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TEMPORAL RELATIONSHIPS BETWEEN HISTONE SYNTHESIS AND MODIFICATION AND OTHER METABOLIC EVENTS DURING THE GERMINATION OF *VICIA FABA* EMBRYOS

by

Wayne Scott Stanley

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I hereby recommend that this dissertation prepared under my direction by Wayne Scott Stanley

entitled Temporal Relationships Between Histone Synthesis And Modification And Other Metabolic Events During The Germination of *Vicia Faba* Embryos

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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*This approval and acceptance is contingent on the candidate's adequate performance and defense of this dissertation at the final oral examination. The inclusion of this sheet bound into the library copy of the dissertation is evidence of satisfactory performance at the final examination.

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SIGNED: Wayne Stanley

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ABSTRACT

The temporal relationship of histone synthesis and modification with other metabolic events has been investigated during the early germination stages of excised epicotyls obtained from the dry seeds of *Vicia faba*. The proteins synthesized during the first 12 hours of germination were not affected by the presence of actinomycin D which was responsible for a greater than 50% reduction in protein synthesis at 24 hours of development. The dependence upon RNA synthesis at the 24 hour stage is evident but the source of mRNA translated during the first 12 hours is unclear. The possibility exists that the proteins are synthesized from preformed templates stored in the dormant tissue. However, $H_3{}^{32}PO_4$ was incorporated into the RNA of actinomycin treated embryos to about the same extent as controls at both the 12 and 24 hour stages and, hence, it was not possible to establish the presence of a preformed mRNA. The lack of observable inhibition of $H_3{}^{32}PO_4$ incorporation into the RNA in actinomycin D treated samples could possibly be explained by intensive end labelling of tRNA.

The synthesis and modification of histones was investigated at intervals up to 48 hours of germination. While histone synthesis was evident at all stages, the

highest rate of amino acid incorporation was observed at the earliest stage examined (14 hours). Total histone acetylation was also found to be highest at the earliest stage and to decline with subsequent development. Histones are phosphorylated to a greater extent at 48 hours of germination which also corresponds to elevated levels of DNA synthesis.

The nature of the acetylation occuring at 12 hours of germination was determined. Employing cycloheximide as a translation inhibitor, it was shown that most of the acetylation was due to side chain modification following protein synthesis although there was some N-terminal acetyl incorporation. This post-translational acetylation may be a prerequisite for the greater extent of genomic derepression, reflected in the higher level of protein synthesis, observed at 24 hours of germination.

The particular histone fractions acetylated and phosphorylated were determined after separation of total histones by electrophoresis on polyacrylamide gels. The histones were resolved into four major bands with fractions f3 and f2b migrating with the same mobility. The lysine-rich fraction f1 was slightly acetylated and this was due entirely to N-terminal acetyl incorporation. The other fractions, f2a2, f3 and f2b, and f2a1 were also acetylated to varying degrees, most of this due to post-translational acetyl incorporation. Only the lysine-rich fraction f1 was phosphorylated to any appreciable extent.

INTRODUCTION

Ontogeny is marked by progressive changes, manifested at a morphological level but determined by underlying molecular interactions. The molecular basis of development is often explained by the transfer of genetic information from deoxyribonucleic acid (DNA) to ribonucleic acid (RNA) and finally to the amino acid sequence of proteins. However, this unidirectional flow of information does not, in itself, explain the mechanism(s) of genomic control of development, nor does it necessarily include other types of molecular activities which may participate in shaping early developmental events.

Evidence from both plant and animal sources has suggested an early cytoplasmic direction of development prior to later genomic activity which ultimately is necessary to specify further ontogenetic events. Consequently, knowledge of the macromolecular activities which occur following the initiation of developmental processes offers an opportunity of elucidating some of the sequential changes and mechanisms responsible for directing early morphogenesis.

In this study, the activity of many molecular species has been investigated in an embryonic plant tissue, the epicotyl excised from the seeds of Vicia faba during the course of germination. The investigation was designed to achieve a better understanding of some of the temporal relationships which may exist during early development and thus to shed some light on the types of interactions and activities occuring at both cytoplasmic and nuclear levels. The state of genetic repression evident in the dormant tissue is overcome by imbibition and, hence, the developmental processes initiated during this period of gene activation or derepression are central to the mechanism(s) of gene regulation in eukaryotes.

The temporal patterns of protein and RNA synthesis have been analyzed with respect to other chromosomal activities reflected in the synthesis of histones and DNA. Additionally, side chain modification of total histone proteins by means of acetylation and phosphorylation was examined and the extent of such modification in individual histone fractions was determined. Such information will be necessary not only in ascertaining the general biological role of histones but also the specific functions of individual histone species. Furthermore, the genomic derepression accompanying the initial stages of germination may depend upon the observed modification of histones. The molecular activities determined from this plant tissue have striking parallels with those observed from animal sources. This consequently suggests that the basic mechanisms responsible for morphogenesis in all eukaryotes are essentially the same. This study also clearly demonstrates the capability of this excised tissue to undergo independent developmental processes during the time periods examined. This observation lends support to the concept of programmed events during the initial stages of development.

LITERATURE REVIEW

Evidence for the Existence of Stable Preformed Messenger RNA in Embryonic Tissue

The initiation of growth and the differentiation of embryonic tissues is dependent upon protein synthesis. However, experimental evidence gathered from a number of different organisms has suggested that messenger RNA (mRNA) necessary to direct protein synthesis following fertilization or during the early stages of germination need not be synthesized *de novo*. In sea urchins, Denny and Tyler (1964) have demonstrated that non-nucleated fragments of unfertilized eggs which are activated parthenogenetically are capable of protein synthesis. In addition, Gross and Cousineau (1964) have shown that protein synthesis in fertilized sea urchin eggs is unaffected by concentrations of actinomycin D sufficient to inhibit the synthesis of RNA.

Parallel studies conducted during amphibian development have revealed this same phenomenon. Brachet, Denis, and DeVitry (1964) have observed that actinomycin D has little effect upon cleavage when this antibiotic is microinjected into fertilized amphibian eggs. Further evidence for the existence of a preformed mRNA is gained from the experiments of Smith and Ecker (1965) who demonstrated that tritiated leucine injected into enucleated Rana pipien eggs was significantly incorporated into protein. Denis (1964) has also observed in Pleurodeles development that actinomycin inhibits protein synthesis to a lesser degree in blastula and gastrula cells than during later developmental stages.

In certain coleopteran insects, Lockshin (1966) was able to detect DNA and protein synthesis during early development in the absence of RNA synthesis. During early tunicate development (Smith 1967), a maternally-derived mRNA is apparently also responsible for directing protein synthesis which continues in the presence of actinomycin D despite an inhibition of RNA synthesis. Although actinomycin will arrest early mammalian development, Mintz (1964) has found that protein synthesis does continue for a time with an inhibition of RNA production.

The above examples indicate that early development in many different animal species is accomplished by translating maternally-stored mRNA following fertilization. The utilization of preformed stable mRNA's however is not confined to animal systems. During the early stages of germination of some plant species, mRNA stored in the dry seed appears to be responsible for directing protein synthesis following imbibition. During the first sixteen hours of germination of cotton seeds, Dure and Waters (1965) found no decrease in the amount of protein synthesized when actinomycin D is added to the medium at a concentration which inhibits most all pulse labelled nuclear RNA synthesis. Proteins synthesized during the germination of isolated lima bean axes are also not affected by actinomycin which does inhibit net RNA synthesis (Klein, Barenholz, and Budnik 1971). Using a different approach, Chen, Sarid, and Katchalski (1968) have also presented evidence for a preformed mRNA in the dry wheat embryo. In their study, RNA extracted from dry embryos and used in a cell-free amino acid incorporating system was successful in directing protein synthesis. In addition, this RNA was found to compete with pulse labelled RNA from 24-48 hour germinating embryos in hybridization competition experiments with DNA.

The Delayed Synthesis of Other Gene Products During Early Development

The above experiments indicate that genomic activity with respect to the synthesis of mRNA is, in many cases, not necessary for the early stages of development. The concept of nuclear repression during the initial stages of development is further strengthened by the observation in many species of a delayed synthesis of other gene products.

During the cleavage and early blastula stages of sea urchin development, Comb and Brown (1964) have observed a loss of rRNA and a net decrease in nuclear RNA. In a different species of sea urchins, Nemer and Infante (1967) found a peculiarity of the rRNA stored in the unfertilized egg. Heating of the maternally-derived 18S rRNA briefly at

$60^{o}C$ converted the RNA to a 13S molecule, while newly synthesized embryonic rRNA was stable to this treatment. Using the fragility of this rRNA (18S) as a marker, the authors were able to demonstrate a decrease in maternally-derived rRNA during cleavage and the early blastula stages. It is not until the mesenchyme blastula stage that new embryonic rRNA is synthesized. Wilt (1964) was also unable to detect the incorporation of ^{3}H-uridine into rRNA during cleavage stages. However, in his experiments, he did detect small amounts of labelling in an RNA fraction which sedimented in a heterodisperse fashion, suggesting the synthesis of mRNA. Likewise, Gross, Kraemer, and Malkin (1965) have found that the base composition of the RNA synthesized during cleavage stages is DNA-like and consequently suggest that this species is mRNA. In addition, these authors have found a large degree of end-labelling of tRNA and suggest that this is the fraction of RNA which was previously found to be insensitive to actinomycin (Malkin, Gross, and Romanoff 1964). Experiments performed by Glisin and Glisin (1964) have also demonstrated an uptake of labelled phosphorus into the pCpCpA terminal end of tRNA during cleavage stages. The isolation and purification of RNA obtained from nuclear, microsomal, and ribosomal-transfer RNA fractions by Comb et al. (1965) have indicated that during cleavage stages no detectable RNA was synthesized. Some mRNA was formed during the blastula stage but it was not until after gastrulation that newly synthesized tRNA and rRNA appeared. This delayed synthesis of rRNA has also been reported by Wilt (1964) and Nemer (1963). Thus, the collective weight of the above experiments indicate that pregastrula sea urchin development is largely independent of embryonic genomic control.

Similar studies conducted on the amphibian *Xenopus laevis* have shown a striking similarity to early sea urchin development. Brown and Littna (1966a) were unable to detect any synthesis of 4S RNA during ovulation and the early cleavage stages. However, end labelling of the pCpCpA nucleotides of this species of RNA was apparent during these times. It was not until the late blastula stage that these authors were able to detect the synthesis of tRNA. In a radioautographic study, Bachvarova and Davidson (1966) observed that nuclear RNA synthesis occurred sharply just prior to gastrulation. In a further study, Bachvarova et al. (1966) have described that the onset of gastrulation is accompanied by the synthesis of an RNA which appears to be mRNA. End-group turnover of tRNA was also observed and the synthesis of this species of RNA is delayed until the onset of pregastrular activation.

Like sea urchin development, small amounts of mRNA are synthesized during early development. Brown and Gurdon (1966) have observed in anucleolate embryos of *Xenopus*, the synthesis of a high molecular weight RNA which resembles

DNA in base composition. In another study, Brown and Littna (1966b) have shown that DNA-like RNA (mRNA) is synthesized to the extent of 1 to 6 mμg during ovulation which represents 0.02 to 0.1% of the total RNA present in the egg. Development up to the swimming stage is marked by an increase in this RNA to about 2.0%. The synthesis of rRNA during Xenopus development (Brown and Littna 1964) does not begin until the onset of gastrulation. Additional evidence that protein synthesis is effected on maternally derived ribosomes is provided by Brown and Gurdon (1964) who demonstrated that homozygous anucleolate mutants in Xenopus laevis which are unable to synthesize 28S and 18S rRNA do survive to the swimming tadpole stage. Maternally stockpiled ribosomes may also be responsible for the proteins which are synthesized during the early development of the milkweed bug. Harris and Forrest (1967) have found in Oncopeltus fasciatus that rRNA synthesis does not begin until gastrulation. Knowledge of the synthesis of individual species of RNA during the early stages of plant germination is less complete but there are some indications that tRNA and rRNA can be stored in the dry seed and utilized during the initial stages of germination much like preformed mRNA discussed previously. Waters and Dure (1966) have shown that ribosomes and polysomes are synthesized during the first 16 hours of germination in cotton seeds but the application of actinomycin D to the extent of a 70% inhibition of ^{32}P

incorporation in RNA does not diminish the total ribosomal population or cause a shift of polysomes to ribosomes. Studies by Key and Ingle (1964) and Key (1966) have demonstrated that the pyrimidine base analogue, 5-fluorouracil, is selective in inhibiting the synthesis of ribosomal and transfer RNA. Experiments conducted on soybean hypocotyls excised after three days of germination indicate that 5-fluorouracil does not affect growth or protein synthesis. This treatment, however, does not prevent the synthesis of one species of RNA which, on the basis of a number of criteria, was identified as mRNA. These results, though, cannot distinguish whether the ribosomes and tRNA's used for growth were present in the dry seed. Walton and Soofi (1969) however, found that 5-fluorouracil inhibited the incorporation of ^{32}P into transfer and ribosomal RNA but did not prevent protein synthesis or growth in excised embryonic axes of <u>Phaseolus vulgaris</u> following imbibition. Chen, Shultz, and Katchalski (1971) have shown that in wheat embryos the synthesis of rRNA begins after 2 hours of germination. However, the rRNA transcribed is cleaved and incorporated into immature ribonucleoprotein particles which do not reach maturation until 6-12 hours of germination.

The examples cited above illustrate what may be a general phenomenon in both early plant and animal development. The initiation of ontogenetic processes following fertilization or imbibition may be completely dependent upon previously synthesized and stored gene products which are utilized prior to the activation or derepression of the genome.

One paradox which arises in some cases is the synthesis of small amounts of mRNA which apparently are not necessary for early development. This problem is partially resolved by a qualitative examination of the mRNAs formed. Employing RNA-DNA competition hybridization experiments, Whitely, McCarthy, and Whitely (1966) and Glisin, Glisin, and Doty (1966) have demonstrated that mRNAs synthesized up to the blastula stage are qualitatively similar to those stored in the unfertilized egg. Later development is marked by the gradual disappearance of this population of RNA molecules and the appearance of new mRNAs presumably reflecting new patterns of gene activation necessary for differentiation. In addition, chromatin isolated from pluteus stage sea urchin embryos (Marushige and Ozaki 1967) supported *in vitro* more DNA-dependent RNA synthesis than similar preparations obtained from the blastula stage. It was also observed that pluteus chromatin contains slightly less histone than blastula chromatin, hence suggesting the possible role of these proteins in the repression of the genome.

Evidence That Histones Are Repressors Of Genetic Activity

Histones are a group of basic proteins found electrostatically bonded to the DNA in the chromosomes of all eukaryotes. Although the exact number of histone species cannot be stated with certainty, there are at least five major fractions found in all higher plants and animals. The biological role of these proteins has not been precisely determined but a number of investigations have clearly implied that histones are repressors of gene activity.

Huang and Bonner (1962) have shown that DNA as it is found in chromatin is a poor template for the synthesis of RNA compared with DNA itself. However, when the histone proteins are removed, there is a five fold increase in the DNA-dependent RNA synthesis. In a further study using rat liver chromatin, Marushige and Bonner (1966) demonstrated that RNA polymerase binds as well to chromatin as it does to DNA. Again, in this system, however, the presence of histones lowers the template activity by a factor of five.

Using isolated thymus nuclei, Allfrey, Littau, and Mirsky (1963) were able to show that histones added to the incubation medium caused a reduction in RNA synthesis. Furthermore, the degree of inhibition was related to the species of histone added. The arginine-rich fraction was the most inhibitory while the lysine-rich fraction was the least. Not only have histones been implied in the repression of transcription but Hnilica and Billen (1964) found that histones were also effective in the inhibition of DNA synthesis *in vitro*. In addition, they found that the inhibition differed with different histone fractions. The lysine-rich histone fraction stopped DNA synthesis at the lowest concentration of protein while higher values were required for the moderately lysine-rich and arginine-rich fractions.

If the biological role of histones is to repress genetic activity, then the question arises as to how this inhibition is overcome at times of transcription or replication. One leading hypothesis to explain this situation is that side chain modification of histones by acetylation, phosphorylation, or methylation reduces the net positive charge of the molecules and, hence, loosens the bonds to the DNA. Experimental evidence in support of this idea has come from a number of sources. Allfrey, Faulkner, and Mirsky (1964) studied RNA synthesis in calf thymus nuclei using arginine-rich histones and acetylated arginine-rich histones. It was observed that untreated arginine-rich histones inhibited RNA synthesis by approximately 66% while acetylated ones inhibited transcription only to the extent of 21%. It was further demonstrated that this decrease in inhibition was not due to the inability of the protein to bind to the DNA. Thermal denaturation studies showed similar melting profiles for both types of nucleohistone complexes.

A temporal investigation of histone acetylation and DNA-dependent RNA synthesis during regeneration of the rat liver has been conducted by Pogo et al. (1968). It was demonstrated that maximum acetylation occurred in several arginine-rich fractions at 3-4 hours following partial hepatectomy. During this same time period, there is extensive RNA synthesis which reaches a plateau at 6 hours. Similarly, human and equine lymphocytes stimulated with phytohemagglutin show an increase in histone acetylation just prior to an increase in RNA synthesis (Pogo, Allfrey, and Mirsky 1966).

Ord and Stocken (1968) have also found in regenerating rat liver a correlation between the phosphorylation of the lysine-rich histone f1 and the period of DNA synthesis. These same authors (Ord and Stocken 1969) showed a phosphorylation of the arginine-rich histone fraction f3 early in regenerating rat liver. This initial phosphorylation occurs some 15 hours before the phosphorylation of f1 histones and a second period of phosphorylation occurs concomitantly with f1 histone phosphorylation at the time of DNA synthesis. Sung, Dixon, and Smithies (1971) have also observed in regenerating rat liver the phosphorylation of a slightly lysine-rich fraction at 15 hours following partial hepatectomy.

Hence, the available evidence indicates that genomic repression is mediated by histone proteins while activation or derepression of the DNA could be effected by the modification of histone molecules.

Histone Metabolism During Development

In an attempt to elucidate the mechanisms responsible for the changing patterns of gene activity during development, many investigations have been conducted almost exclusively on animal systems to correlate changes with respect to histones and certain developmental events. Unfortunately, however, there is little unanimity among the results obtained from the same or similar species nor distantly related organisms.

Electrophoretic separation of histones extracted from sea urchin embryos by Vorobyev, Gineitis, and Vinogradova (1969) has shown major differences between the histones extracted at the blastula and gastrula stages. The authors identified a decrease and the disappearance of some fractions belonging to the arginine-rich group of histones in gastrula samples. Ord and Stocken (1970) have also described quantitative changes of the different histone species during development. Among these changes was a decrease in the sulphur-containing proteins (f3) during cleavage and an increase in the lysine-rich fraction f1 through gastrulation. Benttinen and Comb (1971) have also observed quantitative changes during sea urchin development. During the cleavage stages, a slightly lysine-rich histone was found to predominate while there were increases in both lysine-rich and arginine-rich fractions from the 32-cell stage to the mesenchyme blastula stage. Qualitative changes were detected by Spiegel, Spiegel, and Meltzer (1970) during the early development of both amphibians and sea urchins. At gastrulation in both *Rana pipiens* and *Arbacia punctulata* one new basic protein appeared and at this same stage in the salamander *Ambystoma maculatum*, three new proteins were observed.

Orengo and Hnilica (1970) studied the incorporation of labelled amino acids into the acid-soluble nuclear proteins of sea urchins. Their results indicated that between the 4-8 cell stage and early blastula arginine-rich fractions were the first to appear in the nucleus. It was not until later stages, hatching blastula and gastrula, that lysine-rich proteins were detected. Other experiments done on this organism have indicated that there are essentially no qualitative differences among the histones between blastula and pluteus stages (Marushige and Ozaki 1967). Repsis (1967) also did not observe any qualitative changes between these two stages, although before blastulation some differences were apparent.

During chick development, the results from electrophoretic studies performed by Kischer, Gurley, and Shepherd (1966) and Lindsay (1964) have indicated that there are no essential changes in the histones during early developmental stages. However, in contrast to this, Agrell and Christensson (1965) did detect differences during chick development. Between 2 and 5 days of development these authors found a gradual change to an increased amount of the slightly lysine-rich fraction f2 and a lower quantity of the lysine-rich fraction f1 which resembles the pattern they observed in adult tissue.

During plant development, Srivastava (1971) studied changes in RNA synthesizing activity and histones in young leaves, senescent leaves, and pith tissue of tobacco. There were changes observed in the ratio of f1:f2:f3 histone fractions in the 3 tissues examined. Old leaves and pith tissues which represent more differentiated tissues had more f2 and f3 histones than young leaves.

Fambrough, Fujimura, and Bonner (1968) have made a comparative study of histones extracted from different organs of the pea plant, including embryonic tissues, and were unable to detect qualitative differences although quantitative changes were evident. Likewise, quantitative differences (McDaniel 1972) were demonstrated between histones extracted from barley shoots and dry germ preparations. In addition, there was observed a marked decline in the quantity of histones extractable from scutellum tissue during the first four days of germination.

The examples cited above do not allow the organization of the data into any one pattern which may reflect a general scheme of histone changes during developmental periods. Any real differences which may occur have been presumably obscured in the varied techniques employed by different investigations. Additional experiments and different approaches will be necessary to resolve these apparent discrepencies and answer the questions orginally proposed.

MATERIALS AND METHODS

Epicotyls used in all experiments were carefully removed from dissected seeds of *Vicia faba L.* (var. long pod) obtained from W. Allee Burpee Company.

Extraction of Total Cellular Protein

Approximately 60 mg of embryonic tissue was ground in 5.0 ml of 0.05 M Tris buffer, pH 7.5, in a Potter-Elvehjem homogenizer. When fresh tissue (not previously frozen) was employed, 2 mg of unlabelled leucine were added per ml of solution during homogenization. The homogenate was made 5% with trichloroacetic acid (TCA) and stirred on ice for 15 minutes. Following centrifugation at 4000 x g for 10 minutes, the precipitate was washed with 5% TCA and recentrifuged as above. The pellet obtained was heated in a boiling water bath for 15 minutes with 5% TCA and centrifuged at 4000 x g for 10 minutes. The precipitate was then successively washed with 5% TCA and 70% ethanol and then allowed to dry.

Total Protein Synthesis at 12 and 24 Hours of Germination

Epicotyls were incubated in aqueous solutions in small beakers (15-25 ml size) and capped with parafilm.

All incubations were done in the dark and at room temperature unless otherwise noted and the proteins were extracted as described above.

Treatment With Actinomycin D - 12 Hour Stage

Samples containing 10 excised epicotyls were imbibed for 2 hours in either 1.0 ml of sterile water containing 100 units of penicillin G (controls) or in water containing the penicillin G and actinomycin (20 µg/ml). Following this initial imbibition period, the epicotyls were incubated in 1.2 ml of sterile water containing 0.1 µc of ^{14}C-L-leucine (sp. act. 312 mc/m mole; Schwarz/Mann) and 100 units of penicillin G (controls) or this solution containing actinomycin D at a concentration of 20 ug/ml. These incubations continued for 10 more hours.

Treatment With Actinomycin D - 24 Hour Stage

Control epicotyls were treated as above except that the isotope was added at 12 hours of germination and labelling continued until the 24 hour stage. Actinomycin treated samples were germinated for the full 24 hours with the antibiotic at a concentration of 20 ug/ml.

Treatment With Cycloheximide

Epicotyls were treated for both the 12 and 24 hour stages in an identical fashion as the previous experiment with the exception that cycloheximide was the antibiotic substituted and the final concentration was 8 33 ug/ml.

Extraction of Total RNA

During the extraction of RNA, all operations were carried out at 0-4°C unless otherwise noted. The procedure followed is essentially that of Hiatt (1962). The frozen tissue (100 epicotyls) was homogenized at full speed in a Waring blender in 25 ml of a solution consisting of 0.25 M sucrose, 0.05 M Tris buffer, pH 8.0 and 0.001 M $MgCl_2$. Sodium lauryl sulfate was added to the homogenate such that the final concentration was 1% and an equal volume of 90% phenol containing 0.1% 8-hydroxyquinoline was added. The solution was shaken for ½ hour followed by centrifugation for 10 minutes at 8000 x g. The aqueous layer was removed and re-extracted twice more using ½ volume of phenol with shaking for 15 minutes before centrifugation for 10 minutes at 10,000 x g. To the aqueous phase was added NaCl to a concentration of 0.1 M and ethanol to a concentration of 67% before storage at 4°C for at least 24 hours. The precipitates were removed by centrifugation and dissolved in 5.0 ml of 0.01 M Tris buffer, pH 7.4, containing 0.001 M $MgCl_2$. To this was added 100 µg of DNase (Worthington Biochemical Co.) and the solution was incubated for 2 hours at 28°C. After this incubation, sodium lauryl sulfate was added to a concentration of 1% and ½ volume of phenol was added with shaking for 15 minutes. The aqueous phase obtained after centrifugation for 10 minutes at 10,000 x g was treated with NaCl and ethanol as described above. The solution was stored for ½ hour at -15°C and the precipitate obtained after centrifugation was dissolved in 2.0 ml of 0.1 M NaCl - 0.01 M acetate buffer, pH5.0, and then centrifuged for 10 minutes at 10,000 x g. To the supernatant was added potassium acetate to a concentration of 2.0 M and then 95% ethanol was added dropwise until a final concentration of 20% had been reached. The precipitate formed after 30 minutes was collected by centrifugation for 10 minutes at 10,000 x g and then dissolved in 2.0 ml of 0.001 M $MgCl_2$ and precipitated as above with potassium acetate and ethanol. This step was repeated once more and the precipitate was washed twice with 95% ethanol and then dissolved in 0.001 M $MgCl_2$, 0.1 M NaCl and 0.01 M potassium acetate, pH 5.2. The RNA concentration was determined spectrophotometrically at a wavelength of 260 mμ and assuming an extinction coefficient of 25. The 280/260 ratio was also determined and in all cases was less than 0.5. The RNA was precipitated out of solution with 2 volumes of 95% ethanol and transfered to 2.5 cm paper discs for scintillation counting.

Total RNA Synthesis at 12 and 24 Hours of Germination

Treatment With Actinomycin D - 12 Hour Stage

Samples of 100 epicotyls were used throughout this experiment. Control samples were placed in sterile water containing 1000 units of penicillin and after 2 hours of germination, the embryos were transferred to small beakers containing 1.2 ml of sterile water, 1000 units of penicillin, and 200 µc $H_3{}^{32}PO_4$ (carrier-free; New England Nuclear). After 10 hours incubation, the tissues were washed with distilled water, frozen in liquid nitrogen and stored at -15°C. Treatments receiving actinomycin D were initially incubated in 1.2 ml of sterile water containing 1000 units of penicillin and 20 µg of actinomycin D. After 2 hours imbibition, these epicotyls were transferred to beakers containing 1.0 ml of sterile water, 1000 units of penicillin, 0.2 ml of actinomycin (120 ug/ml), and 200 µc $H_3{}^{32}PO_4$. After 10 hours incubation, the epicotyls were washed, frozen, and stored at -15°C.

Treatment With Actinomycin D - 24 Hour Stage

Control samples of 100 epicotyls were germinated in sterile water containing 1000 units of penicillin for 12 hours, then transferred to beakers containing 1.2 ml of sterile water, 1000 units of penicillin, and 200 µc $H_3{}^{32}PO_4$. After 12 hours incubation in this medium the epicotyls were frozen and stored as described above. Actinomycin treated samples were germinated for 12 hours in sterile water containing actinomycin D at a concentration of 20 µg/ml and 1000 units of penicillin before being transferred to beakers containing 1.2 ml of sterile water with 20 µg actinomycin D, 1000 units of penicillin, and 200 µc $H_3{}^{32}PO_4$. After 12 hours of continuous incubation in this medium, the epicotyls were washed, frozen, and stored at -15°C. The RNAs from the above samples were extracted as previously described.

Histone Extraction

The procedure used for the isolation of histones from chromatin is a modification of that of Huang and Bonner (1962). All operations were carried out at 0-4°C. Embryonic tissue was homogenized in 40 ml of a grinding solution (0.25 M sucrose; 0.05 M Tris, pH 8.0; 0.001 M $MgCl_2$) in a Waring blender equipped with an ice jacket at full speed for 1 minute. This homogenate was then successively filtered through a single layer of cheesecloth and a double layer of Kimwipe (Kimberly-Clark) to remove tissue fragments and unbroken cells. The filtered homogenate was centrifuged for 10 minutes at 4000 x g and the pellet containing the nuclei was washed by resuspension in approximately 10 ml of grinding solution followed by centrifugation for 10 minutes at 10,000 x g. The pellet collected was homogenized in approximately 10 ml of 0.01 M Tris, pH8.0 using a Potter-Elvehjem homogenizer with a motor driven pestle. The chromatin was removed from solution by centrifugation for

10 minutes at 10,000 x g. This step was repeated four more times. Before each homogenization, the starch is scraped away from the chromatin pellet and discarded. The Tris washed pellet was homogenized as above in a 0.15 M NaCl, 0.015 M sodium citrate solution which precipitates nucleoprotein and is easily pelleted by centrifugation for 10 minutes at 4000 x g. This step was repeated twice.

Histones were removed from the chromatin obtained as above by incubation of the pellet with 0.4 N H_2SO_4 for 1 hour. During this time the pellet was slightly and occasionally homogenized with either a glass stirring rod or loose fitting glass pestle. The acid soluble histones were obtained in the supernatant following centrifugation for 10 minutes at 4000 x g.

Histone Synthesis During Early Stages Of Germination

Epicotyls (100) were germinated in 5.0 ml of sterile water for 14, 24, 36, and 48 hours. All samples incubated for over 36 hours had the water changed at that time. For the last 12 hours of each germination period, the epicotyls were thoroughly washed with distilled water and transferred to small beakers containing 0.5 ml of sterile water and 2.0 μc of ^{14}C-L-leucine (sp. act. 312 mc/m mole; Schwarz/Mann). After 12 hours of labelling, the epicotyls were washed, frozen in liquid nitrogen, and stored at -15°C. Histones were extracted as previously described and neutralized with

NaOH before being applied to paper discs for scintillation counting. In addition, 0.1 ml samples of the cellular homogenates were applied to discs in order to estimate the relative intracellular concentration of the isotope at these various stages.

Histone Acetylation and Phosphorylation During Early Stages of Germination

Epicotyls (100) were germinated in 5.0 ml of sterile water for 12, 24, 36, and 48 hours. All samples incubated for over 36 hours had the water changed at that time. For the last 2 hours of each germination period, the epicotyls were washed with distilled water and transferred to small beakers containing 0.5 ml of sterile water and 12.5 μc sodium acetate-1-^{14}C (sp. act. 62 mci/m mole; Amersham/Searle) or 400 μc $H_3{}^{32}PO_4$ (carrier-free; New England Nuclear). Following this period of labelling, the epicotyls were washed, frozen in liquid nitrogen, and stored at -15°C. Histones were extracted as previously described and neutralized with NaOH before being applied to paper discs for scintillation counting. In addition, 0.1 ml samples of the cellular homogenates were applied to discs in order to estimate the relative intracellular concentration of the isotope.

DNA Synthesis and Extraction During Early Stages of Germination

The chromatin pellets with the histones removed from the phosphorylation experiments described above were incubated with 0.3N KOH for 1 hour at 37°C. The solution was then acidified with 10% TCA and centrifuged for 10 minutes at 4000 x g. The supernatant containing hydrolyzed chromatin-bound RNA was discarded and to the pellet was added 5% TCA and the mixture was heated for 15 minutes in a boiling water bath. Following centrifugation, 0.1 ml samples of the supernatant were taken for scintillation counting on paper discs to estimate the relative incorporation of the isotope into the DNA.

12 Hour Protein Synthesis - 3 Hour Incubation With Cycloheximide

Epicotyls (20) were germinated in small (15-25 ml size) beakers containing 1.0 ml of sterile water and 100 units of penicillin for 9 hours. The samples were then given 0.2 ml of water or cycloheximide (6 mg/ml) for 1 hour. At the tenth hour of germination, control epicotyls were transferred to beakers containing 1.2 ml of sterile water containing 0.1 µc ^{14}C-L-leucine (sp. act. 312 mc/m mole; Schwarz/Mann) and 100 units of penicillin. Samples treated for 1 hour with cycloheximide were likewise transferred to beakers containing 100 units of penicillin and 0.1 µc ^{14}C-L-leucine in a solution of cycloheximide at a concentration of 1 mg/ml. After two hours of incubation with the isotope, the embryos were washed with distilled water, frozen in liquid nitrogen, and stored at -15°C. The amount of leucine incorporated into cellular protein was determined as previously described.

12 Hour Histone Acetylation - 3 Hour Incubation With Cycloheximide

Epicotyls (100) were germinated in small beakers containing 2.0 ml of sterile water for 9 hours. Cycloheximide was added to experimental samples as described above. At the tenth hour of germination, control epicotyls were transferred to beakers containing 0.5 ml of sterile water and 50 µc sodium acetate-1-C^{14} (sp. act. 62 mci/m mole; Amersham/Searle). Samples treated for 1 hour with cycloheximide were transferred to beakers containing 50 µc of the isotope and cycloheximide at a concentration of 1 mg/ml. Germination continued for 2 additional hours in the isotope before the epicotyls were washed, frozen in liquid nitrogen, and stored at -15°C. Histones were extracted as previously described and aliquots were taken for scintillation counting, gel electrophoresis, and the determination of protein concentration.

DNA and Protein Determinations

DNA concentrations were determined colorimetrically by the diphenylamine method (Dische 1955) using calf thymus DNA (Nutritional Biochemical Co.) as a standard. Likewise, protein concentrations were determined using the procedure of Lowry et al. (1951) and employing bovine serum albumin

(Nutritional Biochemical Co.) as a standard. Acid extracted histones were neutralized with an equivalent amount of base as NaOH before protein determinations were made. The histone concentrations measured in this fashion will be underestimated due to the lower tryptophane content in histones.

Scintillation Counting

Radioactive samples were counted in a Packard Tricarb (Model 3320) liquid scintillation spectrometer. Samples were counted in vials using 10 ml of 0.55% 2,5-dipenloxozole (PPO), 0.01% 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) and a solution of 33% Triton x-100 (Rohm & Haas) and 67% toluene.

Electrophoresis of Histones

The procedure employed was a slightly modified version of Johns (1967). Plexiglass tubing 7-cm in length with a 7 mm inside diameter were used as supports for polymerization of polyacrylamide gels. Prior to use, the gel tubes were immersed in a 1% silicone solution (Siliclad, Clay Adams) and air dried. The stock gel solutions are as follows: (1) Monomer solution consisting of 30% acrylamide (Cyanogum 41 gelling agent, E-C Apparatus Corp.) and 5.0 M urea dissolved in distilled water, (2) catalyst solution A consisting of a 0.6% aqueous solution of ammonium persulfate (AP), and (3) catalyst solution B consisting of a 0.5% solution of N,N,N,'N'-tetramethylethylenediamine (TMED) in

4.6 N acetic acid. The gel solution was prepared by mixing the above reagents in a 2 monomer: 1 catalyst A: 1 catalyst B ratio. This mixture was poured into the plexiglass tubes and was overlayered with a few drops of water. Complete polymerization was accomplished between 1½ - 2 hours.

Polymerized gels were "pre-run" by electrophoresis at room temperature with a 0.01 N acetic acid buffer, pH 3.55, for 1½ hours at a constant current of 2.5 ma/tube employing a regulated high voltage power supply (Heathkit, Model 1P-17).

Histones were prepared for electrophoresis by making the acid extract 0.5 M with respect to 2-mercaptoethanol and incubating this on ice for ½ - 1 hour. Approximately 0.025 mg of histone was layered on top of the gels and electrophoresis was carried out with fresh 0.01 N acetic acid at room temperature for 2 hours at a constant current of 2 ma/tube. During this time the voltage rises from approximately 190 to 280 volts.

Removal of the gels was facilitated by cooling on ice and rimming the edges with water in a syringe fitted with a 22-gauge hypodermic needle. Gels were stained overnight in a 0.1% amido-black - 7% acetic acid solution and destained electrically in 7% acetic acid (Canalco Quick Destainer #1801).

Stained gels were scanned at 600 mμ with a Gilford spectrophotometer (model 240) equipped with a linear transport (model 2410) and chart recorder (Honeywell, Model Y143X). The relative area of histone fractions were determined by cutting out the peaks and weighing the paper. To detect radioactivity in histone fractions, the gels were frozen on dry ice and individual bands were cut out with a razor blade and digested in scintillation vials with 0.5 ml of 30% H_2O_2 overnight at 60^oC. These samples were counted as previously noted.

RESULTS AND DISCUSSION

Protein and RNA Synthesis

It is generally viewed that dormancy in seeds is accompanied by genetic repression. Studies by Tuan and Bonner (1964) have demonstrated that chromatin obtained from dormant potato tubers is ineffective in the support of DNA-dependent RNA synthesis. However, when dormancy is broken with ethylene chlorohydrin, isolated chromatin does support RNA transcription with the addition of exogenous RNA polymerase. In addition, Marcus and Feeley (1964) have shown that microsomal preparations from dry peanut seeds are ineffective in the incorporation of amino acids into protein.

The repression which grips dormant tissue in seeds can be easily overcome by imbibition. Presumably, the uptake of water sets in motion precise molecular processes necessary for the initiation and direction of early developmental events. As noted previously, there are many instances where development, as measured by protein synthesis, occurs in the absence of RNA production. These experimental observations have led to the concept of preformed stable mRNA's which are activated following fertilization or germination and are utilized to direct protein synthesis during the initial stages of development.

The pattern of protein synthesis was investigated by incubating excised epicotyls from dry seeds in aqueous solutions containing various metabolic inhibitors (Table 1). Cycloheximide, an inhibitor of protein synthesis conducted on 80S cytoplasmic ribosomes, was shown to be effective in blocking protein synthesis at both 12 and 24 hours of germination. Aside from demonstrating the dependence upon protein synthesis during these early developmental periods, knowledge that this antibiotic is permeable to the cells of this tissue provides a useful tool for investigations that will be discussed later. It will be noted in Table 1, however, that actinomycin D, a transcription inhibitor, did not reduce the incorporation of amino acids into protein to any substantial degree at the 12 hour stage. This may be taken to indicate that this antibiotic is not being absorbed into the cells during this time or that protein synthesis is proceeding in the absence of RNA synthesis. If the latter is the case, the proteins synthesized could be accounted for by the translation of preformed mRNA's stored in the dormant epicotyl. When this investigation is extended to 24 hours of germination, the presence of actinomycin D inhibits protein synthesis by better than 50%. It is clear that at this stage, actinomycin D is capable of penetrating the cells and that protein synthesis is at least partly dependent upon RNA synthesis. The datain Table 1 may also indicate an elevated rate of protein synthesis during these developmental

Table 1. Effect of actinomycin D and cycloheximide on the incorporation of ^{14}C-leucine into protein during germination. -- Two replications containing 10 excised epicotyls were used in each treatment. Control samples incubated for 12 hours were initially germinated for 2 hours before being transferred to sterile water with 0.1 µc ^{14}C-leucine and penicillin for an additional 10 hours. Treatments receiving actinomycin or cycloheximide were treated identically except that the inhibitors were added after the first 2 hours of imbibition. Epicotyls germinated for 24 hours were treated similarly except that exposure to the isotope and inhibitors began at 12 hours of germination and continued to 24 hours of imbibition. Protein was extracted as described in Materials and Methods.

Treatment	Hours Imbibed	TCA Precipitate (CPM/30 mg dry tissue)		Mean Value	% Inhibition
Control	12	2145	3731	2938	-
Actinomycin D (20 µg/ml)	12	2154	3671	2912	.9
Cycloheximide (8 33 µg/ml)	12	82	52	68	97.7
Control	24	9857	8121	8989	-
Actinomycin D	24	4209	4053	4131	54.0
Cycloheximide	24	119	65	92	99.0

stages. The incorporation of leucine into protein is approximately 3 times greater at 24 hours of germination than at the 12 hour stage. However, the extension of this incorporation data to a determination of the rates of protein synthesis is complicated by a number of factors. Epicotyls which imbibed for 12 hours were exposed to the isotope for 10 hours whereas epicotyls imbibing for 24 hours were incubated with the radioactive leucine for 12 hours. Another undetermined factor is the intracellular amino acid pool size at these two stages.

In an attempt to determine whether or not actinomycin D was being absorbed into this embryonic tissue during the first 12 hours of germination, epicotyls were germinated in the presence of $H_3{}^{32}PO_4$ to measure the amount of RNA inhibition. As shown in Table 2, there is almost no inhibition (3%) of ^{32}P incorporation into the RNA of actinomycin treated embryos at 12 hours of germination. This would seem to indicate that this inhibitor is incapable of penetrating the cells at this stage. However, at 24 hours of germination, it is observed again that actinomycin is not effective in inhibiting RNA synthesis. This result is surprising in view of the fact that at this same stage and concentration of antibiotic, protein synthesis is inhibited by more than 50%. One possible explanation for this apparent paradox is that the $H_3{}^{32}PO_4$ incorporated into RNA is not the result of RNA transcription but rather of end labelling of transfer

Table 2. Effect of actinomycin D on the incorporation of $H_3{}^{32}PO_4$ into RNA during germination. -- Two replications containing 100 excised epicotyls were used in each treatment. The methods employed were similar to that described in Table 1 except that 200 µc of $H_3{}^{32}PO_4$ was added as isotope. The RNA was extracted as described in Materials and Methods.

Treatment	Hours Imbibed	CPM/1.5 mg RNA		Mean Value	% Inhibition
Control	12	21128	28256	24692	-
Actinomycin D	12	23083	24825	23954	3.0
Control	24	67498	74976	71237	-
Actinomycin D	24	93307	64178	78743	-7.6

RNA. This phenomenon has been described during both sea urchin (Gross, Kraemer, and Malkin, 1965 and Glisin and Glisin, 1964) and amphibian (Brown and Littna, 1966a) development.

Consequently, from the experiments discussed above, it is not possible to discern whether or not a "masked" form of mRNA exists in the dry epicotyl. It should, however, be emphasized that actinomycin is permeable to the cell membranes between 12 and 24 hours of germination.

Again, as in the case of protein synthesis, at 24 hours of germination there may be an elevated rate of $H_3{}^{32}PO_4$ incorporated into RNA compared to the 12 hour stage. The difference in labelling periods (2 hours) between these two stages and the possible differences in pool sizes permit this interpretation to be made, however, only with caution. Thus, development might proceed from low levels of cellular activity to higher ones and this may indicate a gradual derepression of the genome. Although the precise mechanism(s) of genetic activation has not been elucidated, it is generally believed as previously noted that histone proteins are in some fashion involved in the repression-derepression events. Consequently, knowledge of histone metabolism during developmental stages may provide some insight into the general phenomenon of selective gene activity.

Histone Synthesis and Modification

The relative rate of histone synthesis was observed at various developmental stages by following the amount of ^{14}C-leucine incorporated into these proteins. As seen in Table 3, there is synthetic activity occuring at all stages and, furthermore, the maximum value of histone synthesis appears to occur at the earliest stage (14 hours) examined. This type of comparison does not take into consideration changes in the amino acid pool size which may occur during development. Although accurate determinations of pool sizes are difficult to obtain, estimates were made by sampling the cellular homogenates for radioactivity at all time intervals. It would appear that there are no marked differences in the intracellular pool size of leucine which could reflect major differences in the amount of incorporation of this amino acid into the histones.

It should be pointed out that there are some inherent errors in the system being employed. Active growth is most likely confined to the apical end of the embryonic tissue which represents only a small fraction of the total tissue. Variations in the size of the epicotyls and apical ends can lead to differences in the amount of isotope incorporated into cellular components and is manifested when these data are compared to some unit weight of dry tissue or molecular concentration. For this reason, care must be exercised in interpreting the values obtained.

Table 3. The incorporation of ^{14}C-leucine into histones. -- Two replications containing 100 excised epicotyls were used for each time interval. For the last 12 hours of each germination period, the epicotyls were transferred to 0.5 ml of sterile water containing 2.0 μc of ^{14}C-leucine. Histones were extracted as described in Materials and Methods. The radioactivity of 0.1 ml samples of the cellular homogenates were also determined to estimate the relative intracellular pool size of leucine.

Hours Imbibed	Radioactivity (CPM) in Cellular Homogenate		Mean Value	Radioactivity in Histone Protein (CPM/200 mg dry tissue)		Mean Value
14	2905	1926	2416	1471	1358	1415
24	2933	2340	2637	547	771	659
36	2083	1968	2026	1023	883	953
48	2720	2273	2497	828	1414	1121

In a temporal comparison, it is noticed that the high degree of histone synthesis early in development precedes the greater levels of protein synthesis observed at 24 hours of germination. Whether this represents a causative relationship of increased transcriptional activity cannot, however, be ascertained. The possibility remains that these proteins are synthesized for complexing with newly replicated DNA to be incorporated into daughter cells following cell division. As described earlier, there is much speculation that histone modification is a necessary event for gene activation in eukaryotes. Studies of histone acetylation and phosphorylation would, therefore, be more meaningful in terms of relating histone metabolism and increases in cellular activity.

The data for histone acetylation and phosphorylation are presented in Tables 4 and 5 respectively. Again, in both cases the intracellular concentration of acetate and phosphate did not appear to appreciably change during the periods examined. A general trend is apparent with respect to total histone acetylation. The highest value is found at 12 hours of germination and this decreases in subsequent stages. The increased level of protein synthesis observed at 24 hours of germination is preceded by a high degree of histone acetylation observed at the 12 hour stage and, hence, this observation is consistent with the idea that histone acetylation is a necessary prelude to increased levels of RNA transcription.

Table 4. The incorporation of 1-^{14}C-acetate into histones. -- Two replications containing 100 excised epicotyls were used for each time interval. For the last 2 hours of each germination period, the epicotyls were transferred to 0.5 ml of sterile water containing 12.5 µc of sodium acetate-1-^{14}C. Histones were extracted as described in Materials and Methods. In addition, the radioactivity in 0.1 ml samples of the cellular homogenates were also determined.

Hours Imbibed	Radioactivity (CPM) in Cellular Homogenate		Mean Value	Radioactivity in Histone Protein (CPM/0.18 mg histone)		Mean Value
12	13816	13859	13838	793	912	853
24	15913	16999	16456	856	769	813
36	15138	16189	15664	799	680	740
48	13083	13539	13311	424	425	425

Table 5. The incorporation of $H_3{}^{32}PO_4$ into histones. -- The procedure employed in this experiment was the same as that described in Table 4 except that 400 µc $H_3{}^{32}PO_4$ was added as isotope.

Hours Imbibed	Radioactivity (CPM) in Cellular Homogenate		Mean Value	Radioactivity in Histone Protein (CPM/0.12 mg histone)		Mean Value
12	257053	224933	216961	7778	5060	6419
24	181898	212895	189895	7424	8068	7746
36	157064	222726	197397	5762	6870	6316
48	226133	207789	240993	7373	10364	8869

It is apparent from Table 5 that histone phosphorylation also is an early event during germination. It has been observed by Ord and Stocken (1968) that a correlation exists between the timing of DNA synthesis and histone phosphorylation. Consequently, it may be meaningful to also determine the relationship of histone modification and the timing of DNA synthesis. Table 6 depicts the relative incorporation of $H_3{}^{32}PO_4$ into DNA at various developmental stages. Although histone phosphorylation and DNA synthesis are occuring at all stages examined, the highest values recorded in both of these experiments occur at 48 hours of germination. This may be more than fortuitous and indeed indicate a positive correlation between histone phosphorylation and DNA synthesis.

Post-Translational Acetylation

As indicated by the results shown in Table 4, there is a high level of histone acetylation during the first 12 hours of germination. Data of Table 3 suggest also that the amount of histone synthesis is higher during the first 12 hours of germination than in subsequent 12 hour periods up to 48 hours. Studies by Phillips (1966 and 1968) have indicated an acetylation of N-terminal amino acids of some histone fractions. The results of his investigations have suggested that fractions f2a1, f2a2, and perhaps f1 are acetylated at this position. The structure of the N-terminal

Table 6. The incorporation of $H_3{}^{32}PO_4$ into DNA. -- The chromatin pellets obtained from the histone phosphorylation experiments (Table 5) were treated with 0.3 N KOH for 1 hour at 37°C to hydrolyze chromatin-bound RNA. The pellets obtained after these solutions were acidified with 10% TCA and centrifuged were heated for 15 minutes in a boiling water bath with 5% TCA. Samples (0.1 ml) of the supernatant obtained following centrifugation were counted for radioactivity.

Hours Imbibed	CPM/25 µg DNA		Mean Value
12	4746	4349	4548
24	4426	3974	4200
36	6310	5073	5692
48	8867	8576	8722

peptides for both fractions f2a1 and f2a2 were identified as N-acetyseryl-gly-arg. The N-terminal amino acid for fraction f3 was alanine and for f2b, proline. Fambrough and Bonner (1969) have also shown blocked N-terminal amino acids for fractions f1, f2a1, and f2a2 obtained from pea plants. It is assumed that acetylation of this type occurs at the time of histone synthesis. In fact, Liew, Haslett, and Allfrey (1970) have presented evidence to indicate that N-acetyl-seryl-tRNA is involved in the initiation of the synthesis of f2a histones. If this is indeed the case, this represents a slight departure from protein synthesis as it is observed in prokaryotes.

It thus becomes important to know whether the acetylation observed during the first 12 hours of germination represents acetylation coupled with histone synthesis or reflects post-translational modification of the protein. To distinguish between these possibilities, epicotyls were germinated for 9 hours in water before cycloheximide at a concentration of 1 mg/ml was added. At the tenth hour of germination, the epicotyls were transferred to solutions containing cycloheximide and sodium acetate-1-C^{14} for 2 additional hours. As previously noted, cycloheximide is permeable to the cells of this tissue. However, whether the one hour of incubation with cycloheximide prior to the addition of the label was sufficient to effectively stop translation was questionable. A longer pre-incubation with cycloheximide could have been performed but at the risk, however, of blocking the synthesis of enzymes necessary for the post-translational acetylation of histones and, thus, obscuring the results. Evidence for the presence of enzymatic systems for post-translational acetylation has been gained from the experiments of Bondy, Roberts, and Morelos (1970) on rat liver and brain tissue and those of Nohara, Takahashi, and Ogata (1968) on pigeon liver fractions. In addition, Inoue and Fujimoto (1969) have described an enzyme activity from an extract of calf thymus for the deacetylation of histones.

Consequently, it was necessary to establish whether the cycloheximide treatment described above was sufficient to block protein synthesis. For this, embryos were pre-treated for one hour with cycloheximide and then transferred to beakers containing cycloheximide solution and ^{14}C-leucine at the tenth hour of incubation and labelling continued for 2 additional hours. As shown in Table 7, there is approximately a 93% inhibition of total protein synthesis similar to that observed for longer periods of incubation with this antibiotic (Table 1). Having firmly established the effectiveness of this inhibitor for this relatively short exposure time, epicotyls were treated in the same fashion with 1-^{14}C-acetate. As described in Table 8, there is little inhibition (6%) of histone acetylation in the presence of cycloheximide. These data then establish that the major fraction of the acetylation observed, at least during the

Table 7. Measurement of ^{14}C-leucine incorporated into protein with a 3 hour incubation with cycloheximide. -- The 2 control replications and the 3 treated samples each contained 20 excised epicotyls. These samples were germinated in 1.0 ml of sterile water containing 100 units of penicillin for 9 hours. The samples were then given 0.2 ml of water or cycloheximide for 1 hour before being transferred to 1.2 ml of sterile water or cycloheximide solution containing 0.1 µc ^{14}C-leucine and penicillin for 2 additional hours. Protein was extracted as described in Materials and Methods.

Treatment	CPM/14 mg TCA Precipitate			Mean Value	% Inhibition
Control	1595	1392	-	1495	-
Cycloheximide (1 mg/ml)	147	41	142	110	92.7

Table 8. Measurement of 1-^{14}C-acetate incorporated into histones with a 3 hour incubation with cycloheximide. -- Four replications containing 100 excised epicotyls were used in each treatment. The epicotyls were germinated for 9 hours in 2.0 ml of sterile water. The samples were then given 0.4 ml of water or cycloheximide for 1 hour before being transferred to 0.5 ml of water or cycloheximide solution containing 50 µc of isotope for 2 additional hours. Histones were extracted as described in Materials and Methods.

Treatment	CPM/.10 mg Histone Protein				Mean Value	% Inhibition
Control	1375	1724	1097	890	1272	-
Cycloheximide (1 mg/ml)	1203	1333	1294	954	1196	6.0

first 12 hours, is due to post-translational modification of the protein. It is also interesting to note that this inhibition in protein synthesis has not appreciably affected the enzymatic process leading to this acetylation. It is suggested, therefore, that the acetylating enzymes are synthesized prior to the ninth hour of germination or possibly stored in the dry tissue.

Side chain modification of histones is a common event and was first detected by Gershey, Vidali, and Allfrey (1968) and Vidali, Gershey, and Allfrey (1968). Their studies indicated that arginine-rich fractions f2al and f3 extracted from calf thymus nuclei incubated in the presence of sodium acetate-1-^{14}C were appreciably labelled. Following enzymatic digestion and column chromatography, the label was found in amino acids identified as ϵ-N-acetyllysine. This result in calf thymus histone f2al has been confirmed and extended by the amino acid sequence investigations of DeLange et al. (1969) who identified the lysine residue at position 16 to be the one acetylated. Moreover, this residue was found to be acetylated approximately 50% of the time, thus allowing for at least two molecular species of this fraction. However, investigations of pea histone f2al by these same authors indicated that two lysyl residues are capable of modification. In addition, DeLange, Smith, and Bonner (1970) have also indicated that there are two sites for ϵ-N-acetylation in calf thymus histone fraction f3.

The distribution of the acetate into the various histone fractions was also followed in untreated and cycloheximide treated samples which had imbibed for 12 hours. The fractionation was accomplished by disc gel electrophoresis followed by sectioning of the gel and counting. A typical histone containing gel is depicted in Figure 1. The identification of the bands as indicated in that figure was arrived at by assuming the same order of migration as that observed with calf thymus histones. The electrophoretic procedure was essentially that of Johns (1967) and, hence, it is assumed that the four major fractions observed in these gels correspond to the same four he has reported. As shown in that figure, there are some minor components migrating slower than the f1 fraction. It is proposed that these represent protein contaminations in the chromatin preparation. These bands can easily be removed by more vigorous homogenization without affecting the major histone fractions during the preparation of the chromatin. This contamination when present is considered slight since the mass ratio of histone to DNA is approximately 1.0 which is characteristic of nucleoprotein. Fambrough, Fujimura, and Bonner (1968) have also described minor components with low electrophoretic mobility from pea chromatin and have attributed this to contamination by ribosomal proteins. It is also clear in Figure 1 that the f1 fraction is composed of at least 2 and perhaps 3 subfractions. This is consistent with

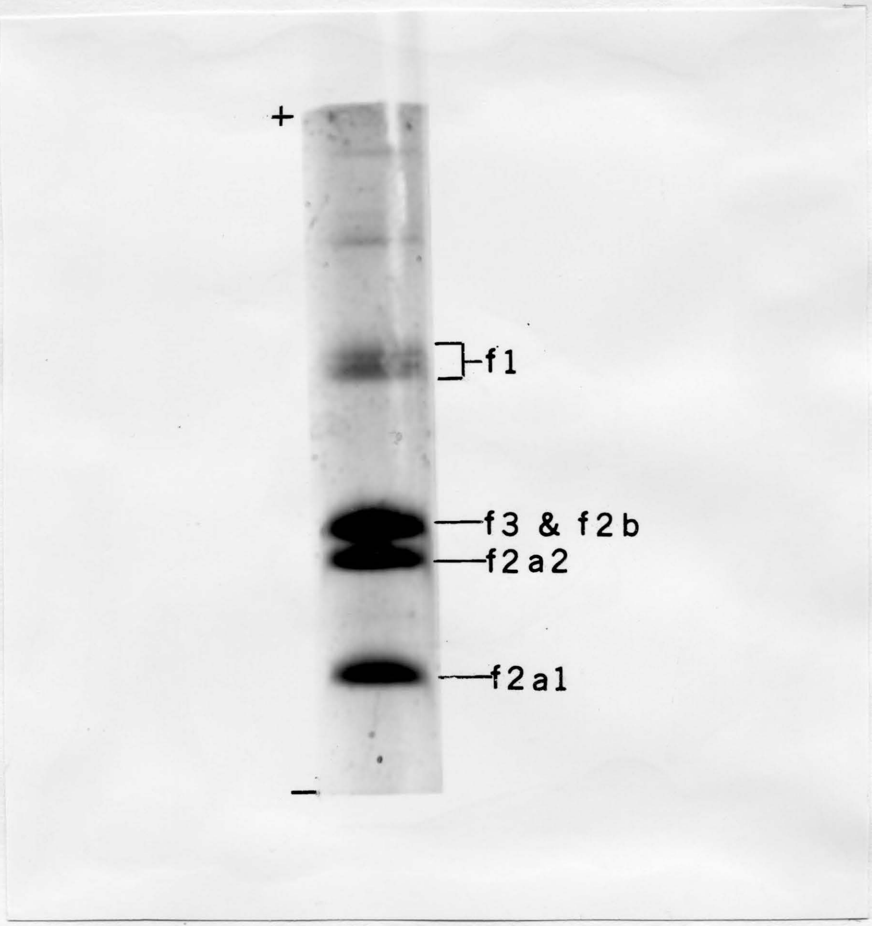

Figure 1 Electrophoretic separation of histones on gel. -- This banding pattern is representative of the type of separation obtained when the histones are prepared and electrophoresis is conducted as described in Materials and Methods.

the results of Panyim and Chalkley (1969) who have also observed multiple banding of this fraction as well as others upon electrophoresis and, hence, further confirms the identification of this histone along with the gray appearance of these bands which is characteristic of this fraction. The quantitative distribution of histone fractions can be appreciated in the spectrophotometric profile of a typical histone gel as shown in Figure 2. Electrophoresis was conducted on histones extracted from samples germinated for 12, 24, 36, and 48 hours. Although gel scans were not performed at all these stages, visual observations permit me to conclude that there are no marked quantitative changes and no qualitative changes during these developmental periods.

Electrophoresis of samples of acetate labelled histones and the determination of the area and radioactivity of the histone fractions was done as previously noted. It can be seen in Table 9 that all the fractions are labelled to various degrees. Side chain modification by ϵ-N-acetylation of lysine residues, as previously described, has thus far only been observed in the arginine-rich fractions (f2a1 and f3). It will be noticed in this table, however, that the slightly-lysine rich fraction f2a2 has the highest amount of labelling on a counts per unit area basis. This result would indicate that either there is an intense synthesis of this fraction with N-terminal acetylation as previously reported, or there is side chain modification not before

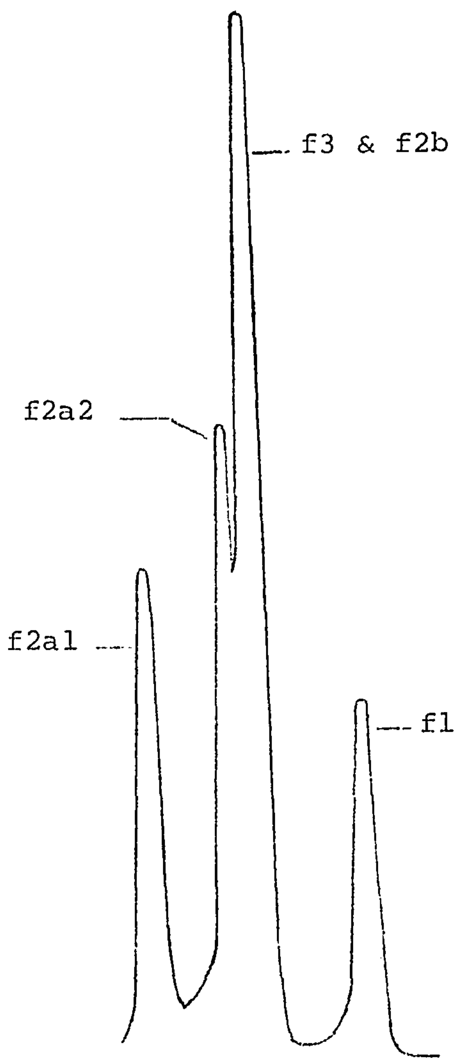

Figure 2. Spectrophotometric profile of a histone gel

Table 9. Percent incorporation of 1-^{14}C-acetate into histone fractions. -- Two replications of acetylated histones obtained as described in Table 8 (control) were separated by electrophoresis on polyacrylamide gels as described in Materials and Methods. The gels were stained and scanned at 600 mμ with a spectrophotometer equipped with a linear transport and chart recorder. The determination of the radioactivity in the histone fractions was done as described in Materials and Methods. The area units correspond to the weight of the paper obtained from cutting out the tracings of the various histone peaks.

Fraction	Area Units		Cts./2 min.		Ratio Cts./Unit Area		Mean Ratio
f1	38.0	44.9	23	64	.61	1.43	1.02
f3 & f2b	104.8	145.2	196	165	1.87	1.14	1.51
f2a2	69.9	94.9	214	286	3.06	3.05	3.06
f2a1	52.1	66.3	118	89	2.26	1.34	1.80

detected in this fraction. The lowest amount of labelling is found in the lysine-rich fraction f1 which in the case of calf thymus studies is found acetylated only at the N-terminal amino acid. When acetylated f1 histones are extracted from cycloheximide treated samples, the average ratio of counts per unit area (Table 10) is zero. It would appear, therefore, that the small amount of acetylation observed in fraction f1 from untreated samples was due to N-terminal labelling. There is also a slight general depression of the ratios obtained for the other fractions from cycloheximide treated embryos but the relative distribution of the labelling in the various fractions has remained the same. If the N-terminal amino acids in *Vicia* histone fractions f3 and f2a1 are the same as those of calf and pea histones, then these lower ratios observed can be explained by the cycloheximide block of protein synthesis. Fraction f2a2 still retains an appreciable degree of labelling in the presence of cycloheximide and it is suggested this histone is acetylated post-translationally.

The distribution of ^{32}P in histone fractions obtained from 12 hour germinated epicotyls was also investigated. The samples used, however, were not initially highly labelled and radioactive decay prior to counting contributed to the low counts. For this reason, reliable estimates of the incorporation of this isotope into the histone fractions could only be made after a counting period of 10 minutes.

Table 10. Percent incorporation of 1-^{14}C-acetate into histone fraction when incubated with cycloheximide. -- This experiment is identical to that described in Table 9 except that cycloheximide at a concentration of 1 mg/ml was present for the last 3 hours of germination.

Fraction	Area Units		Cts./2 min.		Ratio Cts./Unit Area		Mean Ratio
f1	60.0	70.0	0	0	0	0	0
f3 & f2b	151.8	178.3	118	252	.78	1.41	1.10
f2a2	81.9	110.1	224	296	2.74	2.69	2.72
f2a1	60.2	81.7	76	163	1.26	2.00	1.63

The data presented in Table 11 clearly show that only one fraction, the lysine-rich histone f1, is appreciable phosphorylated. This observation is consistent with the reports of Langan (1969) of an increase in phosphorylation of a serine residue in the lysine-rich fraction f1 of rat liver histones following the injection of glucagon into these animals and the studies of Ord and Stocken (1966) who observed phosphoserine in fraction f1. In a later study, Ord and Stocken (1967) also detected phosphate in histone fractions f3 obtained from rat livers and thymus nuclei. Experiments conducted by Jergil and Dixon (1970) and Meisler and Langan (1969) have provided evidence that this phosphorylation is accomplished by an enzymatic process as in the case of side chain acetylation.

It should also be pointed out that the patterns observed with respect to total histone protein and DNA synthesis, as well as histone modification by means of acetylation and phosphorylation in excised epicotyls suggest that this tissue is capable of initiating developmental events during these early stages of germination. Although the influence of other tissues such as cotyledons, cannot be excluded from the in vivo developmental pattern, the data indicates the capacity for independent development during the initial stages.

Table 11. Percent incorporation of $H_3{}^{32}PO_4$ into histone fractions. -- Two replications containing 100 excised epicotyls were germinated in sterile water for 10 hours before being transferred to 0.5 ml of sterile water containing 200 μc $H_3{}^{32}PO_4$ for an additional 2 hours. Histones from these epicotyls germinated for 12 hours were extracted as outlined in Materials and Methods, and analyzed as described in Table 9.

Fraction	Area Units		Cts./10 min.		Ratio Cts./Unit Area		Mean Ratio
f1	16.2	32.4	183	255	11.30	7.87	9.59
f3 & f2b	52.1	105.7	59	76	1.13	.72	.93
f2a2	31.0	69.8	14	32	.45	.46	.46
f2a1	28.8	52.1	15	15	.52	.29	.41

SUMMARY

The temporal relationship of the synthesis of different macromolecules has been investigated during the early germination stages of excised epicotyls obtained from the dry seeds of *Vicia faba*. The metabolism of certain macromolecules has been studied in the hopes of better understanding the developmental processes which are initiated following imbibition. Protein synthesis begins at a low level and increases with germination. During the first 12 hours of development, actinomycin D did not appreciably decrease the amount of ^{14}C-leucine incorporated into protein but at 24 hours of germination, a reduction of greater than 50% was obtained with this inhibitor. Protein synthesis at this later stage is evidently dependent to some degree on the synthesis of RNA. However, this may not be the case at the 12 hour germination stage. The amount of $H_3{}^{32}PO_4$ incorporated into RNA in the presence of actinomycin D was examined to determine if protein synthesis during the first 12 hours of development was accomplished by the translation of preformed mRNA stored in the dormant tissue. This isotope was, however, incorporated into the RNA of actinomycin treated epicotyls to about the same extent as controls at both the 12 and 24 hour stages and, hence, it was not

possible to establish the presence of pre-formed mRNA. The absence of an inhibition of $H_3{}^{32}PO_4$ incorporation into the RNA from actinomycin D treated samples at 24 hours of germination despite a reduction of protein synthesis of greater than 50% can be explained by end labelling of tRNA, although proof of this is lacking.

Since much experimental evidence has indicated that histones are repressors of gene activity, the synthesis and modification of these proteins was investigated at intervals up to 48 hours of germination. While histone synthesis was evident at all stages, the highest rate of amino acid incorporation was observed at the earliest stage examined (14 hours). Likewise, histone acetylation and phosphorylation was found to occur at all stages. In other systems investigated there has been observed a positive correlation between DNA synthesis and histone synthesis. In this study, there was also a tendency for increased phosphorylation at times of greater DNA synthesis. Histones were acetylated to a greater extent at the earlier stages examined than at the later ones. It was determined that the acetylation which occurs at 12 hours of germination is mostly due to post-translational side chain modification, although there is some N-terminal acetyl incorporation. It is suggested that the greater extent of genomic derepression reflected in the higher level of protein synthesis observed at 24 hours of germination may depend upon earlier modification of histones.

The distribution of acetate and phosphate into the various histone fractions was determined after separation of the different molecular species by electrophoresis on polyacrylamide gels. The histones were resolved into four major bands with fractions f3 and f2b migrating with the same mobility. The acetylation in the lysine-rich fraction f1 was due to N-terminal acetyl incorporation during protein synthesis. The other fractions f2a2, f3 & f2b, and f2al are also acetylated to varying degrees and the greater part of this is due to post-translational acetyl incorporation. Side chain modification of fraction f2a2 is particularly noteworthy since this type of acetylation has not been previously detected. Phosphate is almost exclusively incorporated into the lysine-rcih fraction f1 which is consistent with the observations of other experimentors.

The data clearly demonstrates that excised epicotyls do undergo independent development patterns exclusive of other supporting tissue. The gradual derepression of the genome reflected in increasing levels of protein and DNA synthesis may be accomplished or facilitated by the observed modification of histones.

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