>
<td>675 ± 141</td>
<td>4</td>
<td>712 ± 14.4</td>
</tr>
</tbody>
</table>

a. Plants were harvested at indicated periods after incubation and analyzed for DNA and total nucleic acid (TNA) as outlined in Materials and Methods. The results indicated are means of Na observations for DNA and RNA and Nb observations for total nucleic acids. Figures following ± refer to standard deviation from the mean.
Figure 1. Compositional changes in cotyledons of developing seedlings.

The DNA and RNA per cotyledons and the proportional composition of the histone complement were computed as described in Materials and Methods. The mean values were plotted for the corresponding time period.
and 4, was calculated by the application of a "T" test to the values given in Table II. There was, however, a significant increase in both DNA and RNA content between days 2 and 3. These data confirm Pegelow's results and demonstrate that pumpkin cotyledons do undergo changes in the rate of DNA synthesis and in template activity.

Isolation and Characterization of Basic Proteins

In early experiments, considerable difficulty was encountered in resolving and identifying the various histone fractions. Since bands characteristic of histones were often diffuse or nonexistent, it was felt that the lack of resolution may have been partly due to proteolytic activity, and all techniques and solutions were subsequently modified to minimize the effect of proteolytic enzymes. Difficulties in the identification of the bands also occurred. In many cases, replicate samples run simultaneously yielded bands in slightly different locations on the gel. This was believed to have been caused partly by small intrinsic compositional irregularities in the gels and partly due to the effect of sample concentration on the electrophoretic mobility of proteins. Proteins present in high concentrations migrate with lower electrophoretic mobilities in gels than when present in lower concentrations (29). The problem of identification was resolved in part by extreme caution in gel preparation and partly by providing an internal reference for calculation of relative migrations. Bovine serum albumin (BSA, Sigma catalog A-4378, lot 52C-8090) was added to each gel as suggested by Johns (29), and the histone bands were
identified by comparing the relative electrophoretic mobilities (REM), with respect to BSA, of pumpkin histone bands to those exhibited by whole calf thymus histone (Worthington Code H).

Figure 2 compares electrophorograms of an extract from cotyledons of 4-day-old seedlings and calf thymus histone. Panyim and Chalkley (39) characterized calf thymus histones by separation on gels made to 4.5 M of urea and demonstrated that the fastest moving band was f2al. Panyim et al. (41) also demonstrated that f2al isolated from plants has an identical electrophoretic mobility to calf thymus f2al. My results, shown on Figure 2, are in good agreement with this. The mean REM with respect to BSA for f2al in whole calf thymus was calculated to be 2.23. The fastest moving band in the characteristic histone pattern on the electrophorogram tracings of basic pumpkin protein also had a REM with respect to BSA of 2.23. It was concluded that this band was in fact f2al and the remainder of the bands were identified by their position as indicated by Panyim and Chalkley (39) for gels made with 4.5 M urea.

Spiker and Chalkley (51) demonstrated that the slightly lysine-rich fractions of peas and other plants have lower REM's than that of the corresponding fraction in calf thymus histone. The data presented here are in good agreement with Spiker's results. The calculated mean REM with respect to BSA for calf thymus slightly lysine-rich histones was 2.05 while the corresponding value for pumpkin histones was 1.88. Although the two electrophorogram tracings presented in Figure 2 were
Figure 2. Comparison of electrophorogram tracings of histones from pumpkin and calf thymus.

Histones from 4-day-old pumpkin (top) and calf thymus (bottom) were mixed with bovine serum albumin and electrophoresed as outlined in Materials and Methods. Calculated REM's for calf thymus histones, using BSA as reference, were: $f_{2a1}, 2.23$; $f_{2a2}$ and $f_{2b}, 2.05$; $f_{3}, 1.66$; $f_{1}, 1.30$. Calculated REM's for pumpkin were: $f_{2a1}, 2.23$; $f_{2a2}$ and $f_{2b}, 1.88$; $f_{3}, 1.65$; $f_{1}, 1.25$; and $\alpha 2.50$. 
Figure 2. Comparison of electrophorogram tracings of histones from pumpkin and calf thymus.
not run simultaneously and, therefore, cannot be directly superimposed, this result is readily observable.

Examination of Basic Proteins Extracted from Cotyledons of Developing Seedlings

Examination of the electrophorograms, Figure 5 (see p. 35) and the banding patterns, Figure 6 (see p. 36), of acid soluble proteins isolated from cotyledons of 1-, 2-, and 3-day-old seedlings reveals the presence of a large amount of basic, but apparently nonhistone, protein that sedimented with the chromatin pellets. It is not known if the protein (labeled α protein in Figure 3 and Figure 5) is chromosomal in origin or if it became associated with the chromatin during extraction. The α protein can be separated into three electrophoretic bands with characteristic REM values of 2.5, 2.8, and 3.0 (labeled α₁, α₂, and α₃ on Figure 3) which are considerably higher than the 2.23 reported for the fastest moving histone band. From the standpoint of identification and quantification the α protein did much to mask the histone bands. Because of the great quantity present, the α protein complex is the most significant feature in the banding patterns and electrophorograms of one and two-day-old seedlings, as shown on Figures 5 and 6.

Several techniques were employed in an attempt to remove a significant portion of the protein complex from the histones of 2-day-old cotyledons. Results of these attempts are shown in Figures 3 and 4. Examination of Figure 3b reveals that the massive α protein complex almost entirely masks the presence of the histone bands, which are just
Figure 3. Comparison of electrophorogram tracings of basic proteins extracted from chromatin of cotyledons from 2-day-old pumpkin seedlings purified by different techniques.

Chromatin was purified as outlined in Materials and Methods except that the semidiscontinuous sucrose density gradient was either omitted or included and a 1 M NaCl was either added or omitted. a) omitted the gradient but included the 1 M NaCl extraction, b) omitted both the gradient and the 1 M NaCl extraction, c) included the gradient but omitted the 1 M NaCl extraction, d) included both the gradient and the 1 M NaCl extraction.
Figure 3. Comparison of electrophorogram tracings of basic proteins extracted from chromatin of cotyledons from 2-day-old pumpkin seedlings purified by different techniques.
Figure 4. Banding patterns of basic proteins extracted from chromatin of cotyledons from 2-day-old pumpkin seedlings purified by different techniques.

Chromatin was purified as outlined in Materials and Methods except that the semidiscontinuous sucrose density gradient was either omitted or included and a 1 M NaCl extraction was either added or omitted. a) omitted the gradient but included the 1 M NaCl extraction, b) omitted both the gradient and the 1 M NaCl extraction, c) included the gradient but omitted the 1 M NaCl extraction, d) included both the gradient and the 1 M NaCl extraction.
Figure 4. Banding patterns of basic proteins extracted from chromatin of cotyledons from 2-day-old pumpkin seedlings purified by different techniques.
discernible on the left side of the α protein complex. Comparison of Figure 3 (b to c) demonstrates the improved resolution afforded by the two semidiscontinuous sucrose density gradients as mentioned in Materials and Methods. This was the technique used to obtain the data for this report despite the partial masking to the f2al band by the α protein complex, as marked on Figure 3d. Comparison of Figure 3 (c to d) demonstrates the effect of an attempt to remove the α protein complex by suspending the chromatin in 1 M NaCl as outlined in Materials and Methods and described by Mirsky and Pollister (32) and Srivastava (54). It is evident, in comparing 3c to 3d, that a large portion of the α protein complex was removed by this technique, but closer observation will reveal that there is also a loss of f1 and apparently some slightly lysine-rich f2a2 and f2b were also lost. Figure 3d shows the presence of a large band with a mobility slower than that of BSA. This band was labeled f3 dimer in accordance with similar bands shown by Panyim and Chalkley (39). It is not known if this band is an artifact of the salt extraction technique or if the other techniques employed reduced the disulfide bonds that were present, but dimers were not present as a rule in extracts of 2-day-old cotyledons. The salt extraction also led to a loss of the sharpness of the bands which indicated that proteins are somewhat degraded by this technique. However, for future histone studies on cotyledons of 1-, 2-, and 3-day-old seedlings, a modification of this salt extraction technique may eliminate the problems presented by the presence of the α protein complex.
Quantification of the Histone Fraction

The electrophorograms and banding patterns of basic proteins extracted from cotyledons of 1-, 2-, 3-, and 4-day-old seedlings, as shown on Figures 5 and 6, reveal a dramatic change as germination continues. The most obvious change observed is a reduction of the α protein with respect to the histone fractions. This reduction, as also indicated on Figure 1, is not noticeable between days 1 and 2, but by day 4 the α protein complex has almost disappeared, comprising only 14% of the total basic protein.

The most significant change noted in the histone proteins was an increase in the slightly lysine-rich fractions between days 2 and 4 as shown on Figure 1 and Table III. The lysine-rich f1, as shown on Figure 1 and in Table III, increased between days 1 and 2, followed by a decrease between days 2 and 3 and subsequently remaining constant. The arginine-rich histones showed no significant change on a day-to-day basis, but there was a noted continual decrease in the proportion of arginine-rich histones as time progressed. Data were insufficient to determine if this decrease reflected an actual decrease in the arginine-rich histone : DNA ratio or if it reflected a dilution of the arginine-rich histones by increasing amounts of slightly lysine-rich histones.

In an attempt to get a better understanding of the significance of these changes with respect to genetic activity, the percent composition of the histone fractions was compared to the total nucleic acid composition. The total nucleic acid content, instead of RNA
Figure 5. A comparison of electrophorogram tracings of histones extracted from cotyledons of 1-, 2-, 3-, and 4-day-old pumpkin seedlings.

Histones were prepared as described, omitting the 1 M NaCl extraction, and gels were electrophoresed, stained, destained, and scanned as described in Materials and Methods. Scan a is of histones from cotyledons of seedlings 1 day old, b from seedlings 2 days old, c from seedlings 3 days old, and d from seedlings 4 days old.
Figure 5. A comparison of electrophorogram tracings of histones extracted from cotyledons of 1-, 2-, 3-, and 4-day-old pumpkin seedlings.
Figure 6. Banding patterns of basic proteins extracted from the cotyledons of 1-, 2-, 3-, and 4-day-old pumpkin seedlings.

Histones were prepared as described, omitting the 1 M NaCl extraction, and gels were electrophoresed, stained, destained, and scanned as described in Materials and Methods. a is of histones from cotyledons of seedlings 1 day old, b from seedlings 2 days old, c from 3 day olds, and d from seedlings 4 days old, e from calf thymus.
Figure 6. Banding patterns of basic proteins extracted from the cotyledons of 1-, 2-, 3-, and 4-day-old pumpkin seedlings.
Table III. Basic protein composition in cotyledons of pumpkin seedlings of various ages.

<table>
<thead>
<tr>
<th>Age of cotyledon</th>
<th>% α/total protein</th>
<th>% f1/total histone</th>
<th>% f3/total histone</th>
<th>% f2a2 + f2b/total histone</th>
<th>% f2al/total histone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>70.5</td>
<td>24.4</td>
<td>15.8</td>
<td>40.3</td>
<td>19.5</td>
</tr>
<tr>
<td>2 day</td>
<td>66.7 ± 7.29</td>
<td>27.3 ± 5.47</td>
<td>16.5 ± 4.01</td>
<td>37.8 ± 3.71</td>
<td>18.5 ± 2.93</td>
</tr>
<tr>
<td>2 day salt</td>
<td>31.3</td>
<td>8.8</td>
<td>6.1</td>
<td>61.0</td>
<td>14.7</td>
</tr>
<tr>
<td>3 day</td>
<td>56.3 ± 10.83</td>
<td>20.6 ± 4.84</td>
<td>14.6 ± 3.33</td>
<td>45.1 ± 6.74</td>
<td>19.4 ± 5.4</td>
</tr>
<tr>
<td>3 day salt</td>
<td>18.6</td>
<td>19.9</td>
<td>6.1</td>
<td>62.5</td>
<td>11.5</td>
</tr>
<tr>
<td>4 day</td>
<td>14.0 ± 8.42</td>
<td>21.5 ± 6.48</td>
<td>10.4 ± 1.05</td>
<td>52.2 ± 3.38</td>
<td>16.1 ± 4.8</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>4.1 ± 1.64</td>
<td>10.4 ± 3.71</td>
<td>64.3 ± 5.9</td>
<td>21.0 ± 3.37</td>
<td></td>
</tr>
</tbody>
</table>

a. Plants were harvested at indicated periods after incubation and analyzed for basic proteins as outlined in Materials and Methods. Figures are for 7 extractions for 1- and 2-day cotyledons, 2 for 2½-day cotyledons, 10 for 3-day cotyledons and 4 for 4-day cotyledons.
content, was chosen as a reflection of template activity because more data were available for total nucleic acid content. Each specific repetition was plotted as opposed to plotting mean values. The results of this analysis are shown on Figure 7. As with the other comparison, Figure 1, the arginine-rich histones show a slight but continual decrease in their proportion of total histone as germination progresses.

As indicated on Figure 7, the curve representing the percentage of f1 in the total histone is nearly biphasic, remaining relatively constant around a level of 26% until the total nucleic acid per cotyledon value exceeds 400 μg after which it rapidly decreases, then remains constant around 20%. The total nucleic acid value at the 400 μg point corresponds to a time between the second day and the sixtieth hour, as indicated in Table II. This is near the beginning of a rapid increase in total DNA content per cotyledon, as indicated on Figure 1, and reported by Pegelow (43).

The line representing the percentage of slightly lysine-rich histone (f2a2 and f2b) is also nearly biphasic, remaining constant around 38% until the total nucleic acid per cotyledon value reaches a point around 450 μg, after which it remains reasonably constant at an increased level around 50%. The total nucleic acid content of 450 μg per cotyledon corresponds to approximately the sixtieth hour of germination, as indicated on Table II.
The proportional composition of the total histone complement of cotyledons form 1-, 2-, 3-, and 4-day-old pumpkins, and the total nucleic content of the representative cotyledons for each respective harvest were calculated as described in Materials and Methods. The ratio of each fraction to the total histone was then plotted against the corresponding total nucleic acid composition for each sample.

\[ f_1 \]
\[ f_3 \]
\[ f_{2a} + f_{2b} \]
\[ f_{2a_1} \]
Figure 7. Proportional composition of histones from pumpkin cotyledons compared to the total nucleic acid composition.
CHAPTER 5

DISCUSSION AND CONCLUSIONS

The main objective of this study was to determine if there would be a quantitative change in the histone complement of a rapidly developing plant tissue and if this change would correlate to any change in the synthesis rate of DNA or RNA. The cotyledons of germinating pumpkin seedlings were chosen for this study since Pegelow (43) had previously shown a 5- to 7-fold increase in RNA content between the first and fourth days, and a 3-fold increase in DNA content between the second and third days.

The available data (Figure 1 and Table II) show that the DNA content, during this test, increased 2.5-fold between the first and fourth days. The increase was significant between the second and third days but not between the first and second days or the third and fourth days. These data are in good agreement with Pegelow's (43) results and, since DNA is a stable material, they establish that the rate of DNA synthesis underwent two changes during the time interval studied.

The interpretation of the RNA data was somewhat more complex. Although some RNA analyses were made (Figure 1 and Table II), the analyses of total nucleic acids, which consists of 90 to 95% of RNA, provided a more valid index of the change in RNA content because a number of samples set aside for RNA analysis were lost after the analysis for total nucleic acids were completed. The data (Table II) show that the
total nucleic acids doubled between days 1 and 2, and again between days 2 and 3, but did not significantly change between days 3 and 4. Although analyses for the total nucleic acids for seeds were not included in these data, a comparison was made by Pegelow (43) who detected 150 μg of total nucleic acids in both seeds and cotyledons of 1-day-old seedlings. Since there were significant increases in total nucleic acids content at each tested interval between days 1 and 3, these data were interpreted to indicate that cotyledons had a low template activity around the 24-hr interval and that the activity had subsequently increased. The template activity appeared to have decreased after day 3; however, the lack of a significant increase between days 3 and 4 may have been caused either by increased RNase activity or decreased template activity.

In attempting to relate changes in histone content to changes in template activity, it was found that cotyledons of young seedlings contained a large amount of basic but apparently nonhistone protein, referred to as α protein. The most obvious change of the basic protein composition of cotyledons of germination pumpkin seedlings (Figure 5) was the loss of this protein. The significance of this loss was not clear as the origin and nature of the α protein were not determined. However, it was determined that α protein were not "typical" histones by the electrophoretic mobility, and because Mirsky and Pollister (32) had determined that nucleohistone was soluble in 1 M NaCl while α was only slightly soluble. It was decided that α protein was not a breakdown product of histones because it was present despite modifications
of technique designed to prevent proteolytic activity, also because α was present to a greater extent when less time-consuming procedures were used than when more lengthy techniques were used.

A number of authors (7,16,18,53) have reported the loss of a basic arginine-rich storage protein from cotyledons of developing plants, primarily from cytoplasmic sources. It was decided that α could have been cytoplasmic in origin since it was not tightly bound to DNA, as indicated by its significant reduction by sucrose density gradient centrifugation.

The presence of the α protein undoubtedly added to the error of the quantification technique used for proteins, as gels containing a high amount of α also had a higher background and the base lines were often not well defined. Although the electrophorogram tracings used for quantification purposes were approximately 12 times the size of those shown in Results (Figures 2 and 5), the placement of the base line still had considerable effect on the proportion of the total area under the curve that was under each peak. This was believed to have been the major source of the reported error (Table III).

The values reported here for the proportion of histones in each of the fractions are higher for f1 and lower for f3 than those reported for Dwarf peas by Spiker and Chalkley (51) or for wheat by Spiker and Krishnaswany (52). They are, however, well within the range reported by Panyim and Chalkley (40) for various calf tissues. The value reported here for calf thymus cannot be compared to that reported by Panyim and Chalkley because the histone used here (Worthington Code
H) had been treated with a salt solution and was therefore low in f1 content.

Slightly lysine-rich histone (f2a2 + f2b) increased significantly between the second and fourth days (Figure 1) while the proportion of the other histone fractions remained relatively constant. A more specific time for this change was difficult to assess since the deviation within the 3-day-old sample was so great. In an attempt to clarify the time for this change, the proportional histone composition was plotted as a function of total nucleic acid content (Figure 7). It has been determined (Table III) that the total nucleic acid composition was almost directly related to the seedling age. Each sample was plotted as a function of its own nucleic acid content. This analysis resulted in a more continual spectrum of points that resulted in an almost straight line for the arginine-rich histones and lines with only one break for the lysine-rich and slightly lysine-rich histones. The breaking points occurred as the total nucleic acid contents approached 450 μg per cotyledon. This corresponds to a period between 60 and 72 hr. These results could indicate that there was some degree of internal variance within the 3-day-old samples.

Although changes in the rate of RNA synthesis were not as clearly defined as had been hoped, some information regarding histone composition and nucleic acid synthesis rate was obtained. While all animal systems tested (9, 57, 60, 61) indicated tissues with high template activity also had high proportions of arginine-rich histones, this system showed no apparent quantitative change in the proportion
of either arginine-rich histone (f2a1 + f3) or lysine-rich histone (f1). The relative proportion of the slightly lysine-rich histones (f2a2 + f2b) did not change between days 1 and 2, but increased significantly between days 2 and 4.

Due to the nature of expressing the proportion of the histone complement in each fraction as a percentage of the whole histone, there must be a decrease in one of the other fractions associated with the increase in the slightly lysine-rich fractions. Figure 7 implies that this decrease was primarily associated with f1; however, every fraction appeared to decrease to some degree, and no statistically significant decrease was observed in any of them.

To understand the effect of a change in histone composition, it is necessary to know the manner in which the change in the ratio was brought about. One possibility is that slightly lysine-rich histones were replaced by arginine-rich histones. The other possibility is that the amount of arginine-rich histones remained constant and slightly lysine-rich histones were added to or removed from the total complement of histones. Since these data show the proportion of arginine-rich histone is relatively constant, the increased proportion of arginine-rich histones reported in animal systems (9,57,59,61), and associated with increased template activity, may have been caused by removal of slightly lysine-rich histones while the ratio of arginine-rich histones to DNA remained constant.

In summary, these data indicate that there was no quantitative change in the arginine-rich histones associated with probable changes
in template activity. There was a significant increase in the slightly lysine-rich histones during a period of rapid changes in nucleic acid synthesis rates, but the histone change could not be linked to any specific change in nucleic acid metabolism. Additional information concerning the sequence of events occurring between the second and fourth days about the degree of phosphorylation of the histones and regarding the ratios of each specific histone fraction to DNA is required to fully analyze the relationship of the histones to template activity in cotyledons of germinating seedlings.
REFERENCES CITED


