CYTOLOGICAL STUDY OF SOME CHROMATIN BOUND ENZYMES IN **ALLIUM CEPA** L. BY **IN VITRO** AUTORADIOGRAPHY

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Cytological Study of Some Chromatin Bound Enzymes in *Allium cepa* L. by *in vitro* Autoradiography

by

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"The biologist who is interested in cell physiology should not be a morphologist, or a physiologist, or a biochemist: He should not only be capable of using physiological and biochemical methods as well as the microscope, but he should utilize them all in attacking his problem."

Jean Brachet

ABSTRACT

Autoradiography has been used with tritiated precursors to cytologically demonstrate *in vitro* nuclear RNA polymerase, DNA polymerase, poly(ADP-ribose)polymerase and RNA methylase in frozen sections of plant tissue (*Allium cepa* L. cultivar white Barletta). DNA polymerase, poly(ADP-ribose)polymerase and RNA methylase have been cytologically established for the first time (from any tissue source). This is also the first report of the presence of poly(ADP-ribose)polymerase in higher plants.

Different RNA polymerase activities can be reliably demonstrated in cytologic sections. Both nucleolar and nucleoplasmic RNA polymerases appear preformed in onion seed and bulb root tissues. Nucleolar and nucleoplasmic activity is observed in the endosperm from the beginning of hydration while in the embryo axis, distinct nucleolar activity is preceded by general nucleoplasmic labelling. Both nucleolar and nucleoplasmic polymerases were found in bulb roots before sprouting.

No G_2 cells were found in the ungerminated seed, while a small population occurred in the bulb roots. Rare occurrence of S phase nuclei and mitotic figures indicated that slow growth is maintained in the bulb roots and the G_2 cells do not represent a 'true'

physiologically blocked population. Ultrastructural studies substantiated possible slow growth and (or) degenerative changes in the roots of stored bulbs.

DNA polymerase activity can be adequately retained in apparent S-phase nuclei of root meristems. A low level of activity was demonstrated in the ungerminated seed in the apparent absence of *de novo* protein synthesis.

Nuclei in storage cotyledonary cells, in contrast to other tissues, were not efficient templates for exogenous DNA polymerase.

In vitro amino acid incorporation in cytologic sections was decreased with unlabelled precursors and polyuridylic acid, but not with protein synthesis inhibitors.

RNA methylase activity was cytologically detected in root meristematic nuclei. Activity was found over entire nuclei with no preferential nucleolar activity.

An enzyme tentatively identified biochemically as poly(ADP-ribose)polymerase has been recorded for the first time from a higher plant tissue. Incorporation from nicotinamide adenine dinucleotide was demonstrated in cytologic sections of root meristematic cells. Incorporation was distributed over whole nuclei with no preferential nucleolar labelling. Intense activity was found in all tissues of ungerminated seed embryos.

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ADPr	Adenosine diphosphate ribose
ATP	Adenonsine-5-triphosphate
^{o}C	degrees centigrade
Ci	curies
cRNA	chromosomal RNA
CTP	cytidine-5-triphosphate
dATP	deoxyadenosine-5-triphosphate
2,4-D	2,4 dichlorophenoxyacetic acid
dCTP	deoxycytidine-5-triphosphate
dGTP	deoxyguanosine-5-triphosphate
DNA	deoxyribonucleic acid
DNAseI	deoxyribonuclease I
dTT	dithiothreitol
GTP	guanosine-5-triphosphate
Hepes	Hydroxyethylpiperazine ethanesulfonic acid
hnRNA	heterogenous RNA
mRNA	messenger RNA
NAD	nicotinamide adenine dinucleotide
NEM	N-ethyl maleimide
NOR	nucleolar organizing region
pCMB	p-chloromercuribenzoate
PEI	polyethyleneimine
poly (ADP-ribose)	poly (adenosine diphosphate ribose)
poly U	polyuridylic acid
RNA	ribonucleic acid

rRNA	ribosomal RNA
s.a.	specific activity
SAM	S-adenosyl-methionine
snRNA	small molecular weight, monodisperse RNA
Tris	tris (hydroxymethyl) methylamine
tRNA	transfer RNA
TTP	thymidine-5-trisphosphate
UTP	uridine-5-triphosphate

INTRODUCTION

General introduction

The understanding of the role of nucleic acids in the flow of information from gene to protein has been significantly advanced since the early 1920's when it was still believed that ribonucleic acid was a unique "plant nucleic acid" and the only function suggested for deoxyribonucleic acid was to act as a pH buffer within the nucleus (Brachet, 1957). Most of the basic concepts of gene activity, however, has been gained from biochemical studies of DNA, RNA and protein syntheses in microbes coupled with elegant experiments in microbical genetics. Although much remains to be learned about the control mechanisms of gene activity and expression in prokaryotes, an understanding of basic concepts has burgeoned under an intensive study.

Similarly, there are still many problems to be solved in understanding regulatory mechanisms for DNA synthesis and the transcription of genetic information in eukaryotic cells. A great effort is being centered on the structure and function of chromatin. Research areas include delineating specific chromatin molecules, analyzing their physiochemical properties either pure or in reconstituted mixtures, and studying chromatin enzymology. From these studies it is hoped to gain insight on the role of chromatin in cell growth and differentiation.

The term chromatin was used as early as 1882 (Flemming, 1882) to describe the basic staining material of the cell nucleus, and in current use denotes the diffuse interphase form of the chromosomes of eukaryotic cells. It is now well recognized as a complex containing, in addition to DNA, small amounts of RNA, basic proteins (histones) about equal in mass to DNA, and a variable amount of highly heterogeneous acidic proteins (Simpson, 1973). There are also scattered reports of the possible presence of lipid and saccharide components forming various complexes as liporibonucleoprotein or mucopolysaccharides. While differences exist, particularly for acidic proteins, similar complexes of DNA with five main classes of histone have been isolated from almost all types of cells, both in animals and plants. Despite this similar constant chromatin structure, phenotypic variation as different as onion bulb root tips and nerve tissue is found. The major question in chromatin physiology is how control of expression of genetic information occurs in eukaryotic cells so as to allow such diversity.

It is well known that chromatin serves as a substrate for a number of enzymes involved in DNA replication, RNA transcription, DNA repair processes and chemical modifications of nuclear proteins (Simpson, 1973). The best known of these enzymes are the DNA dependent RNA and DNA polymerases. Other enzymes known to be involved in chemical modification of template proteins include the kinases, acetylases, and methylases with the kinases being the ones best characterized. These kinases

are believed to phosphorylate histones altering their interaction with DNA, and thereby modifying template properties. It is now believed, however, that methylation of chromatin proteins is probably the most complex of the modification reactions, with several different enzyme activities being described. In addition to protein methylation reactions, methylases, which have not been well characterized are known to act on chromatin DNA and RNA.

In 1968 work at Jensen's laboratory in California (Fisher, 1968) showed that a reliable level of incorporation from tritiated UTP could be retained in nuclei, when frozen sections of plant tissue were incubated in an assay mixture similar to that used in biochemical studies for the demonstration of the acid insoluble product of RNA polymerase. As noted by Fisher, such 'open' methods can extend the more common autoradiographic studies of isotopic incorporation by living tissues. With the *in vitro* method, problems which may be circumvented include substrate or precursor penetration and metabolism, dilution of isotope by endogenous precursors, rapid changes in synthetic patterns, technical difficulties of incubating, injecting or otherwise administering an isotope *in vivo* in a particular structure or tissue, as well as elimination of the possibility of enzyme product movement within the cell.

This concept of chromatin enzyme demonstration by autoradiography with tritiated precursors on frozen sections was exploited in the studies

reported here. Experiments were first performed to determine if the cytological RNA polymerase method of Fisher could be used with germinating seed tissues to demonstrate the enzyme in different nuclear regions and (or) in different tissues. This was followed by experiments to determine if the products of DNA polymerase, nucleic acid or protein methylase, and the recently discovered polymer forming enzyme poly(ADP-ribose)polymerase could be reliably demonstrated in the nuclear chromatin of frozen sections after incubation and autoradiographic preparation procedures. In addition experiments were carried out to see if protein synthetic activity could be similarly retained in nuclei (it is realized that it is still questionable what components of the presumed nuclear protein synthetic apparatus, is associated with chromatin at the time of protein synthesis). A reliable demonstration of these enzymes could prove fruitful in studies of chromatin physiology in the differentiating cell. Besides affording a direct observation of enzyme activity patterns in different tissues, an open in vitro system offers a potential for studies of the effect of various factors such as hormones and enzyme inhibitors or activators in specific tissues. It is realized that all techniques, these notwithstanding, have their limitations, but it is always hoped that in studies of cell systems undergoing any aspect of development, growth and differentiation, establishing enzyme activities histochemically, can help to bridge the often apparent gap between elegant histologic studies of tissue differentiation and the more sophisticated methods of biological chemistry and physiology.

The use of plant tissues represent not only the author's personal bias towards plant cell physiology but an ungerminated seed embryo is also a convenient model for developmental studies. In the germinating seed a partially differentiated embryo resumes its course of development after a period of 'quiescence'. Populations of cells are 'blocked' in various differentiated states, and thus exhibit a characteristic pattern of metabolism in the germinative phase. Thus, each tissue has a determined metabolic pattern and a stored 'blueprint' for further development when the seed is triggered into growth by environmental stimuli. Storage cotyledon and endosperm tissues are sometimes spoken of as having reached a 'committed' stage of development since they are usually unable to divide and change phase unlike potential meristematic tissues which contain an 'uncommitted' blueprint.

It should be stressed that the primary aim of these studies is not to demonstrate or characterize essential enzyme activities peculiar to seed germination. From the present state of knowledge of the eukaryotic cell there is no reason to believe that the basic concepts of chromatin form and function differ widely in either plant or animal cell differentiation. It is realized, however, that any novel enzyme methodology is best interpreted in relation to the whole organism, as well as in relation to specific cell or subcellular functions. In these studies an attempt was made to relate enzyme activity to resting or growth characteristics of the "functioning" organism. The work thus is involved in the wider context of early seed germination or bulb sprouting.

A large portion of current research on germination is devoted to elucidating the mechanisms of synthesis and activation of enzymes and proteins during the early hours of germination. The techniques used here to demonstrate enzymes in situ will add to these studies.

Nucleic acid metabolism in germinating seeds: An overview

The more general aspects of nucleic acid metabolism in germinating seeds have received some attention and much work has dealt with the protein or nucleic acid synthetic capacity of dry or germinating seeds and the overall picture that has developed is one of a limited amino acid incorporating ability, with both protein and RNA synthesis occurring earlier than DNA replication in germination. Germination characteristics of various species, however, have not been elucidated in terms of overall nucleic acid or protein synthetic capacity.

A review of the literature at the beginning of these studies revealed a lack of knowledge on nucleic acid metabolism in plant growth, specifically in relation to seed germination, typified by cells passing from a resting state, which can last for many years, to a proliferative growth phase. It was apparent that the level of understanding lagged far behind developments in cell growth and differentiation in other fields. It was controversial (and still is) as to what types of RNA, either presumptive ribosomal or messenger, were synthesized at the initiation of germination, while in mammalian systems a whole spectrum of RNA polymers

were being identified in cell and tissue studies. Plant tissue RNA polymerases were little characterized and no information existed on possible different polymerases present in nucleolar and extranucleolar chromatin. At least one study (Barker and Rieber, 1967), however, presented circumstantial evidence for RNA polymerase in ungerminated seeds. Little information which might explain the lag period of DNA synthesis in germination, except the general observation of a requirement for a protein synthesis period, was available. There was little or no data establishing DNA polymerase or precursor enzymes for DNA synthesis in dry or germinating seeds. In 1970 a paper on the partial purification of DNA polymerase in germinating corn reported the first attempt at extensive characterization of the enzyme in germinating seed (Stout and Arens, 1970). There seemed a virtual leak of information on DNA repair replication activities and much evidence has pointed to a need for 'replication' proteins in DNA synthesis, other than those involved in precursor production, or polymerases and ligases; no evidence on these has been forthcoming from seed germination systems. Similarly, there was a limited literature on chromatin availability in relation to RNA and DNA polymerase activity in plant growth, and no data on possible chromatin modification reactions as methylations or ribosylations.

The study reported here involves attempts to demonstrate RNA and DNA polymerase in dry or germinating seeds. In addition to the two chromatin polymerases, two chromatin modification systems were investigated,

methylation reactions by methylases and ribosyl reactions by poly(ADP-ribose)polymerase. Thus, in addition to establishing a histochemical basis for these enzymes in chromatin, it was hoped to gain at least a limited insight into their role in whole organism physiology.

A general problem in seed germination protein synthesis is the lack of distinct protein formation until after several hours germination, even though amino acid incorporation is apparent early in imbibition. An attempt was made to determine if amino acids were incorporated *in vitro* in nuclei of dry or germinating seeds.

RNA polymerase

I RNA synthesis in germinating seeds

A characteristic of germinating seeds is the regeneration of the ribosomal system (Marre, 1967; Barker and Rieber, 1967; Sturani, 1968; Payne and Boulter, 1969; Chen *et al.*, 1971) with concomitant production of enzymes (Chrispeels and Varner, 1967) for mobilization of reserve food. In some plants the first RNA to be synthesized in seed germination is believed to be mostly ribosomal and transfer RNA (Melera, 1971; Frankland *et al*, 1971; Jakob, 1972; Walbot, 1972) while in others it is suggested that m RNA synthesis predominates (van de Walle, 1969; Deltour, 1970). This may, however, be related to the DNA-replicative

state of the presumptive meristematic nuclei. Protein synthesis is activated in germinating wheat embryos after 30 min. imbibition (Marcus et al, 1966) while RNA synthesis is observed in germinating seeds only after a period of several hours (Holdgate and Goodwin, 1964; Chandra and Varner, 1965; Chen and Katchalski, 1968; Chakravorty, 1969; Price and Murray, 1969; Tanifugi et al.,1970; Chen et al.,1971). The earliest incorporation (3 hr.) of a pyrimidine precursor into a seed embryo has been reported for wheat (Chen et al.,1971). Rejman and Buchowicz (1971) have detected immediate incorporation of RNA precursors in intact wheat seeds, after soaking in precursors for 8 hours at 2°C, during which time no incorporation was detected in the acid insoluble fraction. Studies in both dormant and non-dormant tissues (Tuan and Bonner, 1964; Jarvis et al.,1968; Duda and Cherry, 1971) have shown that gibberellic acid may control early germination by gene derepression which increases the amount of DNA template available for transcription and subsequently RNA polymerase activity. Such increased activity, however, has only been apparent after several hours or even a few days germination. Barker and Reiber (1967) reported that the nuclear fraction of dry pea seeds was able to incorporate a low level of ATP into acid precipitable material after 1 hour incubation, suggesting the presence of RNA polymerase activity in resting seeds.

II Establishment of a cytological technique for RNA polymerase activity

An attempt was made to investigate in vitro RNA polymerase activity in dry and germinating onion seeds by frozen section autoradiography. Previous work (Mallery, 1971) characterized nucleic acid metabolism in the root apex during germination and ^{3}H-uridine was not incorporated into RNA in vivo before 18 hours germination.

III Nucleolar morphology in relation to RNA synthesis

Since nucleolar morphology is believed to be related to ribosomal RNA synthesis in the cell cycle, preliminary electron microscopic observations were made on this organelle in both onion seed embryo and bulb root tissues. The nucleolus is known to be associated with a secondary chromosome constriction (McClintock 1934; Ferguson-Smith, 1964), the genes in this region coding for ribosomal RNA (Wallace and Birnsteil, 1966; Ritossa and Spiegelman, 1965). The ultrastructure and cytochemistry of the nucleolar mass in Allium root meristematic cells have been intensely studied (Chouinard, 1966a; Chouinard, 1966b; Chouinard 1970; Lafontaine and Lord, 1975).

Cell cycle studies in seeds in relation to early growth processes

Using DNA synthesis as a marker, cell interphase, can be subdivided into G_1, S, and G_2 periods (Howard and Pelc, 1953). S designates the period of DNA synthesis, while G_1 ("gap 1") and G_2 ("gap 2") denote the intervals of interphase during which no nuclear DNA synthesis takes place, with G_1 preceding and G_2 following the S period. In growth initiation studies (e.g. seed germination) it is important to establish cell cycle states since synthetic patterns depend on the relative proportion of cells in G_1, S, or G_2. For instance, cell cycle biochemical studies have demonstrated a requirement for a period of protein synthesis before a cell at rest in G_1 can enter and continue a DNA synthetic cycle and the required proteins may be programmed from preexisting RNA's or RNA species partially or totally synthesized de novo at the time of metabolic activation. If cells were, however, arrested in G_2 and thus had already gone through their DNA synthetic phase, the initial pattern of macromolecular synthesis could be both qualitatively and quantitatively different.

Of equal interest, besides as a marker for specific synthesis, is the task of establishing a physiological role for G_2 blocked populations. In the dormant or quiescent state some plant species contain mixed G_1 and G_2 populations, while other species contain only G_1 cells (Bryant, 1969a,b).

Some cells "rest" in S but this is believed to be rare in root meristems of higher plants (Brunori and D'Amato, 1967; Davidson, 1966; Stein and Quastler, 1963). Conditions similar to cell cycle 'resting' periods can be experimentally induced. Excised pea roots may be induced to accumulate exclusively in G_1 or G_2 periods of the mitotic cycle when cultured in medium without exogenous carbohydrate (Van't Hof and Rost, 1972) similar to that found in the dormant dry seed radicle (Brunori et al., 1970). It remains to be determined if cell carbohydrate starvation has a physiological role in vivo.

In mammals, proteinaceous substances (chalones) that affect call arrest or blockage in G_1 or G_2 have been reported (Bullough and Deol, 1971; Frankfurt, 1971). A recent report (Evans and Van't Hof, 1974) has demonstrated that a non-proteinaceous factor from the cotyledon of peas promotes cell arrest in G_2 in mature root tissue as well as stationary phase, root and shoot meristems. Since this factor is non-proteinaceous, it is doubtful if any homology exists between plant and animal chalones.

In this study, both onion bulb roots and seeds were checked for G_2 cells. Since preliminary studies suggested slow growth or degenerative changes under storage, ultrastructural observations were carried out to investigate such changes.

DNA polymerase

I DNA synthesis in germinating seeds

DNA synthesis occurs after the initiation of protein and (or) RNA synthesis in germinating seeds (Jakob and Bovey, 1969; Chen and Osborne, 1970; Melera, 1971; Mory et al., 1972) and early protein synthesis may be required for subsequent DNA replication. The replication lag period could be due to such general control mechanisms as (a) lack of an active template (b) shortage of enzymes for DNA precursor production or (c) enzymes directly involved in replication, as DNA polymerases and ligases or (d) the presence of inhibitor(s) which may act at any point in the replication process. When this study was initiated there was little or no biochemical information on the presence of DNA polymerases in ungerminated seeds. In 1970 the first report (Stout and Arens, 1970) on the partial purification and characterization of a DNA polymerase from a higher plant (germinating Zea mays) appeared. Experiments were designed to investigate if the DNA polymerase replication complex could be retained in S phase nuclei of germinating onion seed in cytologic sections. The method developed was also used to assess DNA polymerase activity in dry seeds. The germinating onion seed is a suitable organism for studying DNA synthesis requirements, since there is more than a 30 hour lag before in vivo DNA synthesis (Melera, 1971).

II Templates for DNA polymerase in ungerminated onion seed embryos

The lag in DNA replication in germinating seeds could be due to lack of 'active' templates for DNA polymerase. The relation between chromatin states and RNA polymerase activity in plant tissue has received some attention (Jarvis et al., 1968; McComb et al., 1970; Johnson and Purves, 1970; Teissere et al., 1972) and at least one report has demonstrated template modification (in soybean hypocotyl tissue) by a plant hormone (2,4-D) effecting an increase in DNA polymerase activity (Leffler et al., 1971). Experiments were carried out on cytologic sections to see if tissues of the ungerminated onion seed retained active templates for exogenous DNA polymerase.

III Establishment of a cytological technique for DNA polymerase

Experiments were carried out to determine if the DNA replicase complex could be retained in cytologic sections of germinating onion seeds. The events that lead to polymerization of DNA in the cell are poorly defined. DNA polymerases, whose physiological function await elucidation, have been characterized in a number of cell systems (especially bacteria) on the basis of type of primer, thermal stability, chromatographic behaviour, molecular weight, and sensitivity to ionic strength, sulfhydryl reagents, nucleoside triphosphates, nucleases and antisera. Progress in

the study of these enzymes in mammalian cells has been hampered because they are hard to purify, coupled with a loss of activity in separation procedures. In general, DNA polymerase activity of mammalian non-synchronized cells has been shown to be distributed in both cytoplasm and nuclear fractions with most of the activity in the cytoplasm (Jansz et al.,1971). Recent work (Weissbach et al.,1971; Haines et al.,1971; Long and Garren, 1972; Chiu and Sung, 1972) has however, demonstrated differences between the nuclear and cytoplasmic enzymes and even cytoplasmic polymerases have been shown to consist of many molecular species (Yoshida et al.;1974). In vitro DNA polymerase activity, either extensively or partially purified has received little attention in plant tissue (Stout and Arens, 1970; Leffler et al.,1971; Mory et al.,1972; Dunham and Cherry, 1973; Srivastava, 1974). Most of these investigations have appeared since these studies were initiated.

It has been suggested (Stavrianopoulos et al.,1971) that the elucidation of DNA biosynthesis requires the isolation of a multienzyme complex, which can effect the unwinding of the DNA template, as well as its transcription, replication, excision, repair and ligation. Cytological studies at the level of the DNA replication complex could prove fruitful in an understanding of its biological role in the cell cycle and especially its expression in tissue differentiation. With an efficient cytological technique for DNA polymerase, the possible effects of various hormones, subcellular fractions, and the many substances which have been shown to effect DNA synthesis could be investigated, for instance, in the specific cell types of a model differentiating system as a root meristem, whose tissues are not separable by conventional biochemical methods.

Moreover, at the level of electron microscopic autoradiography an opportunity becomes possible for investigation of DNA polymerase activity in the euchromatin and heterochromatin of various tissues.

Protein synthesis

I. Protein Synthesis in germinating seeds

Dry seeds retain the capacity for amino acid incorporation; several studies have shown that seeds contain ribosomes capable of protein synthesis as well as preformed mRNA (reviewed by Boulter, 1970; Zalik and Jones, 1973; Mayer and Shain, 1974). Upon imbibition, the activation of protein synthesis may be due to such processes as the information of polymers from existing ribosomes and mRNA (Marcus, 1969) or ribosomal dissociation into subunits before the attachment of mRNA (App et al., 1971). Although low levels of amino acid incorporation can be detected early in germination, there is doubt as to the early formation of protein products. Preformed mRNA's have been suggested to be present in dry seeds, but there is little evidence of protein formation from such stored templates (Ihle and Dure, 1972) and some studies (Marcus, 1969; Walton and Soofi, 1969) noted no distinct protein formation before radicle protrusion. Experiments were designed to determine amino acid incorporation could be cytologically demonstrated in vitro in the nuclei of either dry or germinating onion seed tissues. Although investigations have been profuse, there still exists a controversy on the ability of cell

nuclei to carry out protein synthesis and there appears to be no information available for the presence of the complex machinery of protein synthesis in the nuclei of ungerminated seed.

II Establishment of a cytological technique for nuclear amino acid incorporation

An attempt was made to determine if the complex machinery of protein biosynthesis (or any of its components as amino acyl synthetases) could be retained in cytologic sections, especially in the cell nuclei. A nuclear capacity for protein synthesis is important because of the probability that many of the proteins synthesized there may be involved in regulation of gene activity, and there is good evidence that within the nuclei of many cell types, protein synthesis proceeds at rates comparable to those measured in the cytoplasm (Goldstein, 1970).

The question of protein synthesis by cell nuclei has been controversial for at least two decades. There have been innumerable reports of amino acid incorporation by isolated nuclei from various species, but the purity of such preparations has often been questioned. Similarly most autoradiographic data has suggested a role for nuclei (and nucleoli) in protein synthesis, but peptide migration to and from nuclei is well known. It has been equally difficult to prove that some proteins known to be preferentially associated with the nucleus (e.g. histones) are actually synthesized there.

There are, however, several good pieces of evidence for nuclear protein synthesis in at least some species. In mammalian liver, nuclear proteins are rapidly labelled *in vivo* and inhibition kinetics differ from those displayed by cytoplasmic proteins (Kuehl, 1967; Kuehl, 1969). Nucleoli of HeLa cells can selectively label proteins (Zimmerman *et al.*, 1969), thus making contamination by cytoplasmic components unlikely artifacts, since in the latter case many types of proteins would have been labelled. One of the strongest arguments for intranuclear protein synthesis is the fact that amino acid uptake in isolated nuclei from calf thymus is not inhibited in the presence of high concentrations of ribonuclease unlike cytoplasmic incorporating systems which are inactivated by trace amounts of the enzymes (McCarty, *et al.*, 1966). This is consistent with a protective role afforded to the nuclear protein synthetic complex by the nuclear membrane. Conversely, the fact that DNAse can enter the nucleus and inhibit amino acid incorporation (Allfrey *et al.*, 1957) by release of nuclear specific inhibiting histones (Allfrey and Mirsky, 1963) further substantiates evidence for nuclear protein synthesis. Strong evidence for protein synthesis in thymus comes from the demonstration of Na^+ dependent protein synthesis, where it has been shown that the Na^+ ions are involved in amino acid transport (Allfrey *et al.*, 1961; Tsuzuki, 1969).

Some *in vivo* autoradiographic studies have also demonstrated evidence for nuclear protein synthesis. For instance, in a thorough

autoradiographic study on the incorporation of several amino acids (labelled with various isotopes) into tissues of different mammalian species, it was shown that after an initial, brief, period of equilibration of free amino acid pools in the nucleus and cytoplasm, the increase in protein radioactivity with time is the same in the nucleus and the cytoplasm (Schultz et al., 1965).

Nevertheless, it can be argued (Goldstein, 1970) that since an average complete protein molecule is synthesized in a minute or two (Dintzis, 1961) and if diffusion across a cell takes only a fraction of a second, a radioactive amino acid incorporated into the C-terminal end of a protein could appear in the nucleus in a few seconds.

Thus, it can be seen that demonstration of an *in vitro* cytologic system for amino acid incorporation could also contribute to the controversy over ability of nuclei to carry out protein synthesis. In these studies, both dry and germinating seeds were used, in attempts to demonstrate *in vitro* amino acid incorporation. In conjunction with labelling displayed by dry seed tissues, ultrastructural observations on possible subcellular incorporation sites were also investigated.

Methylases

I. Nucleic acid and protein methylation reactions in germinating seeds

Little evidence on methylation reactions in either chromatin

nucleic acids or proteins have been obtained from plants. There is a lack of information on the methylation of even RNA species, which have been extensively investigated in animal tissues. It has been demonstrated, however, that the plant hormone gibberellic acid can enhance the methylation of both transfer and ribosomal RNA species in endosperm tissue (Chandra and Duynstee, 1971) and methylation of RNA components in nucleolar rich fractions of pea embryos has been reported (Tanifuji et al., 1970). A recent investigation (Jakob and Tal, 1973) has pointed out the difficulty of studying plant RNA methylation reactions with methylmethionine due to its rapid incorporation into pectin, which is hard to separate electrophoretically from RNA.

II. Establishment of a cytological technique for chromatin methylases

In this study an attempt was made to determine if germinating onion seeds contain cytologically demonstrable chromatin bound methylases. Although RNA methylation has been extensively characterized there is little information on methylation in other macromolecules. The presence of methylated base and sugar components in transfer and ribosomal RNA was well established in the last decade (Borek and Srinivasan, 1966); Srinivasan and Borek, 1966; Starr and Sells, 1969). Methylation processes are believed to play a role in both the amino acid acceptor and

transfer functions of t-RNA as well as ribosome maturation. An intensive effort has also gone into the study of RNA methylase activity during tissue development.

Although DNA methylation has not been intensively studied, both bacterial and eukaryotic DNA's are known to be methylated at the polymer level (Gold et al.,1966; Shapiro, 1968). The function of DNA methylation reactions remain obscure but it has been established that in bacteria, methylases play a role in host range specificity (Boyer, 1971; Meselson et al.,1972). Some research has centered on the relation between DNA synthesis and methylation characteristics in prokaryotic and animal cells. In bacteria it is known that the methylation of newly synthesized DNA occurs near the point of replication (Billen; 1968; Lrck 1968) with only a few seconds elapsing between DNA synthesis and methylation. In animal cells, DNA methylation begins shortly after the start of DNA synthesis, but may continue for several hours or even several generations after DNA synthesis is complete (Adams and Hogarth, 1971). It is however, not known, what this means as far as genetic activity in cell and tissue differentiation is concerned. Genetically active genes are relatively more methylated suggesting that DNA methylation somehow plays a role in mechanisms responsible for transcription (Comings, 1972). Other work (Silber et al.,1966; Vanyushen et al.,1970) has suggested that the degree and pattern of methylation may be related to the degree of cell maturity.

Nucleoproteins are also known to be methylated at the polymer level (Paik and Kim, 1971) with individual amino acids being methylated by specific enzymes. The occurrence of such methylated residues in nature is very diverse but this in itself suggests a unified functional role for methylated proteins. How this role expresses itself in modification of protein structure largely remains to be determined. An interesting aspect of protein methylation is the presumed role of side chain methylation as one of the mechanisms involved in histone control of genetic activity. Isolated nuclei from a number of animal tissues methylate histones *in vitro* at lysine and arginine residues (Stellwagen and Cole, 1969) and it has been suggested (Tidwell *et al.*, 1968) that methylation is somehow involved in condensation of euchromatin in preparation for mitosis. The methylation of histones could stabilize chromatin structure in preparation for cell division. Nucleoprotein methylation has been mainly concerned with histone modifications although there are some reports on non-histone methylation. At least one study has reported a direct correlation between DNA synthesis and non-histone chromosomal protein methylation (Goodman and Benjamin, 1973). Other work with animal tissues (Paik and Kim, 1971) has similarly demonstrated that little methylation occurs throughout the cell cycle, except during late S and G_2 phases.

Thus, although a large number of methylases are known to be

involved in nucleic acid and protein methylation reactions, information on such polymer modifications in plant growth has attracted little attention except for a few reports on RNA methylation. This study is an attempt to demonstrate the occurrence of methylases in germinating seeds by frozen section autoradiography.

Poly(ADP-ribose)polymerase

I Poly(ADP-ribose)polymerase in germinating seeds

Besides methylation, another enzymic process believed to function in chromatin modification by glycosylation reactions was investigated. The nuclear enzyme poly(ADP-ribose)polymerase has not received any attention in plant biochemistry. Initial experiments were attempted to establish the enzyme in germinating seeds by biochemical techniques. This was followed by attempts to demonstrate the enzyme cytologically in germinating and dry seed tissues.

II Establishment of a technique for poly(ADP-ribose)polymerase

In addition to DNA and RNA polymerases, a poly(ADP-ribose)polymerase which catalyzes the formation of an ADP-ribose homopolymer from NAD, had been established in the nuclei of several eukaryotes (Fujimura and Sugimura, 1971; Sugimura, 1973). The polymerase was first discovered (but

identified incorrectly) in 1963 by French workers (Chambon et al., 1963), who observed that the incorporation of radioactivity from ATP into acid insoluble material in avian liver nuclei was enhanced several fold with NAD. The polymer was subsequently shown to be covalently attached to chromosomal proteins (Nishizuka et al., 1969; Otake et al., 1969) and is believed to have a role in regulation of chromatin function.

The primary purpose of this study was to establish histochemical techniques, in vitro, for the chromatin bound enzymes (a) RNA polymerase (b) DNA polymerase (c) nucleic acid and protein methylases and (d) poly (ADP-ribose) polymerase. If enzymes remain bound to chromatin during incubation procedures, and if enzyme products were not solubilized upon incubation or autoradiographic preparation then the enzymes should be cytologically demonstrable with tritiated isotopic precursors.

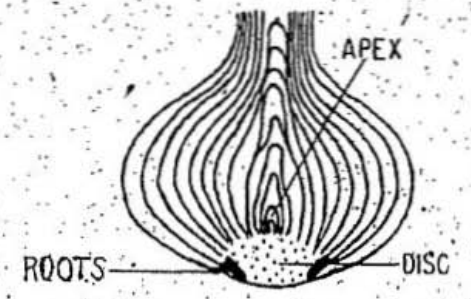
MATERIALS AND METHODS

Dry and germinating onion seeds (Allium cepa, L. cultivar white Barletta) were primarily used in these studies. In the dry seed the embryonic axis which contains recognizable epicotyl, procambial, and cotyledonary zones lies coiled in the endosperm (Figure 1C). In some experiments, unsprouted bulb roots which are embedded to varying degrees in the bulb base were used (Figure 1A). An onion bulb is a modified bud with a reduced stem (disc). In the bulb roots, cap cells, cap initials, and cortical cells can be differentiated (Figure 1B). The germinated seed radicle has a similar anatomy.

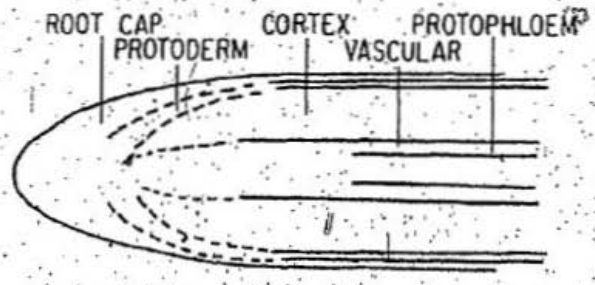
Figure 1. Schematic Diagrams of A. an Onion Bulb B. an Onion Bulb Root Tip and C. an Onion Seed.

The basic anatomy of germinated onion seed root tip is similar to B. The unsprouted bulb roots are embedded in the bulb disc. Throughout this study the term sprouting will be used for root tip protrusion from the bulb base, and germination for root tip protrusion from the seed coat. The term root meristem will refer to a growing root: the term radicle will not be used. By seed embryo will be meant the whole rudimentary plant exclusive of endosperm. The core of the embryo will sometimes for convenience be generally referred to as the vascular cylinder. B. was taken from Jensen and Kavaljian (1966) and C. from Esau (1965).

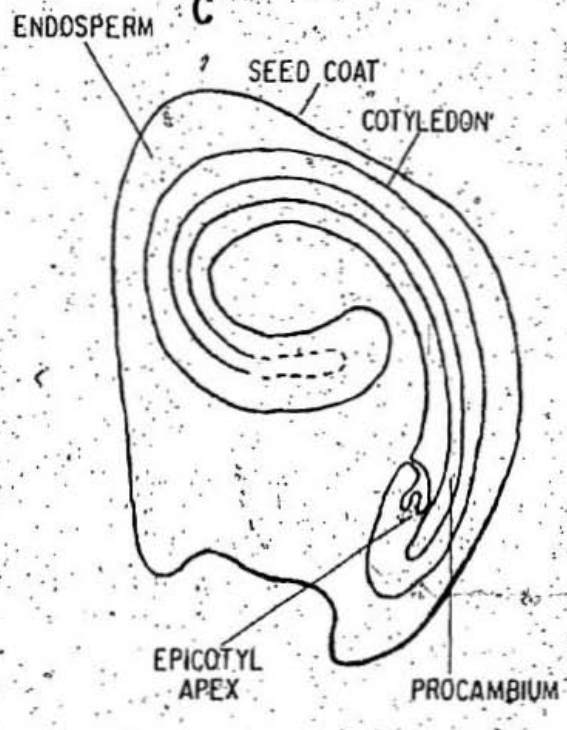
A


APEX
ROOTS
DISC

B


ROOT CAP
PROTODERM
CORTEX
VASCULAR
PROTOPHLOEM

C


ENDOSPERM
SEED COAT
COTYLEDON
EPICOTYL
APEX
PROCAMBIUM

RNA polymerase

RNA polymerase activity was investigated cytologically by a modification of Fisher's method (Fisher, 1968). Whole seeds were sectioned at 8μ in a cryostat (-20°C), placed on microscope slides and dried for 8-10 minutes in a vacuum desiccator at room temperature. Onion bulb discs were excised, frozen in a stream of CO_2 and sectioned. The bulbs used here were grown in the* Departmental green house and stored at room temperature or at 2-4°C. Slides were placed in Petri dishes lined with filter paper soaked in 2mM dithiothreitol (dTT) and approximately 0.25 ml of substrate was added to each slide. The assay mixture contained 40mM Tris-Cl 10mM $MgCl_2$ or $Mg(CH_3COO)_2$, 150mM KCl, 0.5mM EDTA, 1.0mM dTT, 0.25mM each of GTP, ATP, CTP and 50 or 100μ Ci ^{3}H-UTP (s.a. 21 Ci mM^{-1}). The reaction was stopped by immersing slides in acetic acid: ethanol (1:3). The slides were then washed with 5% trichloroacetic acid (TCA) for 30 minutes at 4°C. Autoradiographs were prepared by dipping slides in undiluted NTB_2 liquid emulsion (Kodak). After 14 days the slides were developed and stained with acidic azure B. Controls were carried out with pancreatic RNAse (1 mg ml^{-1} in sodium acetate, pH 5.0 or Tris, pH 7.5 buffers) or pancreatic DNAseI (1 mg ml^{-1} in sodium acetate, pH, 5.0 buffer with 5mM $MgSO_4$).

To investigate *in vivo* RNA synthesis, seeds were hydrated for varying periods with (a) 100 or 1000μCi ml^{-1} ^{3}H-uridine (s.a. 22 Ci mM^{-1})

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(b) the complete nucleotide mixture used for frozen sections except cold nucleotides from 0.25-3mM and ^{3}H-UTP from 100-1000uCi ml^{-1} were tried in three separate experiments using progressively higher concentrations of precursors. In each experiment a batch of seeds was continuously exposed to the nucleotide mixture or alternatively after 3,6,9, and 12 hours of growth, was pulsed for three hours. Seeds were incubated in the dark at 23°C, either intact or after slicing off a portion of the seed coat to eliminate the problem of seed coat nucleotide impermeability. Hydration was on 7 cm^{2} pieces of No. 2 filter paper in Petri dishes with 1.5 ml of precursor. A germination test revealed 95% of the seed stock viable. In all experiments at least 10 seeds were taken at each three hour interval. From seeds fixed in acetic acid: ethanol for at least 2 hours, 5u paraffin sections were processed for autoradiography. Paraffin sections were prepared according to routine procedures (Jensen, 1962). From each seed sample at least 100 sections were examined.* To investigate in vivo RNA synthesis in onion bulb roots, 2 or 8 month old bulbs were grown in 100uCi ^{3}H-uridine (s.a. 22 Ci mM^{-1}) in the dark at 22°C. Grain counts were made from 5u sections of paraffin embedded tissue. Several batches of bulbs were used for ^{3}H-uridine incorporation and rooting tests.

Tissue preparation for electron microscopy

For electron microscopical studies, tissues of dissected seed

* Throughout this thesis the symbol ± refers to the standard error of the mean.

embryos and quiescent roots were fixed in a mixture of paraformaldehyde and glutaraldehyde in 200mM Na_2HPO_4-KH_2PO_4 buffer pH 7.5 (Karnovsky, 1965) at room temperature for at least one hour, rinsed in buffer several times and postfixed with OsO_4 in phosphate buffer. Tissues were dehydrated with ethanol and propylene oxide, embedded in Epon and cured at temperatures from 37°-60°C over approximately 2 days. Sections were cut with glass knives on a Porter Blum MT-I ultramicrotome, stained with urahyl acetate and lead citrate and observed with a Zeiss 9S electron microscope. Half micron sectionsfor light microscopy were stained with basic toluidine blue or azure B.(1% solutions, pH 11.0).

Several procedures were tried to prepare endbsperm tissues for thin sectioning. These included (a) dissolving fixatives in various buffers as phosphate, cacodylate or collidine (b) fixing with different combinations of paraformaldehyde, glutaraldehyde, acrolein, and osmic acid (c) embedding with different proportions of Epon resins (d) varying the epoxy infiltration step from 1 hour to 1 week under vacuum (e) curing at high temperature (100°C) immediately after tissue infiltration (f) substituting Spurr embedding media with a repeat of the above procedures. Methacrylate embedding procedures were not tried.

The methyl green-pyronin method was used to differentiate between RNA and DNA on paraffin sections (Jensen 1962).

Detection of G_2 cell nuclei

Onion bulbs were exposed for varying periods to 10μCi ml^{-1} ^{3}H-thymidine (s.a. 6.7Ci mM^{-1}) dissolved in distilled water. Seeds were similarly germinated on 7 cm^2 pieces of No. 2 filter paper in Petri dishes with 1.5 ml of tritiated precursor. Root tips were excised and fixed in acetic acid:ethanol (1:3) for at least 2 hours. The tips were hydrolyzed in 1N HCl, stained and squashed according to the Feulgen procedure (Jensen, 1962). Cover slips were removed after freezing in liquid nitrogen, the slides dried, and autoradiographed. Slides were developed after 7-10 days. Quiescent roots were also dissected from the bulb disc, exposed to ^{3}H-thymidine for 0.5-1.0 hour and processed for autoradiography. Paraffin sections were prepared from both quiescent and sprouted onion bulb roots exposed to ^{3}H-thymidine, and autoradiographed to see if the first cells to enter S phase were confined to any particular tissue(s). Some quiescent roots were fixed in formalin-acetic acid-ethanol (FAA) (20:1:1), and thick (20μ) paraffin sections prepared to check for mitotic figures.

DNA polymerase

The method used for demonstration of DNA polymerase activity on frozen sections of germinated seed radicles was similar to that used for

RNA polymerase. The assay mixture contained in 0.5 mls 50mM Tris-Cl (pH 8.4), 0.25mM each of d CTP, d GTP, d ATP, 100μCi ^{3}H-TTP (s.a. 18Ci mM^{-1}) 5mM $Mg(CH_3COO)_2$, or $MgCl_2$, 1mM dTT. In one experiment ^{3}H-d ATP was substituted for ^{3}H-TTP and glycine-NaOH (pH 9.0) buffer was used instead of Tris. Separate experiments were performed with the basic incubation mixture minus each of the deoxynucleosides or with ^{3}H-d TMP (s.a. 10Ci mM^{-1}) substituting for ^{3}H-TTP. Ethanol was removed from the isotopes under a gentle stream of N_2 before use. When N_2 was not available compressed air was used. No facilities were available to check for purity after ethanol evaporation. Besides 8μ cryostat sections whole (excised) or slices (60-80μ) of fresh root tips were frozen in* (a) N_2 (b) CO_2 or (c) at -20°C and incubated in the assay medium. Here the concentrations of deoxynucleotides in the incubation mixture were increased from 0.25-2.0mM in successive experiments. Whole or sliced roots were also shaken for 10-15 minutes at 24°C in a suspension of 1% toluene in 70mM K_2HPO_4 (pH 7.4) (Moses and Richardson, 1970), rinsed with buffer and assayed. Tissues were treated with DNAseI (100μg ml^{-1} enzyme, 5mM $MgSO_4$, 50mM Tris-Cl pH 7.4) or RNAse (100μg ml^{-1} enzyme, Tris-Cl pH 7.4) after incubation or alternatively DNAse was included in the incubation mixture. Attempts were made to activate the template with varying concentrations of (a) DNAseI or (b) increase activity with exogenous ATP. The sulfhydryl reagents, N-ethyl maleimide (NEM) (1mM) and p-chloromercuribenzoate (pCMB) (1mM) were tried as inhibitors in separate experiments. *In vivo* labelling was with ^{3}H-thymidine (s.a. 6.7Ci ml^{-1}).

* Liquid nitrogen (N_2) boils at -195.8°C and solid carbon dioxide (CO_2) melts at -56:6°C. For the -20°C temperature a refrigerator was used.

In vivo and *in vitro* experiments were terminated with acetic acid: ethanol after 1 hour. Large pieces of tissue were paraffin embedded and sectioned at 8μ. Autoradiographs were developed in D19 (Kodak) after 14 days exposure.

Root tips, ~1.5mm long, from seeds germinated on moist filter paper in the dark at 24°C were used in the study; preliminary work demonstrated that the labelling index after a 1 hour pulse with ^{3}H-thymidine reached a constant value (~30%) at this length. In *in vitro* experiments (except on the 8μ sections affixed to microscope slides), five whole or sliced (60-80μ) roots were incubated per experiment.

To assess DNA polymerase activity in dry seeds, embryos were individually dissected from dry seeds with a dissecting microscope and sectioned. The incubation mixture was essentially that used for detection of DNA polymerase activity in actively growing root meristems except here the concentrations of nucleotides were increased to 0.5mM. Reactions were terminated with acetic acid: ethanol with 50mM* $Na_4P_2O_7$ after 1 hour incubation at 32°C. Preliminary observations demonstrated that sections of the dry embryo, in contrast to tissues from germinated seeds, retained a greater structural integrity in the hypotonic incubation media. Consequently, sections were not placed on microscope slides for enzyme incubation but were placed directly into vials with 0.5ml of the incubation mixture. After termination of the reaction, the mixture was centrifuged at 500 g to pellet the sections. The sections were then washed several times with cold

* $Na_4P_2O_7.10H_2O$ (sodium pyrophosphate)

5% TCA, 95% ethanol and ethyl ether. Sections were finally suspended in 50% ethanol, spread on microscope slides with a Pasteur pipette, dried and autoradiographs were exposed for 21 days.

In some experiments cycloheximide was added to inhibit protein synthesis (if any were occurring on the tissue sections in the assay medium). Preliminary investigations demonstrated the efficiency of cycloheximide as a protein synthesis inhibitor in actively growing root meristems. Whole roots (5.0-7.0mm long) were exposed to 100μCi ^{3}H-leucine (s.a. 3.2Ci mM^{-1} or ^{3}H-leucine + 25μ ml^{-1} of cycloheximide in 50mM Tris-Cl (pH 7.3) for 1 hour. In autoradiographs exposed for 1 week, provascular, cortical, and root cap tissues were completely 'blackened' (both nuclei and cytoplasm) while in cycloheximide controls only a few grains appeared. In another experiment 100μg ml^{-1} RNAse was added to destroy any functional translational system in the dry seed sections. The sulfhydryl inhibitors NEM and pCMB were used at 2mM concentration. In an attempt to 'activate the template' of the dry seed, concentrations of DNAseI from 25 mg ml^{-1} to 50μg ml^{-1} or alternatively 1-3mM ATP or other nucleoside triphosphates were included in the incubation mixture. Attempts to permeabilize small slices of tissue with toluene (Moses and Richardson, 1970) instead of freeze sectioning, were tried but abandoned because of insufficient activity.

Preliminary experiments indicated a lack of <u>in vivo</u> thymidine or amino acid incorporation for several hours (>30) similar to early reports

for DNA (Bryant, 1969a) and protein (Mallery: 1971) synthesis in germinating Allium cepa. To establish the initiation of protein and DNA synthesis in vivo on this batch of seeds, seeds were hydrated and at varying times a portion of the seed coat was removed exposing the embryo, and either 25μCi ^{3}H-leucine or ^{3}H-arginine was added for protein synthesis and ^{3}H-thymidine for DNA synthesis. Paraffin sections were prepared and autoradiographed.

Nuclei from frozen sections of the dry seed were considered to demonstrate in vitro DNA polymerase activity if they displayed twice as much label as the surrounding cytoplasm. Approximately 20% of the unimbibed seeds contained sufficient activity for analysis.

In all DNA polymerase experiments, assay mixtures with added ^{3}H-TTP were stored frozen at -20°C until used (usually 1-6 weeks).

DNA polymerase template activity

Frozen sections were prepared from ungerminated onion seed tissues and incubated with exogenous micrococcal DNA polymerase. The assay medium contained 50mM Tris-Cl (pH 8.0), 0.5mM each of d CTP, d GTP, d ATP, 50μCi ^{3}H-TTP (s.a. 20Ci mM^{-1}) 5mM $Mg(CH_3COO)_2$, 10mM KCl, 0.5mM dTT, 1 mg purified micrococcal DNA polymerase (Sigma). Tissues were frozen inside the cryostat at approximately -40°C, instead of with CO_2. Sections were not collected until at least most of the embryos (which lie horizontally in the embedding gel) were being simultaneously cut, about one quarter to one

one half way within the axis cylinder. Sections were then alternatively collected for control or experimental incubations at 30°C for 1 hour. Reactions were stopped with acetic acid: ethanol and fixed overnight. Sections least disrupted were collected under a dissecting microscope with fine forceps. Tissues were washed with cold 5% TCA (10 ml volumes) in vials and allowed to settle for 15-20 min. (no centrifugation). The TCA treatment was repeated, and the sections washed with cold distilled water until all acid was diluted (by pH check). After removal of the TCA the sections were brought to room temperature and rinsed twice more with distilled water. It is not known if the long period required for washing in the acid solution causes any product hydrolysis.

To check for the presence of S phase cells in various regions of the embryonic axis, seeds were germinated until the radicle had protruded 0.5-2.0mm from the seed coat and exposed to a high specific activity solution of ^{3}H-thymidine (250μCi ml^{-1}, s.a. 6.7Ci mM^{-1}). After 4 to 6 hours exposure, seeds were fixed in acetic-acid: ethanol, the embryonic axis dissected out, washed with TCA and distilled water, and autoradiographs were prepared from paraffin sections. In in vitro experiments with exogenous DNA polymerase, autoradiographic exposure time was reduced to 7 days (compared with 21 days for endogenous assays) to reduce activity from contaminating endogenous polymerase(s).

Amino acid incorporation

Frozen sections (20μ) from germinated seed radicles (1.5-3.0mm) and endosperm were incubated for *in vitro* amino acid incorporation. The assay mixture contained 50mM Tris-Cl (pH 8.0) 25μCi ml^{-1} of a tritiated amino acid mixture containing the labelled acids, alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine, 6mM $Mg(CH_3COO)_2$, 25mM KCl, 2mM ATP, 1mM GTP. Cycloheximide (100 μg ml^{-1}) was added to some incubations.

Sections (20μ) were cut from dry seed embryos and incubated with the same assay mixture except here 'control' reactions contained an analogous mixture (0.05%) of unlabelled amino acids. The effect of polyuridylic acid (poly-U) on phenylalanine incorporation in embryo sections was tested. 25μCi ml^{-1} of ^{3}H-phenylalanine (s.a.13.4 Ci mM^{-1}) was substituted for amino acid mixture and tissues were incubated with or without 100μg of poly-U.

Frozen sections from germinated seed radicles (1.5-3.0mm) were also incubated in an assay mixture containing 50mM Hepes (pH 7.2), 4mM $Mg(CH_3COO)_2$, 0.4mM $(NH_4)_2SO_4$, 0.6 $MnCl_2$, 1.0mM dTT, 50μCi ml^{-1} ^{3}H-leucine (42.7Ci mM). Control reactions contained a combination of 100μg ml^{-1} each of chloramphenicol, cycloheximide and puromycin.

All reactions were stopped with acetic acid: ethanol after incubating for 1 hour at 30°C, tissues were rinsed with 5% TCA, and autoradiographs exposed for 14 days.

Isotopes solutions were partially neutralized with NaOH before addition to assay mixtures which were stored frozen at -20°C.

To check for possible sites of amino acid incorporation in dry embryos, preliminary histological studies were carried out by light and electron microscopy. Background information on histology was provided by histochemical staining for various substances on frozen or paraffin prepared sections and by inspection of thick Epon sections (0.5-1.0μ) after toluidine blue or azure B staining. For histochemical demonstration of cell substances the following staining reactions were performed (a) Millon for protein (b) periodic acid-Schiff for carbohydrate (c) methyl green-pyronin Y for DNA and RNA (d) sudan-black-B for lipid and (e) acid haematin for phospholipid (Pearse, 1961; Jensen 1962). To check for carbohydrate containing bodies on Epon sections, the method of Jacobsen (Jacobsen et al.,1971) was used. For electron microscopy tissues were processed in the usual manner. Attempts were also made to see if different types of storage bodies (or other organelles) could be differentiated on thick tissue slices by scanning electron microscopy (Scurfield et al., 1970).

Methylases

In initial experiments, methionine was used as substrate. Onion seeds were germinated, radicles (1.5-3.0mm) excised, and longitudinal slices (approximately 100-200μ) were placed in an assay mixture containing

(a) 50mM Tris-Cl (pH 7.4), 50μCi ml^{-1} ^{3}H-methyl-methionine (s.a. 190m Ci mM^{-1}). (b) 50mM Tris-Cl (pH 7.4), 10mM $MgCl_2$, 150mM KCl, 1.0mM dTT, 0.5mM each of GTP, ATP, CTP, UTP, 50μCi ^{3}H-methyl methionine. Alternatively, frozen sections of germinated root tips were incubated in solution (b).

In subsequent experiments, S-adenosyl-methionine (SAM) was used as methyl donor. Ethanol was removed from the isotope under N_2 and the H_2SO_4 partially neturalized with NaOH before buffer addition. The assay mixture contained 50mM Tris-Cl (pH 8.0), 1mM KCl, 0.02mM dTT, 50μCi ml^{-1} S-adenosyl-L-methionine-^{3}H-methyl (s.a. 7.3 Ci mM^{-1}). Frozen sections from sprouted bulb roots (2.0-5.0mm) were also used.

To check for incorporation into DNA or RNA, post fixed tissues were treated with either DNAseI (250μg ml^{-1} enzyme, 0.3mM $Mg(CH_3COO)_2$ in distilled water (pH 5.5 adjusted) or RNAse (200μg ml^{-1} enzyme in distilled water (pH 5.5) for 2.5 hours. In some experiments trypsin (40μg ml^{-1}) was added to 'activate' DNA methylation (Tosi and Scarano, 1973).

All reactions were stopped with acetic acid: ethanol and fixed for at least two hours. Thick tissue slices were embedded in paraffin and autoradiographs prepared. Frozen sections were washed in TCA and rinsed in distilled water over a period of several hours similar to the procedure for DNA polymerase template activity. Autoradiographs were exposed for 7-21 days.

Poly(ADP-ribose)polymerase

Frozen sections were prepared from both dry and germinated seeds. The assay mixture contained 50mM Tris-Cl (pH 8.3), 5mM $Mg(CH_3COO)_2$, 3.3mM NaF, 50mM KCl, 3.0mM dTT 50μCi ml^{-1} nicotinamide adenine dinucleotide ^{3}H-adenine (s.a. 3.46 Ci mM^{-1}) (Lehmann et al.,1974). Incubations were for 1 hour at 25°C; autoradiographs were prepared as for methylase assays and developed after 7-21 days exposure.

In biochemical assays, 20-30 embryos were dissected from dry seeds, homogenized in 2 ml of the assay mixture, and incubated for 10 minutes at 27°C. The reaction was stopped with 5% cold TCA and the precipitate was held at 4°C for 10 minutes. The precipitate collected by centrifugation at 2000g, was resuspended and washed with 5% TCA (repeated 3 times) and dissolved in 0.4 mls 0.1 M NaOH. After 15 minutes the following were added;- 0.4 ml 100mM HCl, 0.4 ml 200mM Tris-Cl (pH 7,5), 0.2 ml 100mM $Mg(CH_3COO)_2$ and type VII snake venom phosphodiesterase (100μg) (Sigma). Possible nucleotidase contaminants in phosphodiesterase were inactivated by preincubation at an elevated temperature and low pH (Sulkowski and Laskowski, 1971). Upon addition of 'purified' phosphodiesterase the assay mixture was incubated for 1 hour at 27°C; 4 mg of pronase were then added and the mixture further incubated for 30 minutes at 27°C. 2 ml of ethanol was then added and the solution stored at -20°C. Aliquots of the solution were applied to thin layer cellulose polyethyleneimine (PEI) plates (pre-washed in 0.9 M acetic acid) and run with 1M acetic acid to the origin followed by 0.9 M acetic acid: 0.3 M LiCl. Radiochromatograms were scanned with a Packard chromatogram scanner.

RESULTS AND DISCUSSION

RNA polymerase

RNA polymerase activity was detected cytologically when frozen sections of dry and imbibed seeds were incubated with ^{3}H-UTP. Incorporation is initiated very rapidly and was evident in 15 minute samples of dry seeds (Table 1). Endosperm cells (approximately 50%) demonstrated heavy nucleolar labelling after 30 minutes hydration. After 1 hour, at least 80% of the cells were labelled, a value of 90% being reached by 2 Hours. In the endosperm all periods of incubation displayed cells with both nucleoplasmic and nucleolar activity (Figure 2A-D), with nucleolar incorporation being characteristically distinct. It is not known to what extent clustered or fused nucleolar masses give the false appearance of heavy nucleoplasmic labelling. No distinct nucleolar labelling was detected in embryonic tissue until after 7 hours hydration; in this period, activity was distributed over the chromatin (Figure 3), displaying a lag of 1 hour before its initiation. Even after 1 hour, however, labelling extended throughout the whole embryonic axis. The exact time of 'initiation' of nucleolar activity in the embryo has not been determined but samples of frozen sections from seeds hydrated for 12, 24 and 30 hours display intense nucleolar incorporation. Only low 'background' was seen in cells when DNAse was included in the enzyme incubation mixture or when tissues were post treated with RNAse.

Table 1. *In Vitro* Incorporation from ^{3}H-UTP in Frozen Sections of Onion Seed.

Presoak time (hr)	Incubation time (hr)	Total hydration (hr)	% cells labelled Endosperm	% cells labelled Embryo and Cotyledon
0.00	0.25	0.25	17 ± 1.9*	-
0.25	0.25	0.50	51 ± 3.0	-
0.00	1.00	1.00	81 ± 0.9	-
1.00	1.00	2.00	89 ± 1.1	90 ± 1.2
6.00	1.00	7.00	90 ± 1.0	92 ± 0.9
12.00	1.00**	13.00	>90	>90
24.00	1.00	25.00	>90	>90
30.00	1.00	30.00	>90	>90

The assay mixture contained 0.25 mls of 0.04 M Tris-Cl buffer pH 7.4, 10mM $MgCl_2$, 150mM KCl, 0.5mM EDTA, 1.0mM dTT, 0.25mM each of GTP, ATP, CTP and 100μCi ^{3}H-UTP (s.a. 21Ci mM^{-1}).

* Mean and ± s.e. for approx. 10.0×10^{3} cells from a sample of 300 sections from 10 seeds.

** Samples from 12-30 hr were also incubated for 0.25 and 3 hr in separate experiments.

Figure 2A. *In Vitro* Incorporation from ^{3}H-UTP into Unhydrated Onion Seed Endosperm after 0.5 hr Enzyme Incubation.

Autoradiographs displaying typical patterns of incorporation from ^{3}H-UTP into endosperm sections. Note the preferential labelling of up to 4 nucleoli (arrows) in some nuclei. Most of the cytoplasmic labelling is probably background. The assay mixture contained 0.25 ml of 0.05 M Tris-Cl buffer pH 7.4, 10mM $MgCl_2$, 150mM KCl, 0.5mM EDTA, 1.0mM dTT, 0.25mM each of GTP, ATP, CTP and 100u. Ci ml^{-1} ^{3}H-UTP (s.a. 21Ci mM^{-1}). Autoradiographs were exposed for 14 days. x700.

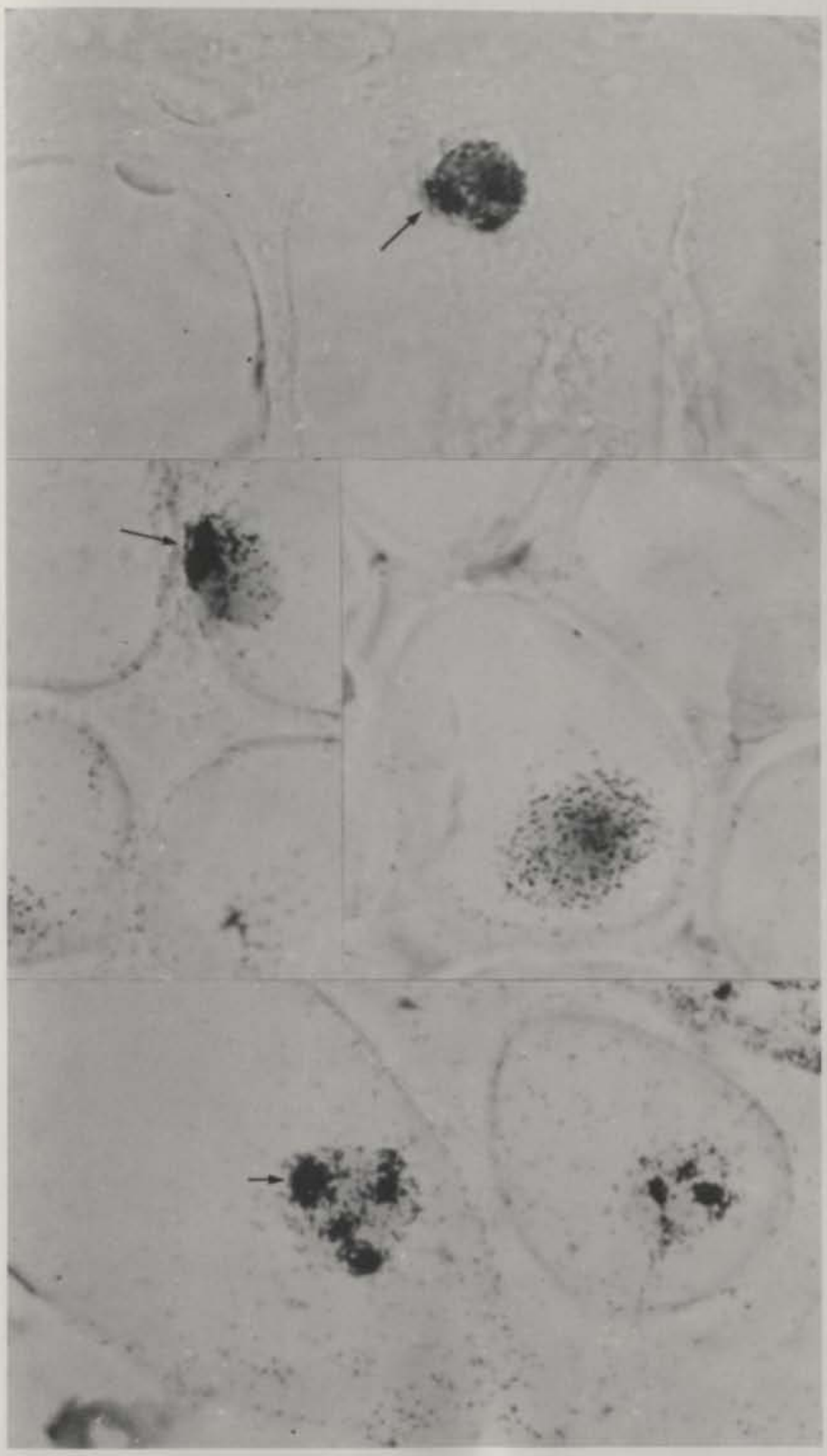


Figure 2B. *In Vitro* Incorporation from ^{3}H-UTP into Unhydrated Onion Seed Endosperm after 1 hr Enzyme Incubation.

Autoradiographs displaying typical patterns of incorporation from ^{3}H-UTP after 1 hr incubation. Note that some nuclei have an intense general distribution of label, while others have preferential nucleolar activity (arrows). Autoradiographic preparation was the same as for Figure 2A. x700.

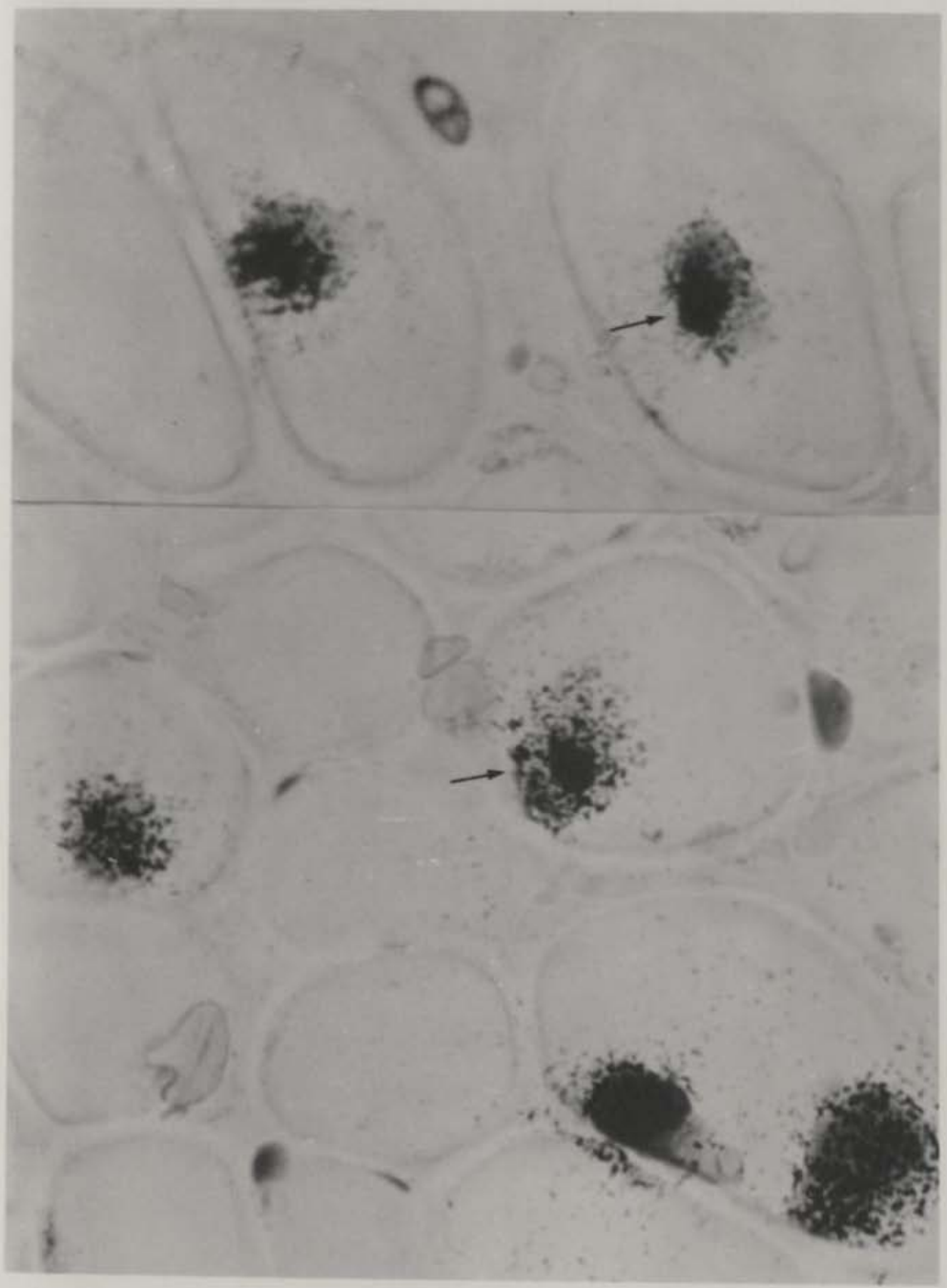


Figure 2C. *In Vitro* Incorporation from ^{3}H-UTP into Onion Seed Endosperm after 2 hrs Hydration.

Autoradiographs displaying typical patterns of incorporation from ^{3}H-UTP after 2 hrs hydration. Note the similarity to shorter hydration periods with both specific nucleolar (arrows) and general nuclear labelling. Note dislodged nucleus (top picture); nuclear displacement was not common however. Some nuclei were completely 'blackened'. Autoradiographic preparation was the same as for Figure 2A. x700.

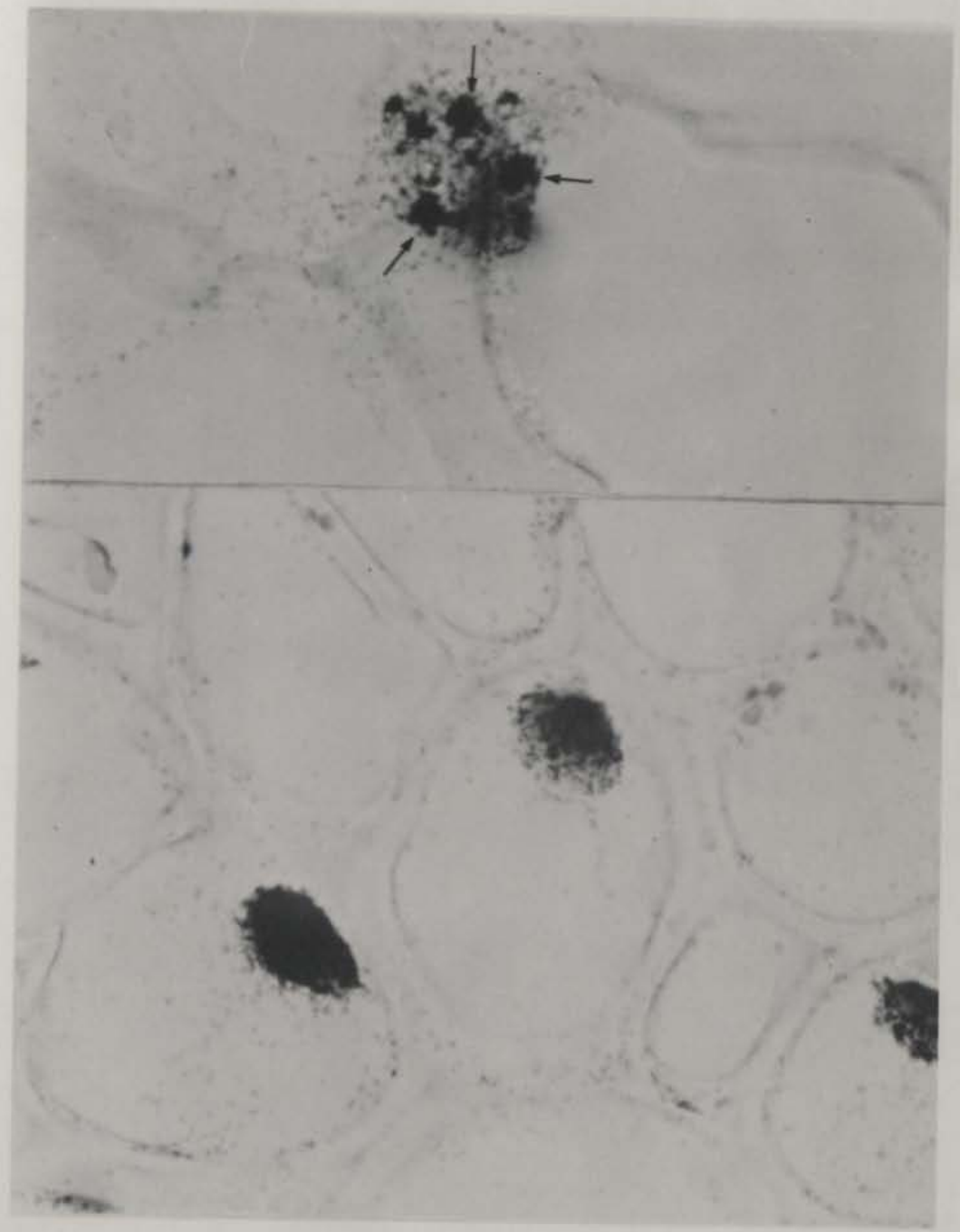


Figure 2D. *In Vitro* Incorporation from ^{3}H-UTP into Onion Seed Endosperm after 7 hrs hydration.

Autoradiographs displaying typical patterns of incorporation from ^{3}H-UTP after 7 hrs hydration. Again note the similarity to shorter hydration periods with specific distinct nucleolar activity (arrows) as well as varying degrees of intensity of general nuclear labelling. The top picture contains a nucleus with approximately 6 labelled regions; this was the largest number seen throughout the experiments. Autoradiographic preparation was the same as for Figure 2A. x700.

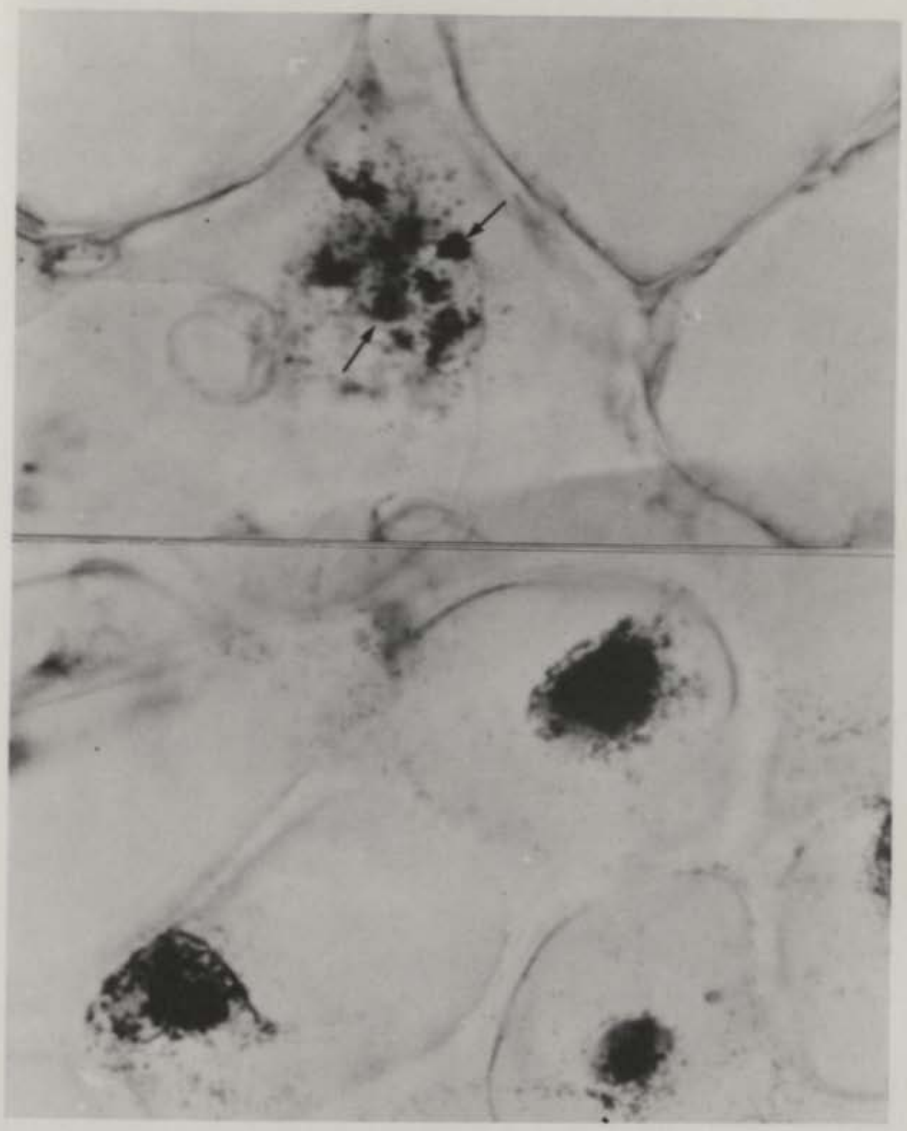
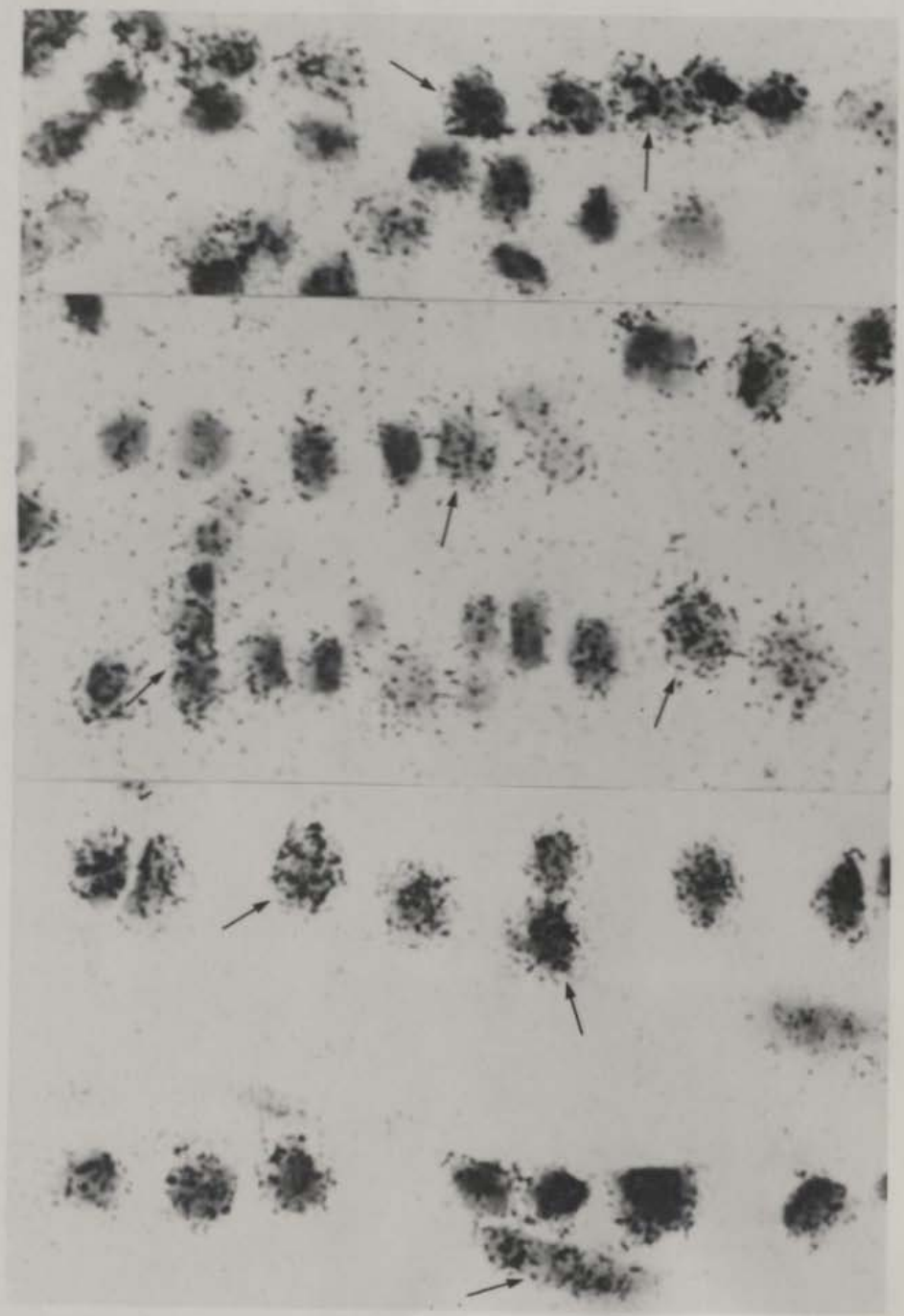


Figure 3. *In Vitro* Incorporation from ^{3}H-UTP in Ungerminated Onion Seed Embryos.

Autoradiographs from different sections displaying incorporation from ^{3}H-UTP into cortical cells of ungerminated embryos (hydrated for 2 hrs). Note the general distribution of nuclear label (arrows). The assay mixture contained 0.25 ml of 0.05 M Tris-Cl buffer pH 7.4, 10mM $MgCl_2$, 150mM KCl, 0.5mM EDTA, 1.0 mM dTT, 0.25 mM each of GTP, ATP, CTP, and 50uCi ^{3}H-UTP (s.a., 21Ci mM^{-1}). Autoradiographs were exposed for 14 days. x700.



Even with ^{3}H-uridine of high specific activity, no incorporation was detected *in vivo* before 18 hours continuous incubation (Table 2), which is in agreement with previous work on *Allium cepa* seed germination (Melera, 1971). Removal of a portion of the seed coat did not increase the rate of incorporation, but permitted an earlier detection of activity. In intact seeds, activity is not detected until the radicle has broken the nucleoside impermeable seed coat (Melera, 1971). Intact seeds incubated for periods to 20 hours in the complete nucleotide media, of varying concentrations and specific activities, did not incorporate any precursors.

In onion bulbs stored 2 months post harvest, rooting and ^{3}H-uridine incorporation was not detected in the most rapidly sprouting bulbs for 30 hours while in those stored for 8 months, RNA synthesis was detected as early as 6 hours (Figure 4). In frozen sections of 2 month old (non-sprouted) bulb roots however, at least 90% of the cells displayed nucleolar and nucleoplasmic RNA polymerase activity (Figure 5). In some cells the nucleoli are preferentially labelled while in others, grains are generally distributed over the whole nucleus. RNA polymerase extends for approximately 1800μ along the root axis, with very low or no incorporation in the root cap zone. In tissues surrounding the quiescent roots some nuclei also displayed a low level of incorporation.

Table 2. *In Vivo* Incorporation from ^{3}H-uridine by Onion Seed Tissues.

	% seeds demonstrating activity in embryo and endosperm (time-hr)		
Precursor	0-18 hr	18-21 hr	21-36 hr
^{3}H-uridine	0	70	95
^{3}H-UTP	0	0	-

Seeds were hydrated in (a) ^{3}H-uridine (100μCi ml^{-1}, 500μCi ml^{-1}, 1000μCi ml^{-1} s.a. 22Ci mM^{-1}, (b) ^{3}H-UTP (100μCi ml^{-1}, 500μCi ml^{-1}, 1000μCi ml^{-1}, s.a. 21Ci mM^{-1} and 18Ci mM^{-1}) 0.25-3.0 mM each of ATP, GTP, CTP, in 3 separate experiments using progressively higher concentrations of precursors. In each experiment exposure to the nucleotide mixture was continuous, or alternatively after 3,6, etc. hr of growth the seeds were pulsed for 3 hr before sampling. Only seeds from which a portion of the seed coat was removed incorporated ^{3}H-uridine at 18 hr.

Figure 4. *In Vivo* Incorporation from ^{3}H-Uridine in Onion Bulb Root Meristems.

Grain counts of *in vivo* ^{3}H-uridine incorporation in a region 800 u from the root apex in quiescent root meristem cells of *Allium cepa* bulbs; 0——0: 8 months post harvest; 0----0: 2 months post harvest. Each point on the graph represents mean grain counts from 5 u paraffin sections prepared from the 7-10 bulbs displaying the first emergent roots. Autoradiographs were exposed for 7 days.

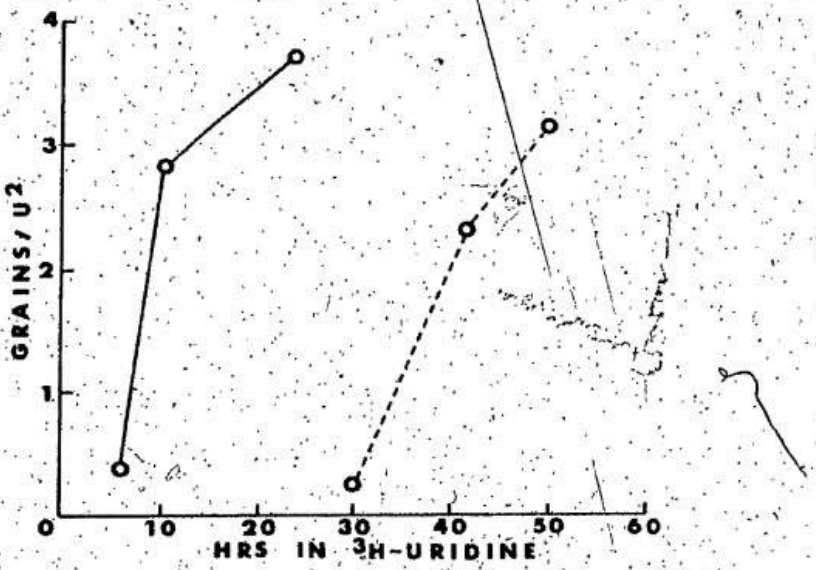
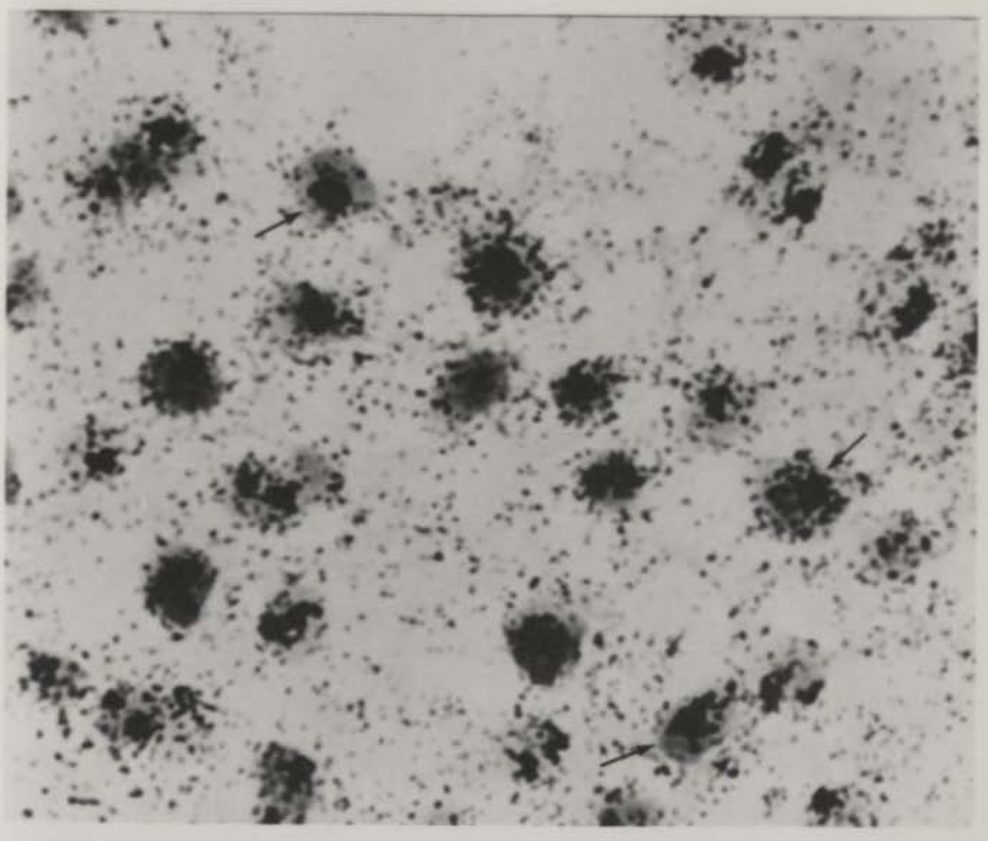

GRAINS / U²
0
1
2
3
4
10
20
30
40
50
60
HRS IN ³H-URIDINE

Figure 5. *In Vitro* Incorporation from ^{3}H-UTP in Onion Bulb Unsprouted Roots.

Autoradiograph displaying incorporation from ^{3}H-UTP into cortical cells. Note the preferential nucleolar (arrows) labelling of some cells. The assay mixture contained 0.25 ml of 0.04 M Tris-Cl buffer pH 7.4, 10mM $MgCl_2$, 150 mM KCl, 0.5 mM EDTA, 1.0 mM dTT, 0.25 mM each of GTP, ATP, CTP and 50uCi ml^{-1} ^{3}H-UTP (s.a. 21Ci mM^{-1}). Autoradiographs were exposed for 14 days. x700.



Some of the cytoplasmic labelling could be removed by post incubation with RNAse, but no attempt was made to critically establish cytoplasmic RNA polymerase activity. Since high concentrations of RNAse can digest total cell RNA, to which even small amounts of radioactivity may be adsorbed, digestive enzymes as RNAse may not be reliable when low levels of incorporation are being assessed (especially on frozen sections). To check for cytoplasmic polymerase, RNA synthesis inhibitors (e.g., actinomycin D, ∝-amanitin) may be useful.

It has been suggested (Mazus and Buchowicz, 1972) that mature seeds may have a complete enzyme system for the immediate initiation of RNA synthesis from low molecular weight precursors, even before protein synthesis begins. Rejman and Buchowicz (1971) demonstrated a more rapid synthesis of RNA in whole wheat seeds as opposed to the excised embryos used by Chen et al., (1968, 1971) in earlier studies. He suggested that this was probably due to the absence of endosperm or aleurone layer which may promote embryonic synthetic activity in the intact seed. The reverse situation, where an embryo factor, gibberellin, increases carbohydrase activity in barley aleurone is known (Radley, 1959; Yomo, 1958). The results of this study suggest that the early RNA synthesis recorded for whole wheat seeds may initially be restricted to aleurone tissue, with a lag before embryo activity commences. It is not known if the enzymes reported for nucleotide synthesis in dry wheat (Duda and Cherry, 1971) and pea seeds (Price and Murray, 1969) can be demonstrated in the excised dry embryo alone.

It is now accepted that nucleolar RNA is not derived from extranucleolar chromosomal activity (Birnstiel et al., 1962; Goldstein and Eastwood, 1966) and it has been shown that the multiple RNA polymerases of rat liver nuclei isolated in high and low salt concentrations have the ionic requirements for ribosomal (nucleolar) or non ribosomal (nucleoplasmic) RNA formation. Eukaryotic RNA polymerases sensitive to α-amanitin are believed to originate in the nucleoplasm as opposed to the nucleolar portion of the nucleus and are believed to be responsible for the transcription of the DNA-like RNA (Lindell et al., 1970; Jacob et al., 1970; Horgen and Griffen, 1971). A recent in vivo autoradiographic study of germinating corn (Deltour, 1970) suggests that early RNA, synthesized in the chromatin, moves to the nucleolus at later stages of growth. The initiation of an intense nucleolar activity would appear to give the same results, unless labelling patterns could be critically quantitated. Onion seeds hydrated for 12, 24 and 36 hours demonstrated embryonic nucleolar RNA polymerase activity, even after short incubation periods. Some onion bulb root cells demonstrated preferential nucleolar activity while in others, grains were more generally distributed over the whole nucleus.

The ribosomal genes in wheat embryos are located entirely within the chromosomal DNA (Chen and Osborne, 1970a) and nucleolar satellites have been reported for pumpkin, barley (Matsuda and Siegel, 1968) and wheat roots (Hotta et al., 1965). It has been shown (since the present work on onion tissues was completed) by cytological hybridization, in situ that ^{3}H-rRNA

fractions from onion root cells are hybridized over nucleoli (Avanzi et al., 1973). The onion seed demonstrates nucleolar activity in endosperm from the beginning of germination and nucleolar activity in the embryo after an initial lag of a few hours, which is characterized by predominantly chromatin labelling. A rapid activation of endosperm nucleoli with subsequent development of the enzymatic machinery for metabolism of polymeric reserves is coincident with evidence from both plant (Bal and Gross, 1964; Das, 1963) and animal systems (Busch and Smetana, 1970) showing rapid growth or high protein production. In such very active cells, nucleolar labelling precedes that of the cytoplasm or the remainder of the nucleus.

It has been suggested (Chen et al., 1971) that early transcription in the wheat embryo may be repressed by a regulatory, or an earlier translational, step involving DNA-dependent RNA polymerase activity. Gibberellin is reported (Chen and Osborne; 1970b) to function only when a cofactor(s) (probably limiting in dry embryos) is available by controlling the expression of preformed messages, but it has also been suggested (Tuan and Bonner, 1964; Jarvis et al., 1968; Duda and Cherry, 1971; Jarvis and Hunter, 1971) that it may act primarily by changes in the transcriptional process. In both onion seed and bulb tissues such enzymes as RNA polymerase, DNA modifying enzymes ('unwindases'), various factors and hormones needed for transcription appear to be preformed. Moreover, if repressive factors are present, their effect is rapidly lost. It is

of interest that an endogenous inhibitor of maize RNA polymerase has been recently purified (Arens and Stout, 1974). Factors responsible for the lag in embryo-transcription and the specific control of nucleolar and chromatin activity remain obscure.

Ultrastructural observations on nucleoli

In Allium cepa the interphase nucleolus may be described (Chouinard, 1966a,b; Chouinard and Leblond, 1966; Chouinard 1970) as consisting of four components intermingled with no definite segregation 1. nucleolar chromatin-dense compact fibrillar patches 2. fibrillar component surrounding the nucleolar chromatin 3. granular component and 4. vacuolar lacunae having a lighter fibrillar matrix. In dry embryonic cells the granular component is poorly defined or lacking and vacuolation is not apparent (Figure 6). The bulk of the nucleolus is made up of fibrillar material, less dense than the surrounding nuclear chromatin. The nucleolar organizing region (NOR) passes through the fibrillar material and is continuous with the nuclear chromatin (Figure 6). There are dense fibrillar areas (about 100Å fibers) embedded in a less dense fibrillar matrix in the nucleolar chromatin; the denser areas which resemble the filamentous chromatin are sometimes arranged in an orderly pattern of 2-4 rows. In the tip of the cotyledon the granular component is again lacking, but the nucleoli are often seen to be extensively vacuolated (Figure 7A). Here the nucleolar material frequently appears as two hemispheres abutting a common NOR (Figure 7B).

Figure 6. Electron Micrograph of a Nucleolus from Onion Seed Embryo Tissue Demonstrating the Continuity of the Nucleolar Chromatin.

The nucleolar chromatin (nch) is continuous with the extra-nucleolar chromatin (ch) material. Note the absence of a granular component and vacuoles. (f, fibrillar material). x27,600.

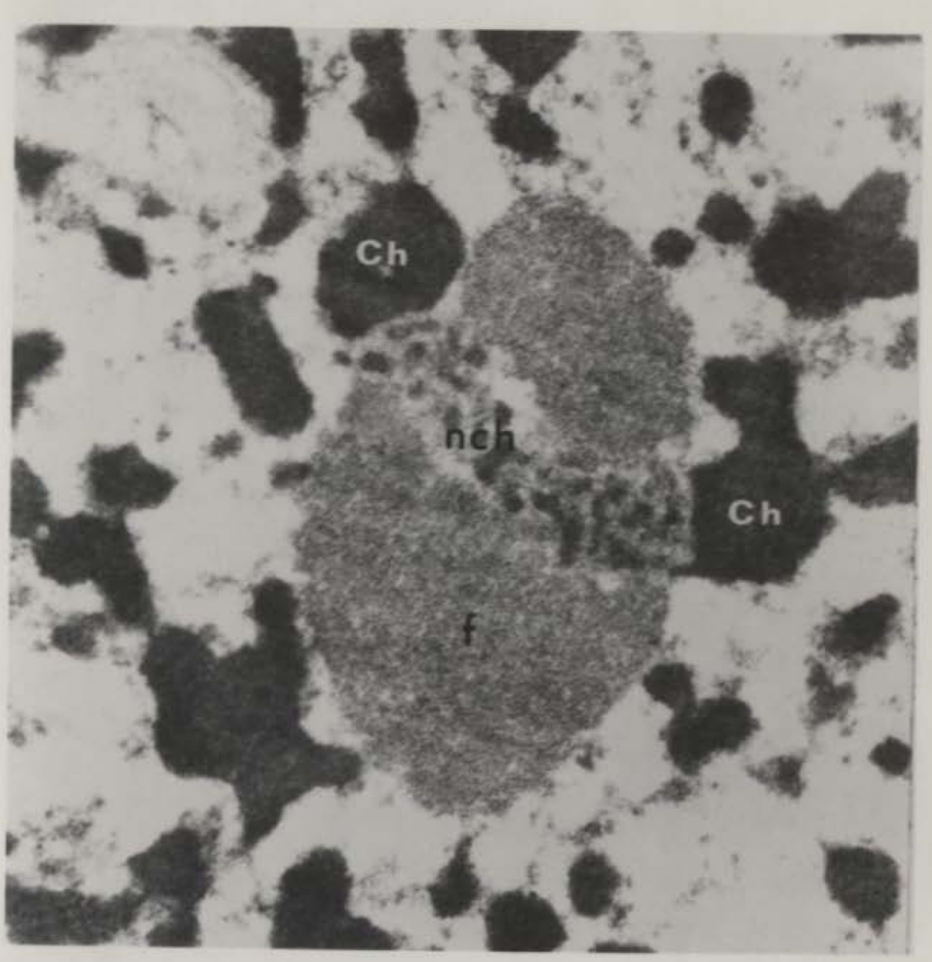
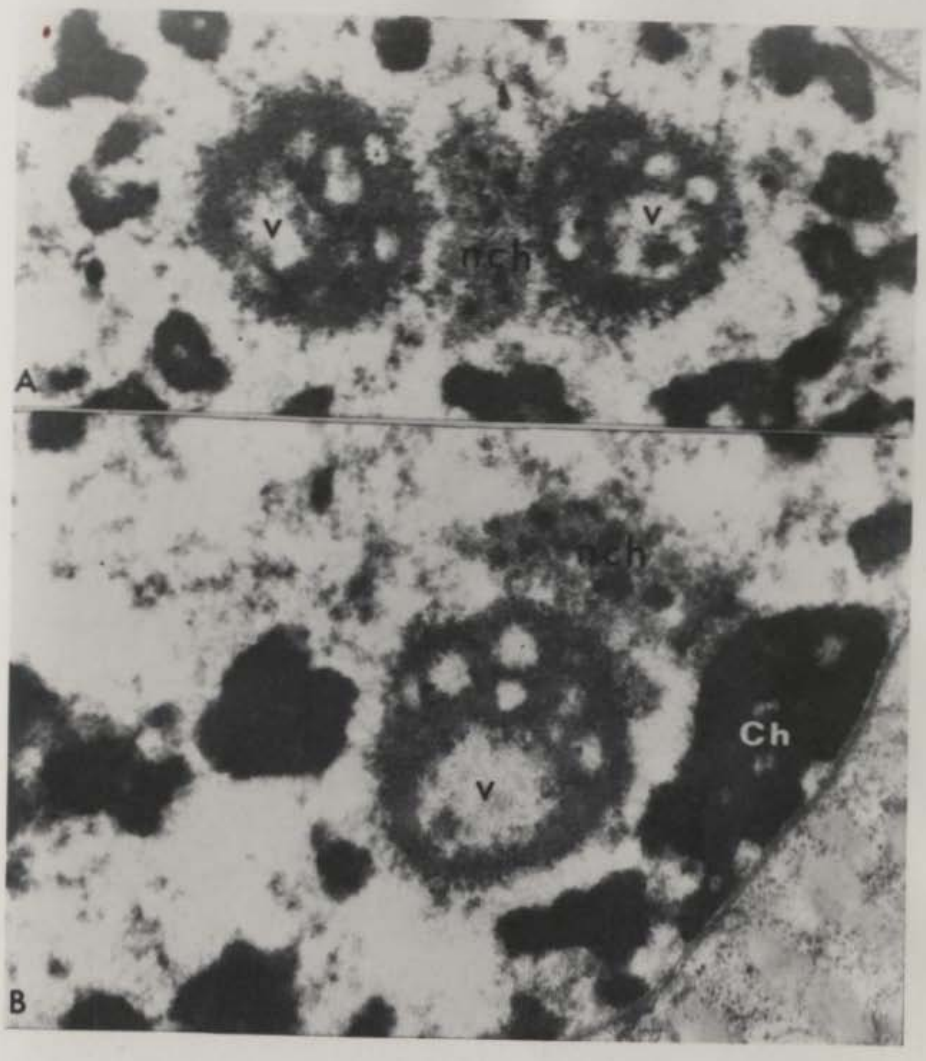

Ch
nch
Ch
f

Figure 7. Electron Micrographs of Nucleoli from the Cotyledon tips of Onion Seed Embryos.

In A. note the absence of a granular component and the separation of nucleolar material into two vacuolated (v) spheres abutting the nucleolar chromatin. In B. the nucleolar chromatin (nch) is continuous with the nuclear chromatin (ch). x27,600.


V
V
nch
A
nch
Ch
V
B

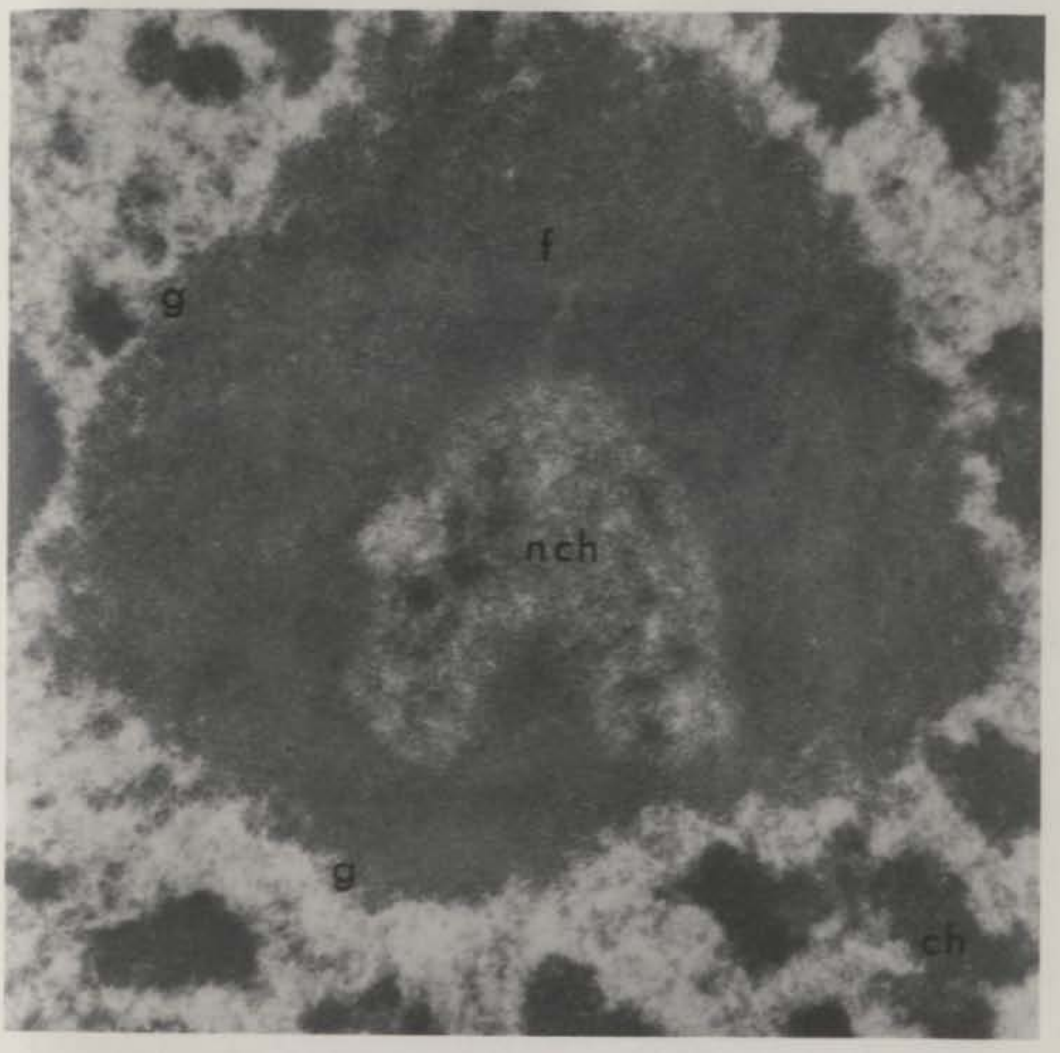
Suitable sections of endosperm tissue could not be obtained for study of nucleolar fine structure. An interesting feature found in half micron Epon sections, however, was the presence of large 'ring shaped nucleoli', the 'ring' periphery characteristically staining with basic dyes. These large nucleoli were not associated with any particular region of the endosperm. When methyl green pryonin was used to differentiate between DNA and RNA on paraffin sections of endosperm, pink staining RNA rich nuclear masses (most likely nucleoli) varied in size and number in different cells.

In the quiescent root some nucleoli have a distinct granular zone (Figure 8) although generally not as marked as in growing roots. Condensed chromatin is associated with the NOR which may be well embedded in the fibrillar matrix (Figure 8). The NOR consists of dense patches surrounded by a less dense matrix which directly abuts the condensed chromatin.

It has been well established that nucleolar morphology is physiologically related to ribosomal RNA synthesis and maturation. In dormant Jerusalem artichoke cells an extensive granular zone develops upon metabolic activation (shaking in distilled water) (Fowke and Setterfield, 1968; Jordan and Chapman, 1971; Rose and Setterfield, 1971) with concomitant nucleolar RNA synthesis (Rose et al.,1972). A predominantly fibrillar nucleous is believed indicative of minimal or no activity in rRNA synthesis in animal or plant tissue. The fibrillar nucleoli of quiescent root tips (Hyde, 1967) have low rates of RNA synthesis (Barlow, 1970). Similar changes in nucleolar activity and RNA synthesis have been noted during the development of the wheat coleoptile (Rose, 1974).

Figure 8. Electron micrograph of a Nucleolus from an Unsprouted Onion Bulb Root.

Note the presence of granular (g) and fibrillar (f) components and electron dense material within the nucleolar chromatin (nch). x42,000.


f
g
nch
g
ch

There have been many reports that nucleolar vacuolation is related to physiological activity (Luehr, 1928; Hopker, 1953; Soudek, 1960; Kordan and Morgenstern 1962; Deltour and Bronchart, 1971) with a correlation between nucleolar vacuolation and incorporation of RNA precursors in both plant (Johnston, 1969) and animal (Zybina, 1968) tissue. In a critical study of vacuolation, however, Barlow (1970) demonstrated that the rate and not the degree of vacuolation was related to RNA synthesis. Similarly, discs cut from Jerusalem artichoke tubers show nucleolar vacuolation before activation (Jordan and Chapman, 1971) while in carrot discs vacuolation occurs only after 24 hour activation (Jordan and Chapman, 1973). The presence of nucleolar vacuoles in the onion seed embryo cotyledon tip is tenable with the hypothesis that the degree of vacuolation need not be dynamically related to nucleolar synthetic activity, but no explanation can be put forth for histological differences with other regions of the embryo.

In both onion seed embryo and quiescent root meristems the NOR is seen to be embedded in the nucleolus. Studies in aritchoke tuber and carrot discs (Jordan and Chapman, 1971; Jordan and Chapman, 1973) show that a region identified as the NOR moves from an extreme external position on the nucleolar periphery into the fibrillar region upon metabolic activation, suggesting that the chromosomal region moves away from the periphery into the nucleolus during periods of increased r-RNA synthesis, with a position

external to the nucleolus representing little or no active RNA synthesis. Support for this view is found in ultrastructural studies of amphibian oocyte nucleoli (Ebstein, 1969) and an external position has also been noted for NOR of quiescent center root tip cells (Hyde, 1967), which are known to be metabolically inactive. The work reported here suggests that a distinct association of the NOR with the nucleolar periphery (e.g. lying on top of the nucleolus) as an indicator of 'repressed' r-RNA synthesis may not be a strict generality applicable to all plant cell activity since in both the onion seed embryo and quiescent root the NOR can often be found partially removed from the nucleolar rim.

Throughout the fibrillar region of many plant nucleoli, runs a coarse skein which is thought to consist of loops of chromosomal origin (La Cour, 1966). Both autoradiographic and cytochemical techniques have shown that such skein containing nucleolar zones are the sites of synthesis of rapidly labelled RNA (La Cour and Crawley, 1965). Work with both animal and plant cells have identified the nucleonema (Estable and Sotelo, 1955) or the corresponding meandering filamentous thread-like structure as the NOR. It is believed that when the organized segments penetrate the nucleolus, they transform into filamentous structures which course throughout the fibrillar zones in a complex fashion. Characteristic of the coarse nucleolar loops is the presence of numerous lacunar spaces which have been shown to contain chromatin and to be continuous with perinucleolar segments of chromosomes. These lacunae contain loose fibrillar material and sometimes within this matrix dense central

cores have been found. High resolution autoradiography has shown DNA in both the dense and light portions of the nucleolar loops (Lafontaine and Lord,1973). An hypothesis has been put forward (Chouinard, 1974) that the state of condensation of the central chromatin core is believed to reflect the degree of unwinding or re-winding of transcriptionally active microfibrils of chromatin extending into the fibrillar region. Thus, when the NOR is active in the synthesis of nucleolar material, its chromatin, including that within the lacunar spaces, would be expected to be 'unwound' and in an extended state. Conversely, a repression of the activity of the nucleolar organizing segment would result in a 'rewinding' of the chromatin microfibrils associated with the dense fibrillar region, and the appearance of chromatin in a condensed state within the lacunar spaces. An earlier report (Bal and Payne, 1972) on the characteristic presence of discrete masses of condensed chromatin arranged in a relatively orderly pattern within the elongated lacunar spaces of nucleoli in onion embryo tissues, has been offered as partial evidence (Chouinard, 1974) for the above hypothesis.

An interesting feature in thick sections of nucleoli in some mature endosperm cells, is prominent central vacuoles occupying most of the nuclear volume having the appearance of "ring shaped nucleoli" (Busch and Smetana, 1970). Such nucleoli frequently occur in blood cells, spermatocytes and oocytes where they seem to be inter-related

with cell maturation and differentiation. The giant ring shaped nucleoli are believed to arise by nucleolar fusion, the number of nucleoli originally produced in each nucleus being constant. This is supported by observations on nucleoli from paraffin sections of onion seed endosperm where nuclear pyronin-positive RNA-containing bodies vary in size and number in different cells. Such heterogeneity in nucleolar size and number is not found, however, in tissues of the embryo axis. An attempt should be made to define more clearly the role of nucleolar fusion in cell differentiation. A recent mathematical model has demonstrated (Hasofer, 1974) that in some cells the frequency of nucleolar fusion is significantly higher than the assumption of initial random location in the nucleus would indicate, suggesting that nucleolar fusion is a controlled physiological process.

Cell cycle

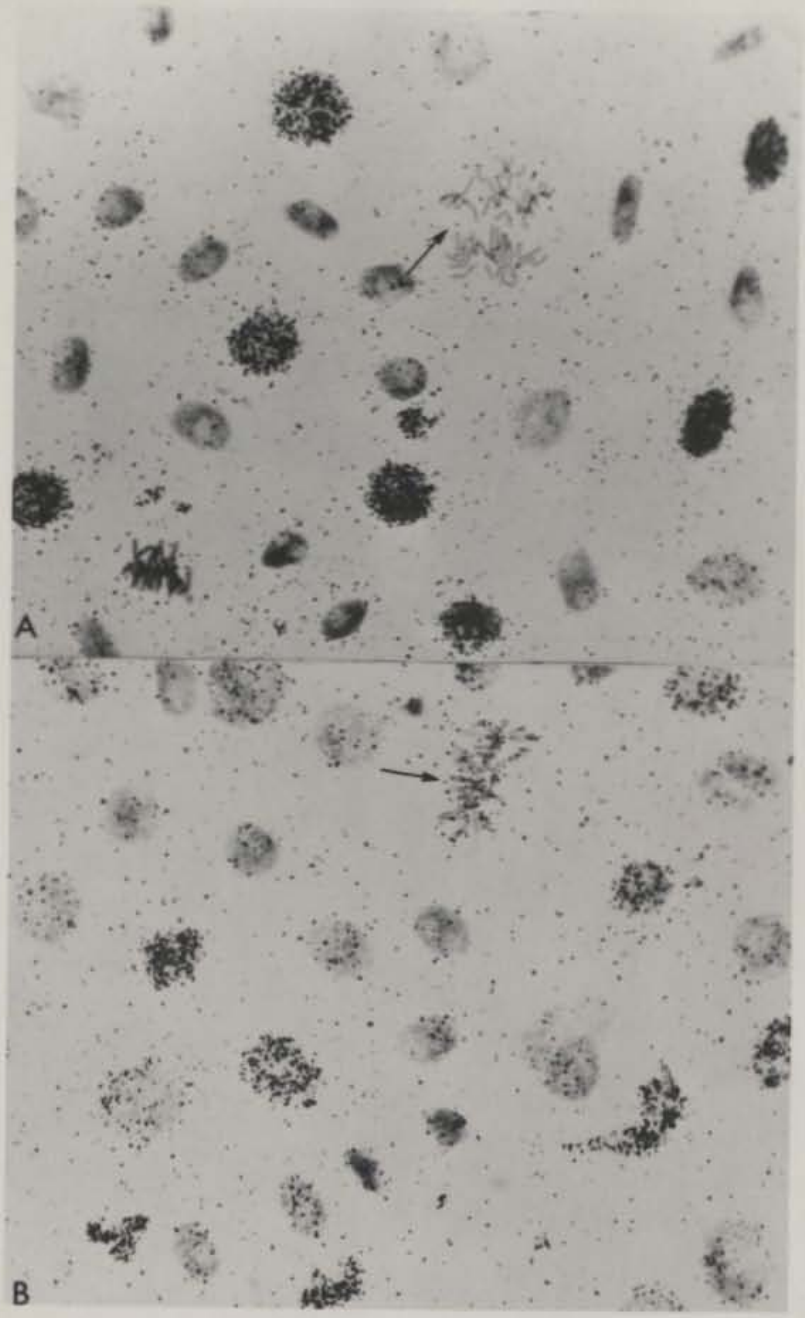
There was no evidence of G_2 arrest in the dry seed, while a proportion of cells in the bulb roots contained a post replicative G_2 population (Figure 9). G_2 cells were found not only at the beginning of sprouting (as early as 3 hours) but were also detected in root tips 12.0-15.0mm long after 24 to 48 hours growth. Similarly G_2 cells were found in root tips that had only protruded 1.0-2.0mm after 48 hours sprouting. Such heterogeneity is not unreasonable since the quiescent roots in the intact bulb display various sizes and degree of development and differentiation. For instance, large roots often appear around the

Figure 9. Identification of Unlabelled and Labelled Mitotic Figures in Sprouted Onion Bulb Roots for G_2 Analysis.

A. Autoradiograph displaying an unlabelled (arrow) mitotic figure (G_2 cell).

B. Autoradiograph displaying a labelled (arrow) mitotic figure.

In Vivo incorporation from ^{3}H-thymidine (10uCi ml^{-1} s.a. 6.7Ci mM^{-1}) into sprouting onion bulb roots with continuous nucleoside exposure. The mitotic figure in A. is considered to be a G_2 cell, similar cells were not found in germinating onion seeds. Autoradiographs were prepared from Feulgen squashes and exposed for 7 days. x500.


A
B

circumference of the bulb base and can be easily recognized with the unaided eye while small non-protruding roots located in the center of the base may only be seen after tissue teasing or paraffin sectioning. Because of the heterogeneity in root protrusion and growth, the total number of cells found in G_2 was compared with percentage labelled classes in S phase (Table 3).

Since G_2 cells can be found a few hours after sprouting to two days, they do not appear to be an homogenous group but have differing G_2-M rates. The duration of the mitotic cycle has been studied in growing root tips of Allium cepa bulbs (MacQuade, 1956; Matagne, 1968) with rates of G_2 from 3-4 hours and M rates from 4-5 hours. It was also reasoned that if an homogenous population of G_2 cells were present in root meristems, it would likely be found associated with a particular tissue region. No such G_2 zones were found on autoradiographs of thick paraffin sections.

Further study demonstrated that in quiescent roots, it is unlikely that there exists 'true' G_2 cells in terms of physiologically blocked populations. Although some cells may be blocked in G_2, their numbers cannot be reliably estimated since the quiescent roots are capable of limited synthetic activity with some cells continuously proceeding through mitosis. When roots were dissected from the bulb base and incubated in ^{3}H-thymidine for 0.5 hours, S phase cells were often seen (Figure 10A). These labelled cells were most frequent in the larger roots near the periphery of the bulb base. The larger roots also frequently displayed mitotic figures in Feulgen stained squashes (Figure 10B).

Table 3. Comparison of Total Number of G_2 Cells in Sprouting Onion Bulb Roots and Percentage of Cells in S Phase

Percentage ^{3}H-thymidine labelled classes	Total number of G_2 cells
0–10	325
11–20	280
21–30	300
>30	150

In vivo incorporation of ^{3}H-thymidine (10μCi ml^{-1} s.a. 6.7 Ci mM^{-1}) into sprouting onion bulb roots with continuous nucleoside exposure. Autoradiographs were prepared from Feulgen squashes and exposed for 7 days. 80 squashes were analyzed and approximately 2000 cells were scored from each. Experiments were performed on the same batch of onion bulbs over a storage period of 4 months (storage was at room temperature under uncontrolled atmospheric humidity.

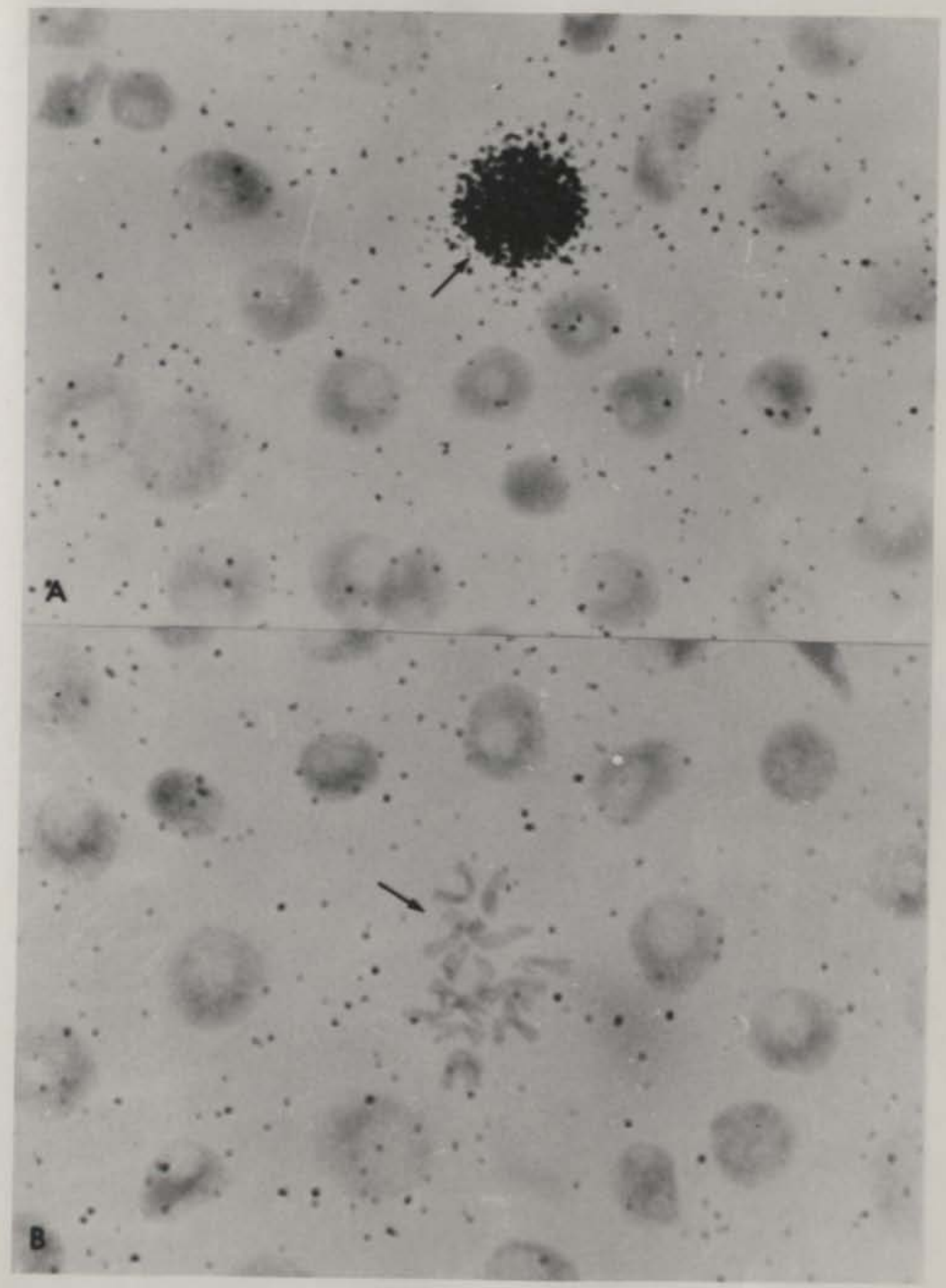
No G_2 cells were detected in 37 squashes of germinated onion seeds.

Figure 10. Detection of S Phase Nuclei and Mitotic Figures in Unsprouted Onion Bulb Roots.

A. Autoradiograph displaying an S phase nucleus (arrow).

B. Autoradiograph displaying a mitotic figure (arrow).

Unsprouted roots were dissected from the bulb base and exposed to 100μCi ml^{-1} ^{3}H-thymidine (s.a. 6.7Ci mM^{-1}) for 0.5 hrs. Both S phase and mitotic figures were found in the 'resting' state. Autoradiographs were prepared from Feulgen squashes and exposed for 7 days. x900.


A
B

Labelled cells were also found in autoradiographs of paraffin sections of quiescent roots incubated for 15 minutes in solutions of tritiated amino acids, consistent with a protein synthetic capability. Such macromolecular synthetic potential is consistent with an elevated metabolic rate. The necessity of a high metabolic rate for the initiation of DNA synthesis has been demonstrated in a number of cell types (Malamud and Baserga, 1968; Polger et al., 1968; Robbins and Morrill, 1969) and in species as diverse as peas and mammals, the higher rate is associated with the synthesis of RNA and protein (Robbins and Morrill, 1969; Webster and van'Hof, 1969).

Ultrastructural studies presented further evidence against the presence of 'true' G_2 blocked cell populations in nuclei of quiescent root meristems. It is more likely that a low level of growth continues in the bulb roots with a few cells continuously entering S phase and dividing. It is not known if there is any relationship between growth and degenerative changes which seem to occur in the quiescent tissue. In bulbs stored for 4-5 months there was cell wall dissolution in cortical and provascular tissues. This dissolution sometimes resulted in the formation of groups of free pycnotic nuclei in an extensively vacuolated cytoplasm (Figure 11). Rough endoplasmic reticulum (RER) was common and in appropriate sections were arranged in unique concentric whorls (Figure 12). In some RER stacks, ribosome attached vesicles appeared to arise by fragmentation from the ends of the cisternae (Figure 13). These vesicles were scattered at random

Figure 11. Electron Micrograph Showing Cell Wall Breakdown in Unsprouted Onion Bulb Roots. Note the formation of masses of 'free floating' pycnotic nuclei (N). x6000.

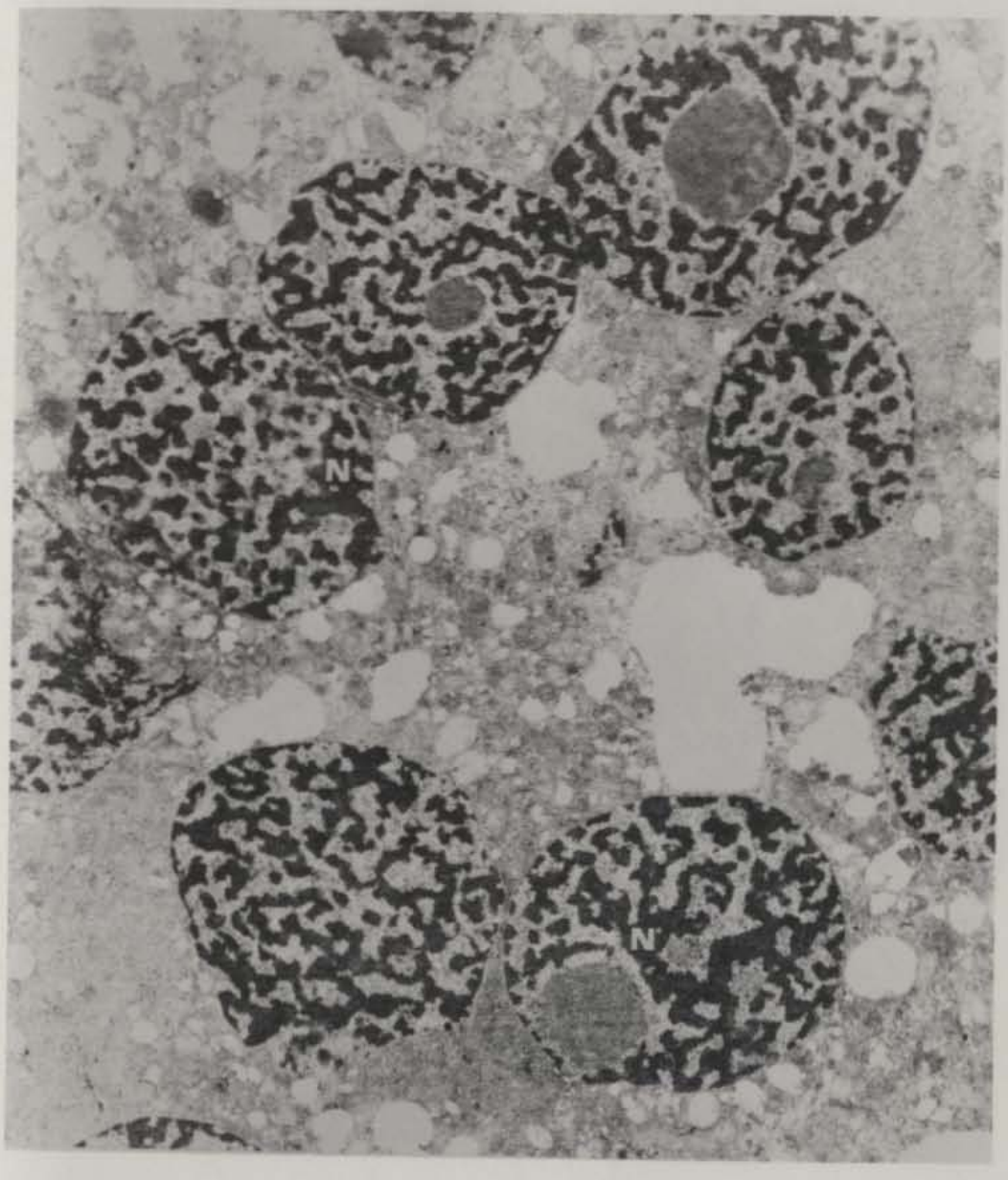

N
N

Figure 12. Electron Micrograph Showing Concentric Whorl of Rough Endoplasmic Reticulum (rer) from an Unsprouted Onion Bulb Root Cell. x18,500.

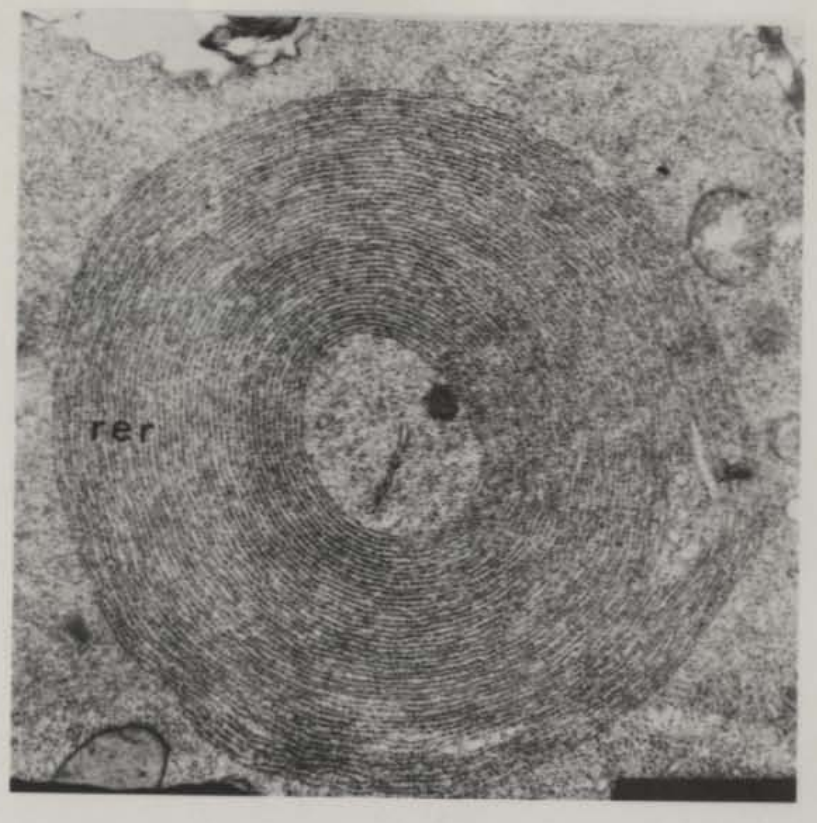

rer

Figure 13. Electron Micrograph Showing Stacks of Rough Endoplasmic Reticulum (rer) and Detached Vesicles (arrows) from an Unsprouted Onion Bulb Root Cell. (c.w., cell wall). x27,500.


rer
cw

throughout the cytoplasm and some were associated with the cell wall. In addition lomasome like configurations and multivesicular bodies were common.

Ultrastructural studies of quiescent roots support the idea of a metabolic state in which synthetic and (or) degradative activities are not completely repressed. It is not known if the RER is involved in the synthesis, packaging, and elaboration of enzymes which may be secreted at the cell wall via ER derived vesicles. Production of ER membranes and the enzyme systems necessary for their formation implies the operation of considerable control at the molecular level. It has been suggested, however, (Sassen, 1965) that during endogenous breakdown of cell walls, Golgi membranes are necessary for the transport of the necessary enzymes. Conversely, it has been well established that during cell wall growth, material is passed into the wall across the plasmalemma by reverse pinocytosis of Golgi derived vesicles (Sievers, 1963; Mollenhauer and Moore, 1966; Pickett-Heaps and Northcote,1966; Wooding, 1968). A view of Golgi membranes as well as rough and smooth ER as distinct morphological and physiological entities may be misleading. For instance, normally in Golgi, ribosomes are not attached to the membranes, but polyribosomes have been recently conclusively demonstrated to be associated with the forming Golgi face (Mollenhauer and Morré, 1974).

A role for ER instead of Golgi in cell wall degradation, however, is not without precedent since in the development of sieve tubes in phloem tissue a functional role has been implicated for such membranes (Northcote and Wooding, 1966; Northcote and Wooding, 1968). It is believed that the

ER serves to remove specific material from the cell wall resulting in pore formation, but it is not known if it acts as a supply route for degrading glycolyases or is involved in transport for the removal of material after its breakdown. Thus, any speculation of a role for ER involvement in cell wall degradation in quiescent roots should be approached with caution at this time.

It is not known if the unique ER whorls in quiescent roots are of any physiological significance. They have been reported before in potato tuber buds (Shih and Rappaport, 1971) and dormant embryos (Nougarede and Pilet, 1964; Varner and Schidlovsky, 1963) and have been induced to form under conditions of low oxygen tension (Whaley et al.,1964; Perner, 1966). They are also known from various types of animal tissue (Ruthmann, 1958; Herman et al.,1962) and are often observed to form after treatment with metabolic inhibitors (Hwang et al.,1974). A critical study of their formation in liver cells in the presence of protein synthesis inhibitors (Hwang et al.,1974), has revealed that whorl-like membrane structures may be indicative of a normal transient stage of membrane formation and (or) repair.

A variety of plasma membrane lomasome-like modifications were frequently found in onion quiescent roots (Bal, 1973; the present work); no such structures were found in seed tissues. Such membrane modifications are known throughout the plant kingdom, but evidence for their presence in 'true' quiescent tissues as seeds and spores is lacking. The function

of various types of lomosomal structures has not been completely elucidated and at least some of the structures are believed to be fixation artifacts (Brushaber and Jenkins, 1971). It has been suggested that lomasomes may function in cell wall synthesis (Marchant and Robards, 1968) which is an attractive role for their presence in those quiescent root cells where division and growth is occurring. It is maintained however, (Fowke and Setterfield, 1968; Brushaber and Jenkins, 1971) that lomasomes may play no part in cell wall formation. There is good ultrastructural and cytochemical evidence for the involvement of plasma membrane invaginations (pinocytotic vesicles) in absorptive processes in roots (MacRobbie, 1969; Hall, 1970; Wheeler and Hanchey, 1971) but such activities would be expected to be minimal or absent in quiescent roots. It is apparent however, that most of the suggested functions are more characteristic of 'active' rather than repressed or quiescent tissues supporting the concept of a depressed synthetic rate in the root meristems. It is tenable that some of the membranous structures are indicators of catabolic degenerative activity since some of them resemble autophagic vacuoles found in growing roots (Mesquita, 1972) and senescing root cap tissue in germinating seeds (Berzak and Villiers, 1970).

DNA polymerase

A significant level of *in vitro* DNA polymerase activity was detected in 8μ cryostat sections of germinating seed root tips (Figure 14),

Figure 14. *In Vitro* Incorporation from ^{3}H-TTP in Onion Seed Root Tip Meristematic Nuclei.

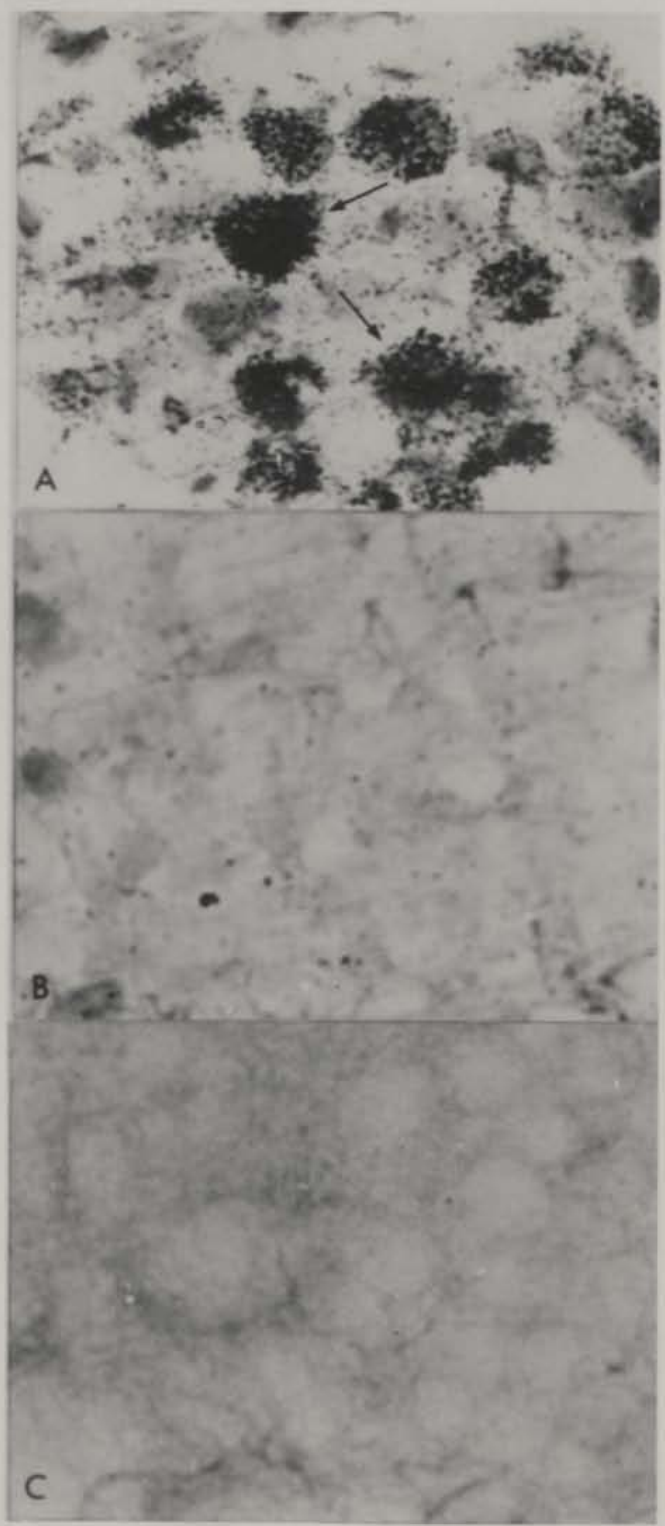
Autoradiographs displaying

A. Incorporation from ^{3}H-TTP into cortical meristematic cells.

B. Incorporation from ^{3}H-TTP with DNAse treatment

C. Incorporation from ^{3}H-TTP with KCl treatment

Frozen sections were prepared from germinated root tips (2-4 mm long). The assay mixture contained 0.05M Tris-Cl buffer pH 8.4, 0.24mM each of d CTP, d GTP, d ATP, 75uCi ml^{-1} ^{3}H-TTP (s.a. 18Ci mM^{-1}), 5mM $MgCl_2$, 1mM d TT. In B. 100ug DNAseI was included in the reaction mixture; in C. 4M KCl was included. A similar lack of incorporation was observed with NEM (1mM) and pCMB (1mM) treated tissues. Autoradiographs were exposed for 14 days. x700.


A
B
C

and approximately 30% of the cells were labelled (Table 4). Enzyme activity could also be detected in whole roots or tissue slices permeabilized with toluene or by freeze thawing, but incorporation was not as intense. Permeabilizing by freezing also allowed sufficient activity to be demonstrable in Feulgen stained squashes (Figure 15). In whole or root slices prefrozen in N_2, CO_2 or at -20°C, activity was often more apparent in peripheral cell layers (Figure 16A), but incorporation was also seen in cortical and provascular tissues (Figure 16B). Similar results were noted for toluene treatments (Figure 17). Successive freeze thaw treatments did not increase the efficiency of the methods (Figure 18). Precursor hydrolysis was cytochemically demonstrated in cell walls, especially in external cell layers and throughout the root cap, but increasing deoxynucleoside concentrations from 0.25-2.0mM and ^{3}H-TTP to 250µCi ml^{-1} did not significantly alter incorporation in the larger pieces of tissue. Although pretreatment with toluene increased the permeability of the tissue to precursors, consistency of results was reduced when sections greater than approximately 200u were used; the method could however, permit a composite picture of incorporation to be attained. Activity was detected if ^{3}H-dATP was substituted for ^{3}H-TTP. At least 90% of the activity disappeared if tissues were pre or post treated with DNAse. Similarly nuclear activity was abolished if tissues were heated to 80-90°C or pretreated with 1% phenol. The latter treatments were especially performed on whole roots or tissue slices to see if the cytoplasmic labelling often found in the root

Table 4. Percentage Onion Seed Root Tip Meristematic Nuclei Displaying Intense *In Vitro* Incorporation from ^{3}H-TTP.

Number of nuclei section^{-1} counted	% nuclei displaying ^{3}H-TTP Incorporation
127.34±56.39	28.32±7.50

Frozen sections were prepared from germinated seeds. Assay mixture same as for Figure 14. 15 sections were analyzed for activity. Nuclei considered to display intense activity are marked by arrows in Figure 14. Autoradiographs were exposed for 14 days.

Figure 15. *In Vitro* Incorporation from ^{3}H-TTP in Feulgen Stained Squashes of Sprouted Onion Bulb Root Meristems

Autoradiographs of Feulgen stained squashes of root meristem cells displaying incorporation from ^{3}H-TTP after permeabilizing by freezing at A. -20°C or B. two successive freeze thaw treatments with nitrogen. The reaction mixture contained 0.05M Tris-Cl buffer pH 8.4, 0.25mM each of d CTP, d GTP, d ATP, 100uCi ml^{-1} ^{3}H-TTP (s.a. 18Ci mM^{-1}) 5mM $MgCl_2$, 1mM dTT. Autoradiographs were exposed for 14 days. x700.

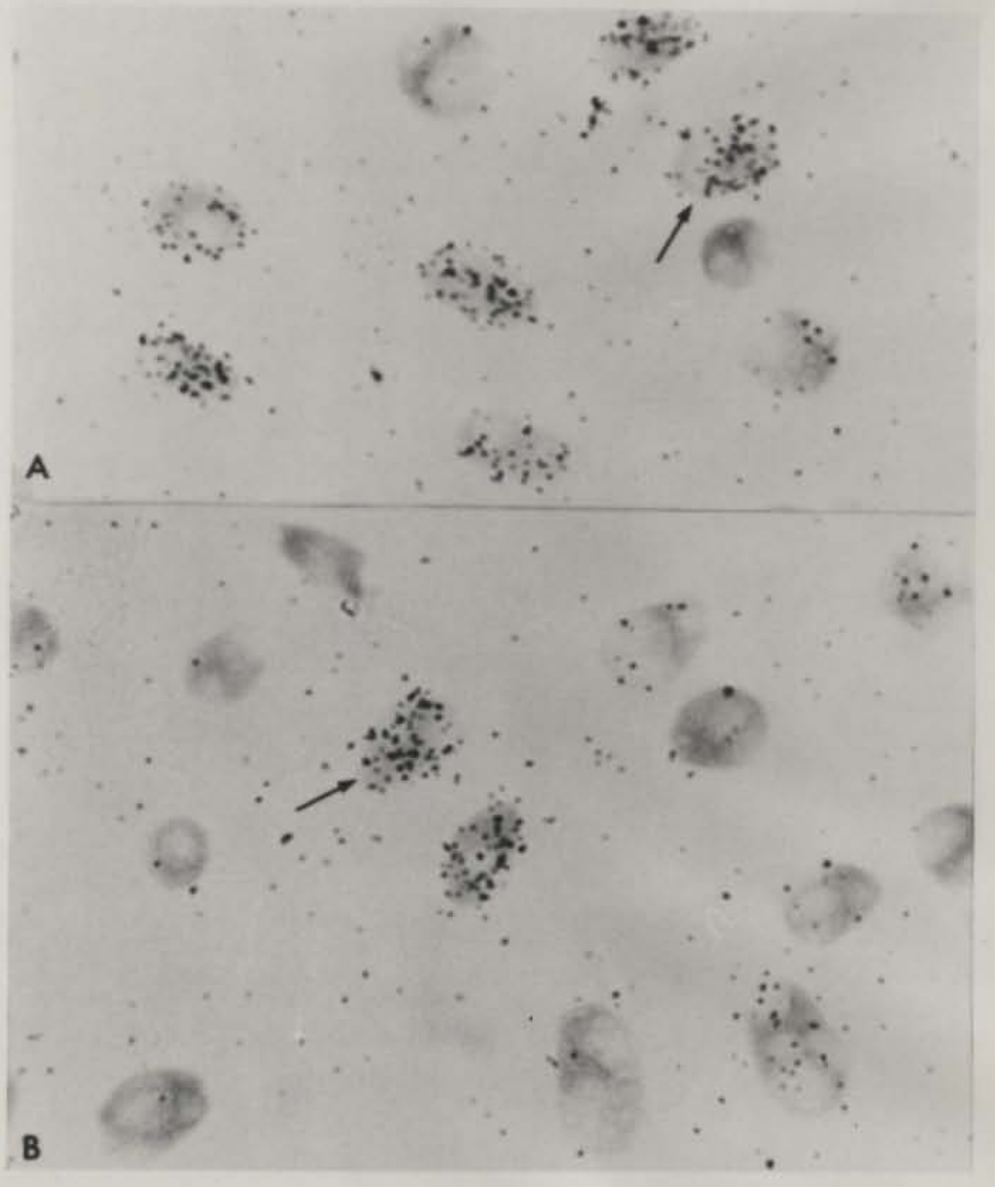

A
B

Figure 16. In Vitro Incorporation from ^{3}H-TTP in Onion Seed Root Tip Meristematic Nuclei after Freeze Permeabilization of Thick Tissue Slices.

Autoradiographs displaying

A. Incorporation from ^{3}H-TTP into cortical cells adjacent to the root periphery (p) after one freeze thaw treatment with nitrogen.

B. Incorporation from ^{3}H-TTP into cortical cells after one freeze thaw treatment at -20°C.

The assay mixture contained 0.05 M Tris-Cl buffer pH 8.4, 0.5mM each of d CTP, d GTP, d ATP, 100uCi ml^{-1} ^{3}H-TTP (s.a. 20Ci mM^{-1}) 5mM Mg $(CH_3COO)_2$, 1mM dTT. Autoradiographs were prepared from paraffin sections and exposed for 14 days. x700.

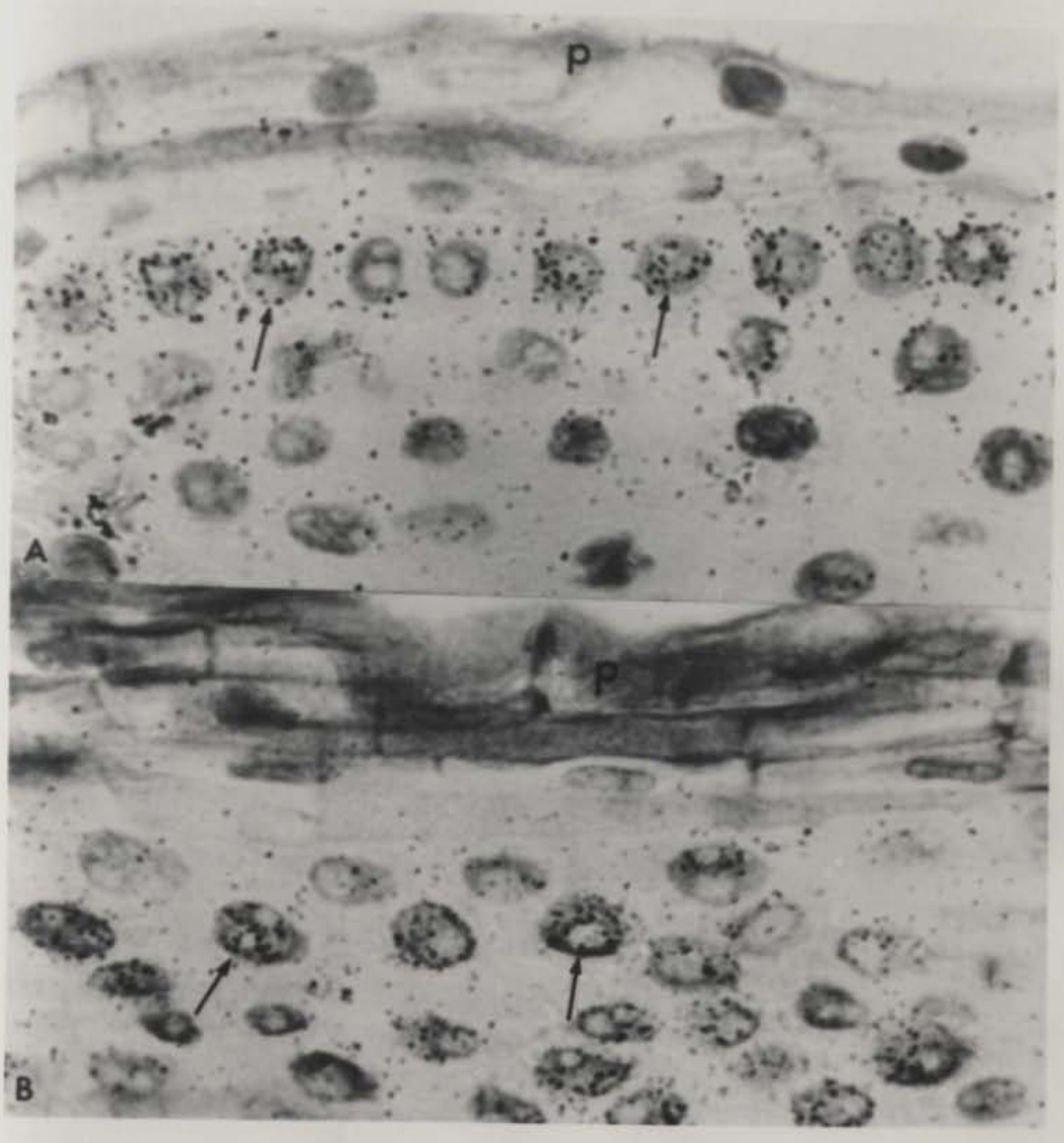

P
A
P
B

Figure 17. *In Vitro* Incorporation from ^{3}H-TTP in Onion Seed Root Tip Meristematic Nuclei after Toluene Permeabilization of Thick Tissue Slices.

Autoradiographs displaying

A. Incorporation from ^{3}H-TTP into cortical cells adjacent to the root periphery (p).

B. Incorporation from ^{3}H-TTP into cells adjacent to the excision surface approximately 1600-2000u from the root apex.

Germinated seed roots (2-4mm long) were excised and shaken in 1% toluene in 0.07M K_2HPO_4 buffer pH 7.4 for 1 min. The assay mixture contained 0.05M Tris-Cl buffer pH 8.4, 0.5mM each of d CTP, d GTP, d ATP, 50uCi ml^{-1} ^{3}H-TTP (s.a. 20 Ci mM^{-1}), 5mM $Mg(CH_3COO)_2$, 1mM d TT. Autoradiographs were prepared from paraffin sections and exposed for 14 days. x700.

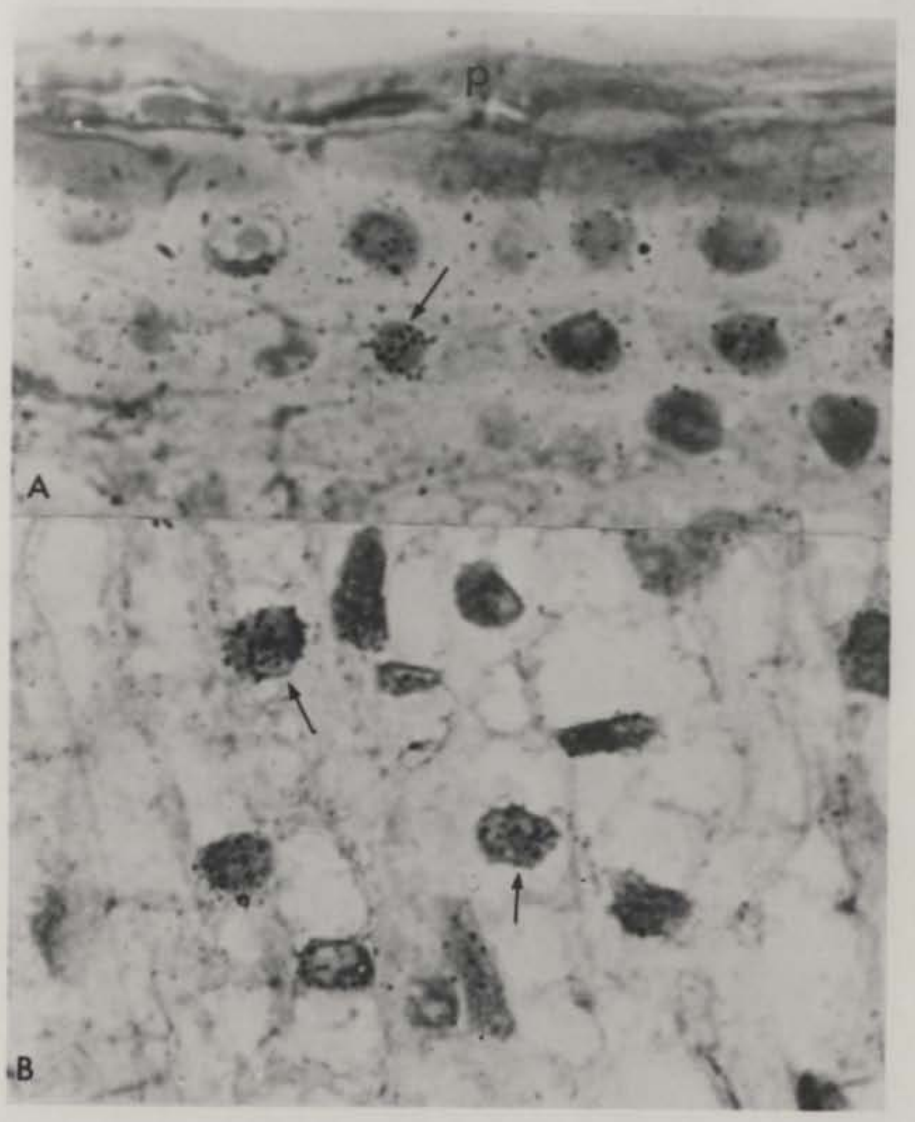

P
A
B

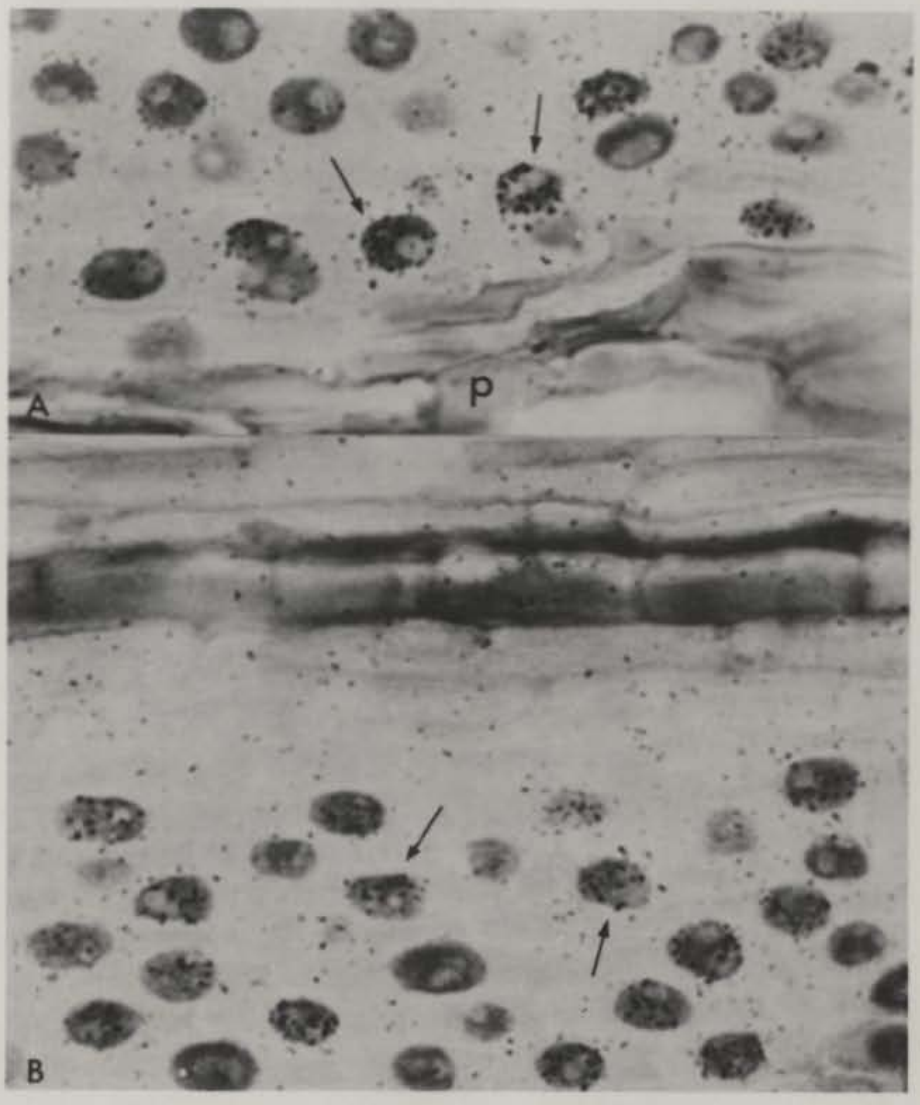
Figure 18: In Vitro Incorporation from ^{3}H-TTP in Onion Seed Root Tip Meristematic Nuclei after Freeze Permeabilization of Thick Tissue Slices.

Autoradiographs displaying

A. Incorporation from ^{3}H-TTP into cortical cells adjacent to the root periphery (p) after two successive freeze thaw treatments with nitrogen.

B. Incorporation from ^{3}H-TTP into cortical cells after two successive freeze thaw treatments at -20°C.

The assay mixture contained 0.05M Tris-Cl buffer pH 8.4, 0.5mM each of d CTP, d GTP, d ATP, 75uCi ml^{-1} ^{3}H-TTP (s.a. 20Ci mM^{-1}) 5mM $MgCl_2$, 1mM dTT. Autoradiographs were prepared from paraffin sections and exposed for 14 days. x700.


A
P
B

cap zone after freezing or toluene permeabilization treatments (Figure 19) was removed. Variability in incorporation was, however, great and a number of experiments would have to be performed before any conclusions could be reached. The exclusion of individual deoxytriphosphates reduced grain densities to approximately one third. This experiment was performed on a set of root tips permeabilized by freezing at -20°C and incubated with one particular batch of ^{3}H-TTP (s.a. 48Ci mM^{-1}). Only one experiment was done and should be repeated with other separate batches of nucleotides. Each experiment has to be done with separate shipments of isotopes and one experiment cannot be compared with another meaningfully unless facilities are available to establish the purity and specific activities of incubation mixtures.

A number of treatments affected enzyme activity. High concentrations of potassium chloride (Kornberg and Gefter, 1970; Chiu and Sung, 1972) inhibited incorporation (Figure 14C); this could be partially due to an indirect 'salting away' of the chromatin as nuclear staining was reduced with increasing molarity. The sulfhydryl compounds, NEM and pCMB were good inhibitors. Activity was not enhanced if small concentrations of DNAse (concentrations were progressively reduced from 50µg ml^{-1}, 135 units, to 0.2µg ml^{-1}, 1 unit, in separate experiments) or if ATP (0.5-3.0mM) were included in the incubation mixture. Concentrations of DNAse as low as 5µg ml^{-1} in the assay mixture abolished activity, and 20µg ml^{-1} solubilized the nuclei to an extent where only fibrous remmants remained; this is

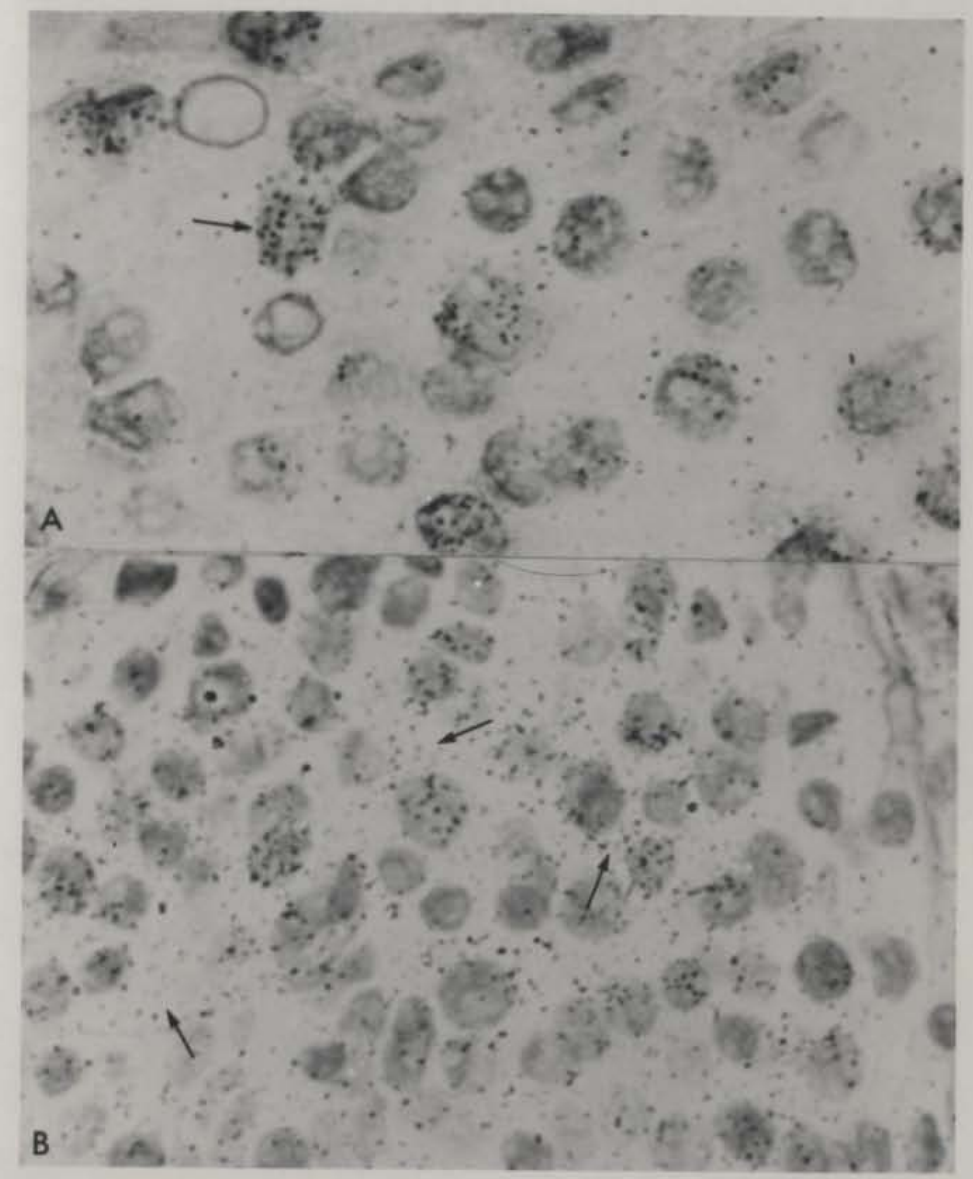
Figure 19. *In Vitro* Incorporation from ^{3}H-TTP in Onion Seed Root Cap Nuclei after Freeze and Toluene Permeabilization.

Autoradiographs displaying

A. Incorporation from ^{3}H-TTP into cells approximately 300-500u from the root apex after freezing at -20°C.

B. Incorporation from ^{3}H-TTP into cells approximately 100-200u from the root apex after toluene treatment. Note the extensive cytoplasmic labelling (arrow).

Treatment and assay mixture were similar to Figure 18 for A and Figure 19 for B.


A
B

contrasted with a partially purified DNA polymerase fraction from corn seedlings (Stout and Arens, 1970) where DNAse concentrations to 50µg ml^{-1} (135 units) in the assay mixture markedly stimulated activity (3 fold) on native primer.

A low level of DNA polymerase was detected in the pre-emergent onion embryo. Activity was not confined to any particular tissue, but could be found in provascular, epicotyl, cotyledonary and root cap zones, with the cotyledonary cells displaying the least incorporation (Figure 20-21). Inclusion of NEM or pCMB in the incubation mixture reduced activity considerably (Table 5) and no significant incorporation was found after DNAse treatment. No increase in activity was found with low concentrations of DNAse or in the presence of high nucleotide concentrations (Table 5). When cycloheximide or RNAse was included in the incubation mixture, activity appeared to be unchanged (Table 5). The low level of polymerase activity in the dry embryo is contrasted with the intense labelling seen in S phase cells.

DNA polymerases are unable to initiate DNA synthesis 'de novo' on a single stranded template, without a primer containing a free -3OH end (Bollum et al.,1969; Baltimore and Smoler, 1971). In vivo experiments demonstrated a population of approximately 30% of meristem cells in S phase. A similar number in the in vitro situation displayed DNA polymerase activity, suggesting that deoxynucleoside polymerization is true replication and is proceeding on the primed template, which has

Figure 20. In Vitro Incorporation from ^{3}H-TTP in Ungerminated Onion Seed Embryos.

Autoradiographs displaying a low level of

A. Incorporation from ^{3}H-TTP into cells adjacent to the embryo periphery approximately 1000-1500u from the root apex.

B. Incorporation from ^{3}H-TTP into presumptive meristematic cells approximately 500-800u from the root apex.

Frozen sections were prepared from dissected embryos. The assay mixture contained 0.05M Tris-Cl buffer pH 8.4, 0.5mM each of d CTP, d GTP, d ATP, 100uCi ml^{-1} ^{3}H-TTP (s.a. 18Ci mM^{-1}), 5mM $Mg(CH_3COO)_2$. Autoradiographs were exposed for 21 days. x700.

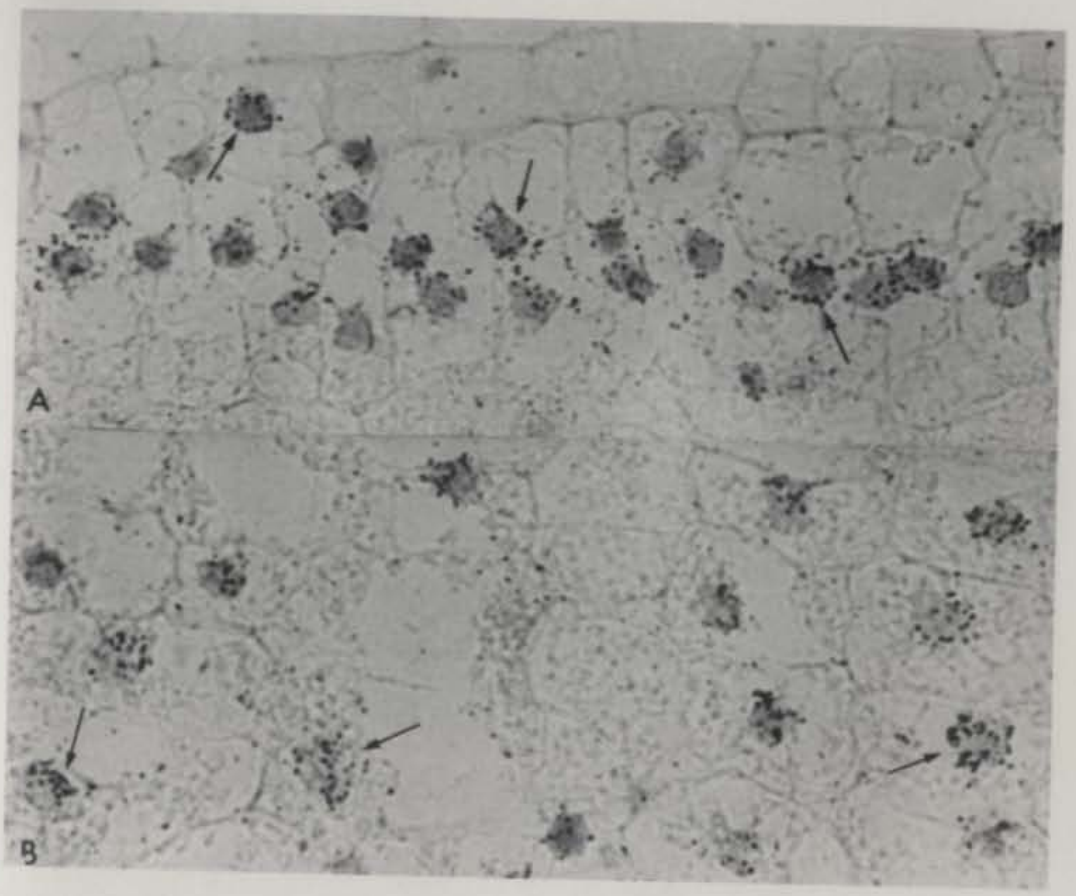

A
B

Figure 21. *In Vitro* Incorporation from ^{3}H-TTP in Ungerminated Onion Seed Embryos.

Autoradiographs displaying a low level of

A. Incorporation from ^{3}H-TTP into provascular cells approximately 1200u from the root apex.

B. Incorporation from ^{3}H-TTP into cells approximately 300u from the root apex.

Autoradiograph preparation was the same as for Figure 20. x700.

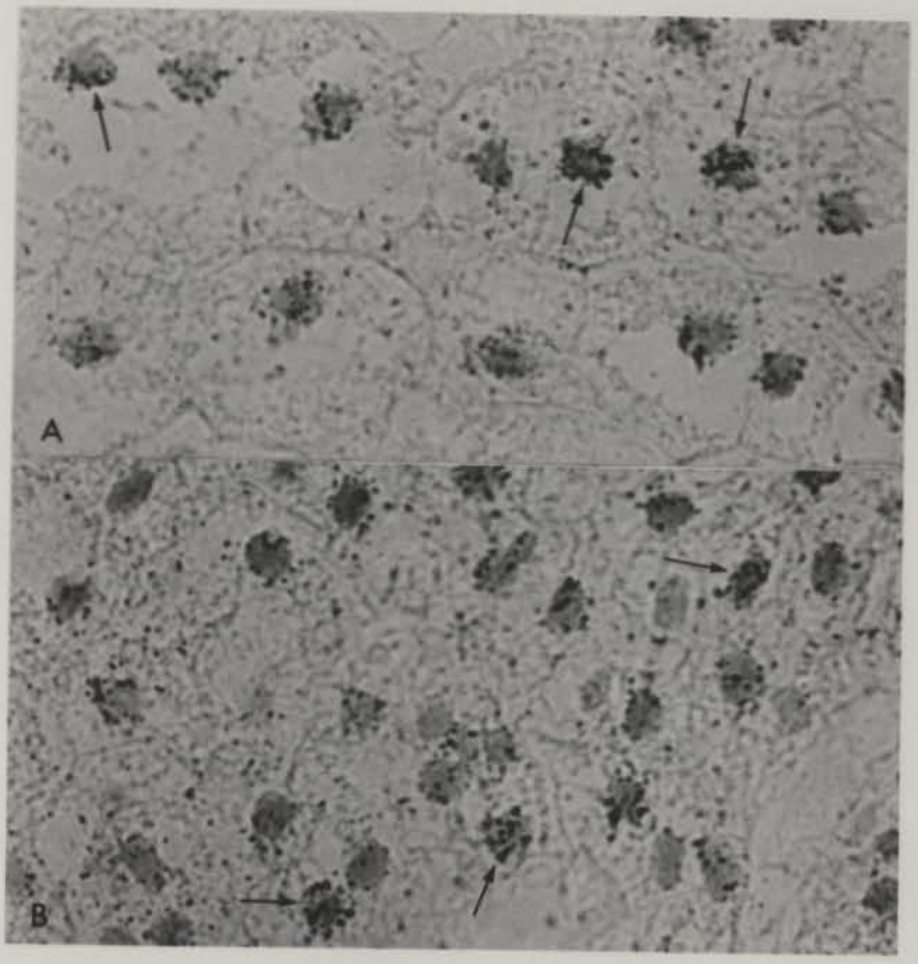

A
B

Table 5. Effect of Changes in Reaction Mixture on *In Vitro* Incorporation from ^{3}H-TTP in Tissues of Ungerminated Onion Seed Embryos.

Reaction mixture	% labelled cells	Reaction Mixture	% labelled cells
complete	19*	+100 µg ml^{-1} RNAse	16
+0.025 µg ml^{-1} DNAseI	18	+2mM pCMB	<2
+0.1 µg ml^{-1} DNAseI	17	+2mM NEM	<2
+50.0 µg ml^{-1} DNAseI	4	+ 1 or 3mM ATP	17
+50.0 µg ml^{-1} cycloheximide	16	+ 1 or 3mM GTP	19
+10.0 µg ml^{-1} cycloheximide	17		

Frozen sections were prepared from dissected embryos. The assay mixture contained 0.05M Tris-Cl buffer pH 8.4, 0.5mM each of d CTP, d GTP, d ATP, 100µCi ^{3}H-TTP (s.a. 18Ci mM^{-1}) 5mM Mg $(CH_3COO)_2$, 1mM dTT. Nuclei considered to be labelled are shown in figures 20 and 21. Autoradiographs were exposed for 21 days.

* Mean from sample of 150 sections.

already initiated a round of DNA replication (Burger, 1971; Howell and Walker, 1972) in the cells at S phase. A favorable level of DNA polymerase activity was demonstrable in these studies, especially on 8μ cryostat sections. In bacterial systems the rate of true DNA replication in vitro is much lower than in vivo rates (Schaller et al., 1972; Smith et al., 1970).

Two types of DNA synthesis are known, one for replication of DNA, the other for repair of DNA. A number of studies with toluene (Moses and Richardson, 1970; Masamichi and Kolber, 1970; Burger, 1971; Matsushita et al., 1971; Howell and Walker, 1972; Brown et al., 1972), sucrose (Wickner and Hurwitz, 1972) or Briz-58 (Ganesan, 1971) have shown that a major part of DNA polymerase activity may reflect normal replication of DNA while, for instance, Tris-EDTA permeabilized cells (Buttin and Wright, 1968) are believed to display predominantly repair synthesis. A frequent characteristic of replicative DNA synthesis is a requirement for ATP (or other nucleoside triphosphates) and an inhibition by sulfhydryl reagents as NEM or p-CMB, whereas repair synthesis does not require exogenous ATP and is not inhibited by sulfhydryl compounds. Also purified preparations of E coli DNA polymerase II (Kornberg and Gefter, 1970) as well as a distinct species of DNA polymerase (other than I or II) isolated from E coli mutants (Kornberg and Gefter, 1971), differ from polymerase I (believed to function mostly in DNA repair) in their sensitivity to sulfhydryl reagents. In eukaryotic systems, such p-CMB or p-HMB (Weissback et al., 1971; Haines et al., 1971; Long and Garren, 1972) and NEM sensitivities (Chiu and Sung, 1972)

have also been noted for DNA polymerases. In the system reported here (both toluene and cryostat sections), NEM or p-CMB inhibited activity whereas inclusion of nucleosides as ATP in the incubation mixture were without effect, similar to the ATP effect reported for Chlamydomonas (Howell and Walker, 1972). The lack of DNAse primed incorporation also argues against the major role of repair replication in the root meristem cells in vitro.

A low level of terminal deoxynucleotidyl transfer activity has been reported for tobacco plant tissue (Srivastava, 1972). Such activity is (a) abolished when other deoxynucleotides are added to the incubation mixture, (b) is not inhibited by the sulfhydryl reagent p-CMB and (c) is markedly inhibited when native chromatin is used as a primer. Thus, it is doubtful if terminal transferase activity is more than minimal in the system reported here.

The presence of a low level of DNA polymerase activity in the ungerminated seed is of interest. Much evidence has been presented (Bonhoeffer and Messer, 1969; Gross, 1972) that protein synthesis is required for the initiation of chromosome replication. In E coli Lark and Renger (1969) observed the existence of at least two regulatory proteins with different sensitivities to chloramphenicol. Both kinds of proteins are necessary for initiation and have to be made anew in each cycle. The ability, however, to sustain DNA replication in the absence of protein synthesis varies according to the system studied (Hotta et al., 1968;

Wanks and Moors, 1970; Wanka et al., 1972). In an earlier study of protein metabolism during Allium germination (Mallery, 1971) a significant amount of ^{3}H-leucine incorporation was shown to occur only after 30-36 hours from the start of imbibition. Similar results were obtained by autoradiography in these studies. These observations and the use of cycloheximide and RNAse in enzyme assay media, suggest that newly translated protein is not required to 'initiate or sustain' the DNA polymerase activity in ungerminated onion seeds.

DNA polymerase template activity

DNA polymerase primed activity was demonstrable in frozen sections of ungerminated onion seed embryos. When DNA polymerase was added, activity increased significantly above 'background' levels. Activity was preferentially found in provascular, epicotyl, and epidermal tissues in preference to storage cotyledonary cells (Figure 22-23, Table 6). Even with a saturating level of exogenous enzyme, activity remained low with no dramatic differences between tissue types (Table 6). Most incorporation was associated with nuclei, and no attempt was made to assess cytoplasmic activity. Since an excess of DNA polymerase was used in these experiments, it suggests that more than increased polymerase levels are required to stimulate the nuclear template of the ungerminated seed tissue.

Figure 22: In Vitro Incorporation from ^{3}H-TTP with Exogenous DNA Polymerase in Ungerminated Onion Seed Embryos.

Autoradiographs displaying

A. Preferential Incorporation from ^{3}H-TTP in epidermal (e) cells of the cotyledon(c).

B. Preferential Incorporation from ^{3}H-TTP in provascular (py) cells.

Frozen sections were prepared from dissected embryos. The assay mixture contained 0.05M Tris-Cl buffer pH 8.0, 0.5mM each of d CTP, d GTP, d ATP, 50uCi ml^{-1} ^{3}H-TTP (s.a. 28Ci mM^{-1}), 5mM $Mg(CH_3COO)_2$, 10mM KCl, 0.5mM dTT, 1 mg micrococcal DNA polymerase. Autoradiographs were exposed for 7 days. x700.

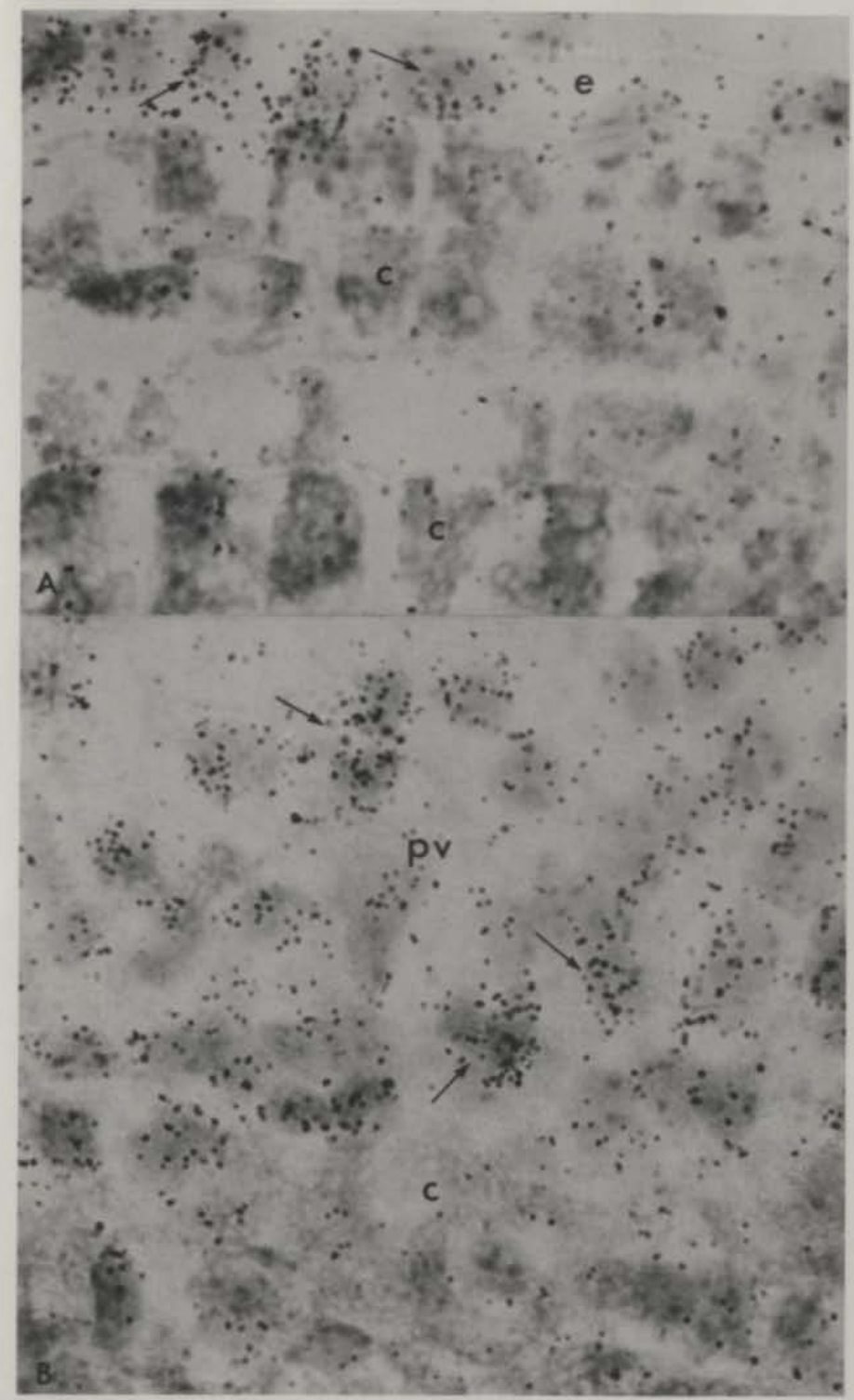

e
c
c
A
pv
c
B

Figure 23. *In Vitro* Incorporation from ^{3}H-TTP with Exogenous DNA Polymerase in Ungerminated Onion Seed Embryos.

Autoradiograph displaying preferential incorporation from ^{3}H-TTP into epicotyl cells. Autoradiograph preparation was the same as for Figure 22. x700.

Table 6. Effect of Exogenous DNA Polymerase on Incorporation from ^{3}H-TTP in Ungerminated Onion Seed Embryos.

	plus DNA polymerase (grains nucleus^{-1})	minus DNA polymerase (grains nucleus^{-1})
Provascular + epicotyl cells	5.06±1.89	0.44±0.17
epidermal cells	4.0 ±1.19	0.47±0.23

Frozen sections were prepared from dissected embryos. The assay mixture contained 0.05M Tris-Cl buffer pH 8.0, 0.5mM each of d CTP, d GTP, d ATP, 50μ Ci ^{3}H-TTP (s.a. 28Ci mM^{-1}) 5mM $Mg(CH_3COO)_2$, 10mM KCl, 0.4mM dTT, 1 mg micrococcal DNA polymerase. Autoradiographs were exposed for 7 days. For grain counts of provascular and epicotyl cells 19 sections were analyzed from both control and experimental slides. For counts of epidermal cells 8 sections were analyzed. Here pieces of tissue having intact root caps were used and counts were made on the epidermal cell layer on both sides of the cap.

Exogenous DNA polymerase primed activity in ungerminated seed tissues implies the retention of accessible 3'-termini (initiator-DNA), since all known DNA polymerases use free-3OH ends of denatured DNA as initiator sites (Schekman et al.,1974). The purified micrococcal DNA polymerase used in the present study requires a primer and template and is incapable of initiating new polynucleotide strands (Harwood and Wells, 1970). It cannot add random nucleotides into a DNA template by an end addition mechanism like terminal transferases. The length of denatured DNA required for the micrococcal enzyme is not known, but at least 20 nucleotides are required for calf thymus DNA polymerase (Bollum, 1959) in contrast to terminal deoxynucleotidyl transferase which has much shorter chain length requirements (Bollum et al.,1964; Kato et al.,1967).

It would be interesting to establish the physicochemical state of the chromatin in nuclei displaying preferential primed activity. In(mouse) degenerating lens nuclei, the DNA after denaturation acts as a better template for exogenous calf thymus DNA polymerase (Modak and Bollum, 1970; Modak et al.,1968; Modak et al.,1969) and this has been interpreted as an indicator of DNA strand scission (Modak et al.,1968; Modak et al.,1969) resulting in liberation of 3-OH ends (Modak and Bollum, 1970). Cytological studies with exogenous DNA polymerase on brain tissues(mice) of various ages (Modak and Price, 1971) as well as similar studies with other mammalian cells have led to the suggestion (Modak and Price, 1971) that during aging DNA undergoes strand breaks which are accumulated and exogenous polymerases can detect such template breaks.

It would be of interest to test seeds of varying ages or seeds stored under different environmental regimes for (strand break) genome damage. It is not known if seeds contain a DNA repair replication mechanism; repair replication occurs in non-replicative tissues (Devik and Halverson, 1963); but no studies have been done on tissues which simulate the metabolic state of seeds. The concept of somatic mutations as major factors in ageing tissues is not new (Curtis, 1966). Visible damage to chromosomes occur in post mitotic animal tissues and has been found to be an index of seed age (Abdalla and Roberts, 1968). Studies of accelerated ageing treatment in seeds (Berjak and Villiers, 1972a; Berjak and Villiers, 1972b) suggest that loss of seed viability may be due to general damage to macromolecules including nucleic acids. There is also biochemical evidence for RNA damage in aged seeds (Roberts et al., 1973).

Since protein synthesis is not initiated in germinating onion seeds before several hours hydration, it would be interesting to see if primer termini (measured with exogenous DNA polymerase) only increase concurrently with such synthesis. An increase of termini in the absence of protein synthesis would imply that activation of preexisting enzymes or other non enzymatic control mechanisms associated with hydration, play a role in DNA polymerase template activation.

Ultrastructural observations on chromatin.

Since studies with exogenous DNA polymerases indicated possible tissue differences in the state of the chromatin, a preliminary fine structural study of nuclear chromatin in various regions of the dry embryo was made. All regions were generally characterized by large dense chromatin masses of various sizes and shapes interspersed with electron transparent regions of dispersed fibrillar material (Figure 24). In some sections heterochromatic masses were reticulate and tortuous while in others they were much more discontinuous. The electron dense material was sometimes extensively vacuolated in storage tissue cells. In presumptive meristematic zones e.g., the epicotyl, the degree of condensation of the chromatin often appeared to be less than in cotyledonary cells, but no conclusions could be reached without a detailed analysis of serial sections. An interesting feature of epicotyl chromatin is the presence of sharply delimited light portions frequently found dispersed within the electron dense chromatin (Figure 25). These light portions appear as distinct chromatin entities separable from the relatively electron transparent lacunar spaces which though present in the dry embryo, are much more common in chromatin of actively growing tissues. It is quite probable, however, that the small amounts of fibrillar material sometimes observed in lacunae represent an altered state of the same light chromatin masses. No tissues of the onion embryo contained nuclear chromatin

Figure 24. Electron Micrograph Showing Condensation State of the Chromatin in Onion Seed Embryo Tissue.

Electron Micrograph demonstrating general condensation state of the chromatin (ch) in onion seed embryo tissue. This section was taken from a cotyledonary cell. Similar and greater degrees of condensation were common for nuclear chromatin in both cells of the vascular cylinder and cotyledon throughout the embryo axis. x26,000.

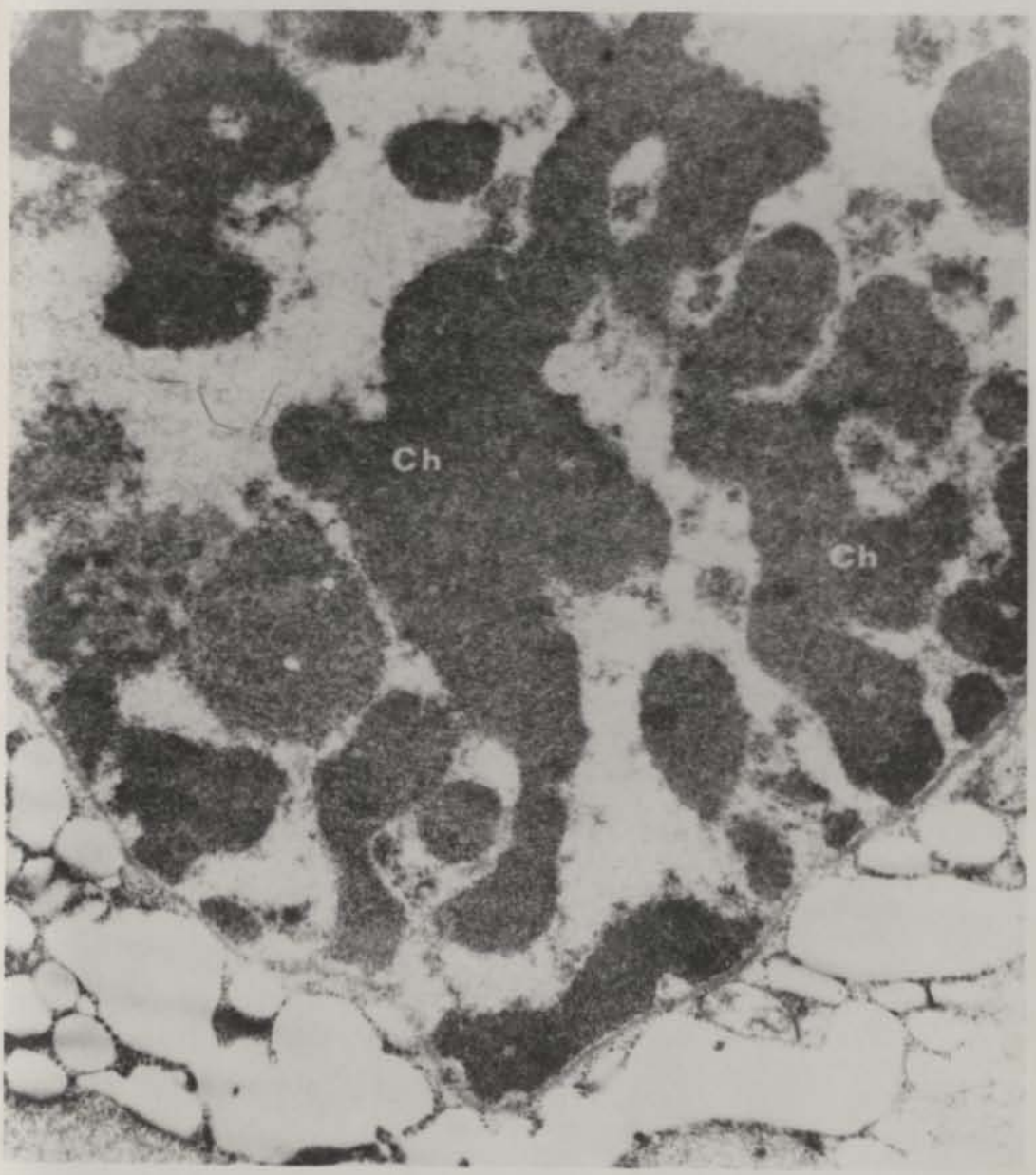
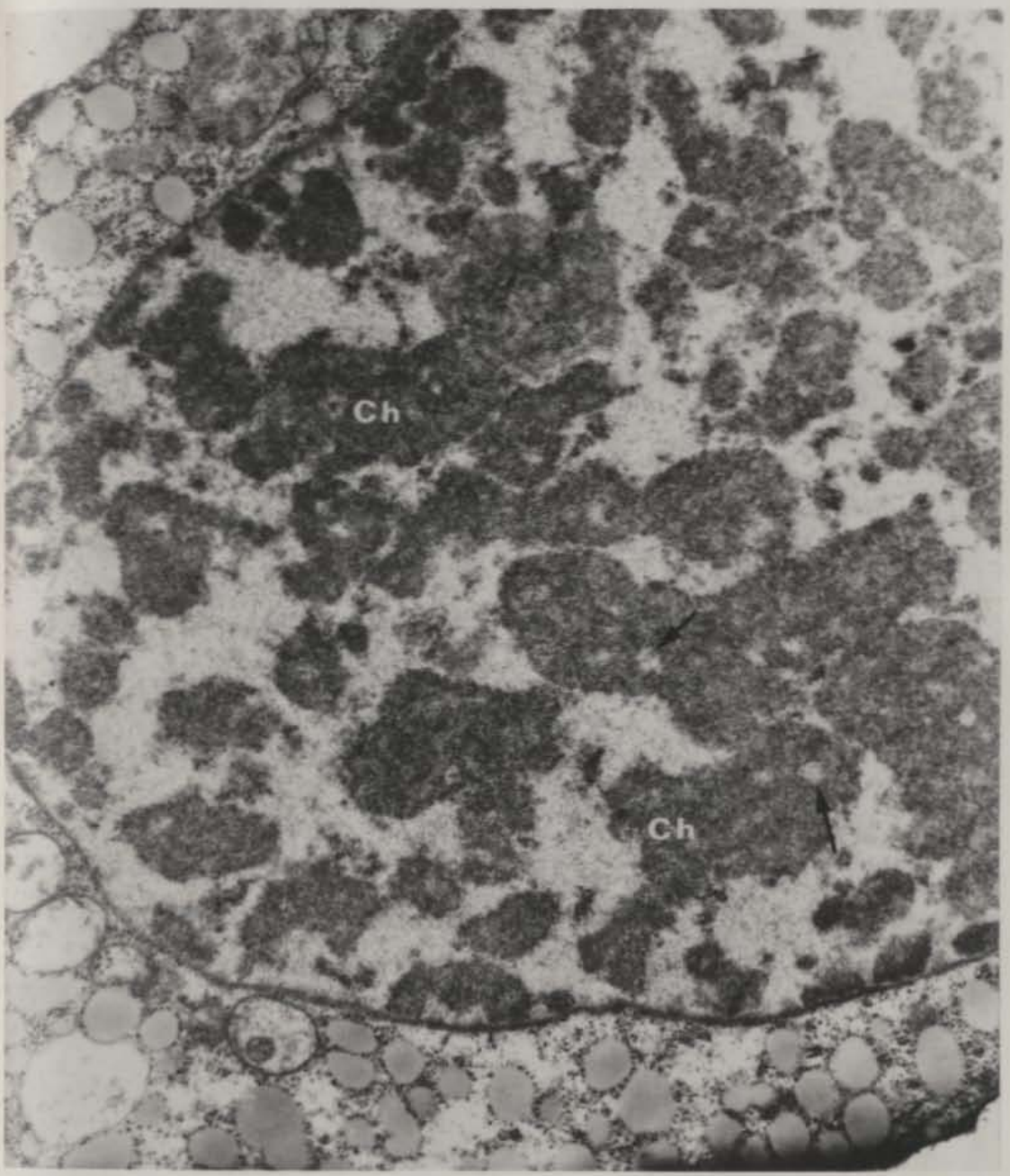

Ch
Ch

Figure 25. Electron Micrograph Showing Condensation State of the Chromatin in Onion Seed Embryo Epicotyl Nuclei.

Electron Micrograph of an epicotyl nucleus demonstrating sharply delimited electron transparent portions (arrows) dispersed within the electron dense chromatin (ch). x26,000.


Ch
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comparable to the decondensed states common in growing root tips.

From preliminary studies of nuclear chromatin, no (readily) apparent relation between ultrastructure and rate of incorporation by exogenous DNA polymerase in various cell types could be observed. Electron microscope autoradiography, however, with exogenous polymerase or transferase may be useful in defining template sites. Ultrastructural cytochemical probes of DNA template states, especially in relation to RNA synthesis, have been developed (Frenster et al., 1974) and single stranded DNA have recently been identified immunologically at the ultrastructural level (Suzuki et al.,1974).

There is some information on the fine structural sites of nuclear DNA synthesis in eukaryotes. Evidence has been presented for the synthesis of DNA on dispersed (Hay and Revel, 1963; Milner and Hayoe, 1968; Blondel, 1968; Littau et al.,1964) and dense chromatin (Comings, 1967; Klinger et al.,1967) although it is now generally believed that early replicating DNA is synthesized in a dispersed form (Kuroiwa, 1973; Kuroiwa, 1974; Lafontaine and Lord, 1974). Even heterchromatin containing late replicating DNA is synthesized in a dispersed form with rapid organization into dense chromatin (Lafontaine and Lord, 1974). In onion seed tissues, no nuclei having chromatin states comparable to meristematic cells were observed. Dry seed nuclear chromatin is similar to that present in G_1 interphase cells in growing Allium porrum L., (Lafontaine and Lord, 1974) but in the seed, chromatin condensation is undoubtedly more pronounced. Studies on chromatin ultrastructure in different seed tissues at varying periods of germination could be

fruitful in elucidating template modification, especially if coupled with cytochemical probe techniques.

Amino acid incorporation

When root tip sections from germinated seeds were assayed in the complete amino acid mixture, incorporation was distinct but unaffected by cycloheximide. In dry seed sections assayed in the complete mixture, cycloheximide was replaced by a mixture of unlabelled amino acids ('cold chase'), and this was effective in distinctly reducing activity (Table 7). Instead of an increase, there was a small but significant reduction in activity when dry seed sections were assayed with ^{3}H-phenylalanine in the presence of the artificial 'messenger' poly-U (Table 8) ^{3}H-leucine incorporation was not affected by a combination of protein synthesis inhibitors (Table 9). In all amino acid incorporation experiments, activity was distributed over both cytoplasm and nuclei with no preferential labelling (Figure 26).

Since there was no apparent reduction in activity with inhibitors, it is unlikely that the labelling found here represents amino acid incorporation into protein. Reduction of activity with 'cold' amino acids could be due to decreased enzymatic amino acid charging to endogenous t-RNA but this cannot be presently established. Similarly, it is not known if the polyanion, polyuridylic acid, acts

Table 7. Effect of Unlabelled Amino Acids on *In Vitro* Incorporation from an ^{3}H-amino acid mixture in Ungerminated Onion Seed Embryos.

	minus 'cold' amino acids (grains/50u^2)	plus 'cold' amino acids (grains/50u^2)
cotyledonary tissue	48.79±14.73*	4.72±2.15

Frozen sections were prepared from dissected embryos. The assay mixture contained 0.05M Tris-Cl buffer pH 8.0, 25uCi ml^{-1} of an ^{3}H-amino acid mixture containing alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryosine, valine, 6mM $Mg(CH_3COO)_2$, 25mM KCl, 2mM ATP, 1mM GTP. Control reactions contained a 0.1% solution of unlabelled amino acids. Autoradiographs were exposed for 14 days. For grain counts 16 sections were analyzed. Counts were made in an area 1000-1500u from the root apex on both sides of the vascular cylinder.

* For calculation of mean and standard deviation 40% of the areas observed had grain counts greater than 60 per 50u^2 but were included as containing 60 per 50u^2 in these calculations. Counting difficulty was experienced with grain counts greater than 60 per 50u^2.

Table 8. Effect of Polyuridylic Acid on *In Vitro* Incorporation from ^{3}H-Phenylalanine in Ungerminated Onion Seed Embryos.

	minus Poly-U (grains/50u^2)	plus Poly-U (grains/50u^2)
cotyledonary tissue	30.43±7.12	12.36±3.08

Frozen sections were prepared from dissected embryos. The assay mixture contained 0.05 M Tris-Cl buffer pH 8.0, ^{3}H-phenylalanine 25uCi ml^{-1} (s.a. 13.4Ci mM^{-1}), 6mM $Mg(CH_3COO)_2$, 25mM KCl, 2mM ATP, 1mM GTP, 100ug poly-U ml^{-1} was added in some experiments. Autoradiographs were exposed for 14 days. For grain counts, 16 sections were analyzed. Counts were made in an area 1000-1500u from the root apex on both sides of the vascular cylinder.

Table 9. Effect of a Combination of Protein Synthesis Inhibitors on *In Vitro* Incorporation from ^{3}H-leucine in Germinated Onion Seed Root Tips.

	minus inhibitors (grains/50u^2)	plus inhibitors (grains/50u^2)
Cortical tissue	25.71±6.01	23.47±4.37

Frozen sections were prepared from germinated onion seed root tips (2-4 mm long). The assay mixture contained 0.05M Hepes buffer pH 7.2, 50uCi ^{3}H-leucine (s.a. 42.7Ci mM^{-1}), 4mM $Mg(CH_3COO)_2$, 0.4mM $(NH_4)_2SO_4$, 0.6mM $MnCl_2$, 2.0mM dTT. Control reactions contained a combination of 100ug ml^{-1} each of chloramphenicol, cycloheximide, and puromycin. Autoradiographs were exposed for 14 days. For grain counts 16 sections were analyzed. Counts were made over cortical tissue.

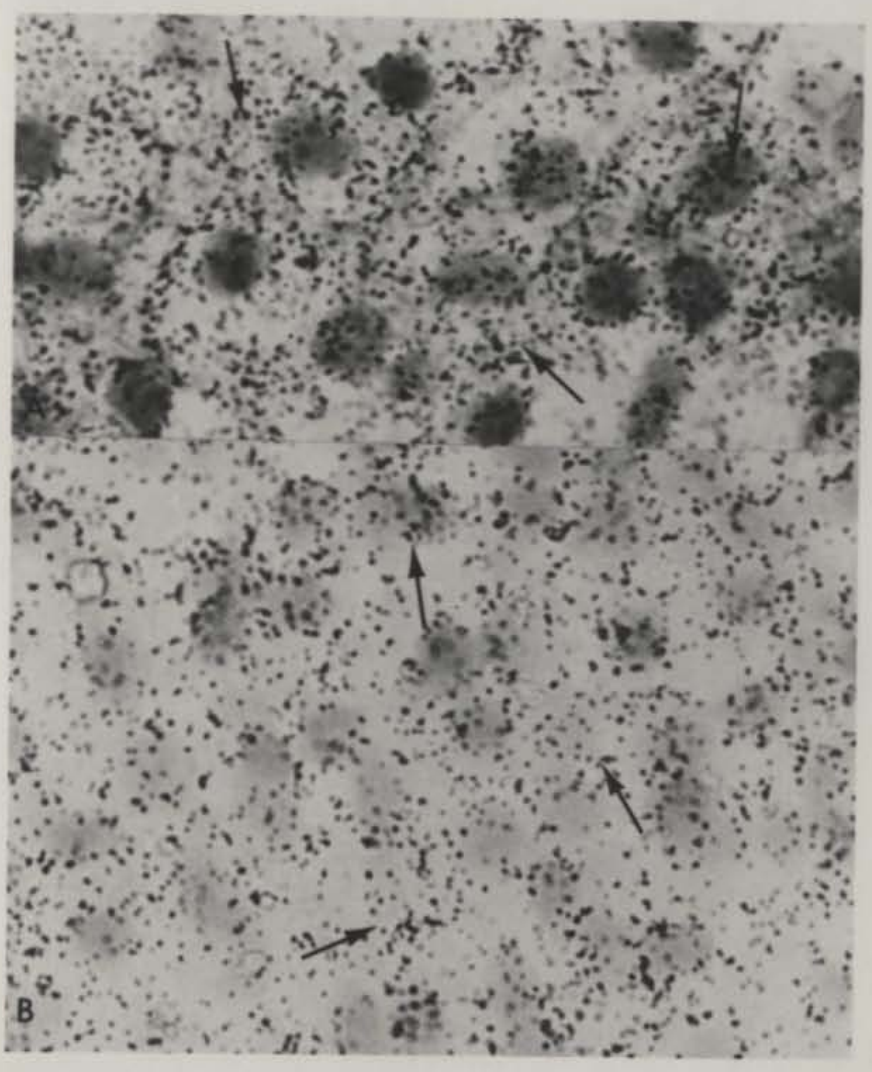
Figure 26. In Vitro Incorporation from ^{3}H-leucine in Onion Seed Root Tip Meristematic Nuclei

Autoradiographs displaying

A. Incorporation from ^{3}H-leucine into cortical tissue

B. Incorporation from ^{3}H-leucine into epicotyl tissue

Note that grains were found over nuclei and cytoplasm (arrows). Frozen sections were prepared from germinated onion seed root tips (2-4 mm long). The assay mixture contained 0.05M Hepes buffer pH 7.2, 50uCi ml^{-1} ^{3}H-leucine (s.a. 42.7 Ci mM^{-1}). 4mM $Mg(CH_3COO)_2$, 0.4mM $(NH_4)_2SO_4$ 0.6mM $MnCl_2$, 2.0mM dTT. Autoradiographs were exposed for 14 days. A similar grain distribution over nuclear and cytoplasmic regions was found in all in vitro amino acid incorporation experiments. x700.


B

by inhibiting an enzymatic reaction as amino acyl synthetase. If amino acid acylation reactions are responsible for the activity on cytologic sections, nuclear and cytoplasmic incorporation patterns, as well as amino acid localization in both presumptive meristematic and storage tissues of the ungerminated seed, are of interest.

One explanation for the lack of protein synthesis in cytologic sections, could be that essential components of the protein synthesizing apparatus are diluted and disrupted into the incubation media. Factors such as those involved in binding of aminoacyl-t RNA to the ribosomes are found in soluble fractions of cell homogenates (Lucas-Lenard and Haenni, 1968) while others as peptidyl transferase may remain associated with macromolecules as ribosomal subunits (Vasquez et al., 1969). Dilution and disruption of essential components would not be surprising in view of the large number of highly coordinated events involved in protein synthesis including aminoacylation of t RNA, the initiation of polypeptide chains, their elongation and termination. Several biochemical studies have reported amino acid incorporation in vitro within the confines of the nuclear membrane and this supports the argument that on tissue sections, nuclear rupture with the loss of essential components, accounts for failure to demonstrate protein synthesis. Besides 'physical' disruption of essential components, degradative enzymes as ribonucleases and proteases could play a role (Payne and Loening, 1970; Payne and Boulter, 1974).

If protein synthesis were detectable in autoradiographs, it would likely consist of ribosome bound peptide fragments. By virtue of the

experimental method, peptide fragments released from ribosomes would be lost into the incubation media and (or) removed by washing. (It is realized, however, that some 'non specific' adsorption is possible). Although in the present studies there was no evidence for protein synthesis in acid insoluble 'particulate' components retained in tissue sections, it is possible that amino acid incorporation into 'soluble' proteins can be demonstrated biochemically.

Although the efficiency of amino acid incorporation in different cell fractions depends on a number of factors as type of buffer, pH, concentrations of various nucleotides and ions, etc., the design used here (in some experiments) contained the components of a typical "cell free" ribosomal system consisting of K^+, Mg^{++}, ATP, GTP, labelled and unlabelled amino acids, and the various endogenous components such as t-RNAs and ribosomes. In addition 'messenger' RNA was presumed to be present either endogenously or supplied as a synthetic i.e., poly U.

Three different inhibitors were used in combination because of reported inconsistencies of their effectiveness in different tissues and subcellular fractions. Cycloheximide is generally recognized as an inhibitor of the translocation of t RNA on 80 S ribosomes (Kaji, 1973) but its effectiveness as a protein synthesis inhibitor has been reported to be variable in plants (Boulter, 1970). Chloramphenicol is regarded as an inhibitor of peptidyl transferase on bacterial ribosomes and is known to be an effective inhibitor of chloroplastic and mitochondrial protein synthesis (Boulter, 1970). Puromycin, which acts on normal

peptide chain elongation by participating in peptidyl transferase reactions, inhibits amino acid incorporation by cytoplasmic, chloroplastic, mitochondrial and nuclear moieties. Rat liver amino acid incorporation is reported to be insensitive to puromycin and cycloheximide, but completely inhibited by chloramphenicol (Ono and Tarayama, 1968), while Dipteran nuclei are repotedly sensitive to chloramphenicol and puromycin but not cycloheximide (Helmsing, 1970). In preliminary studies, cycloheximide sensitive in vivo ^{3}H-arginine incorporation in onion root meristems, was well established, but because of reported differences for protein synthesis inhibitor effectiveness in vitro, they were used in combination in some experiments reported here.

It is not known if the inhibition of ^{3}H-phenylalanine incorporation by poly-U is enzymatic; most evidence points to a stimulatory role for the polymer and this has been demonstrated in plant tissues (Ellis and MacDonald, 1968; Payne et al., 1971). Inhibition of cell free phage induced protein synthesis by homopolynucleotides has been reported for a bacterial system (Moller and Ehrenstein, 1963). Polyribonucleotides are also known to react with DNA (Quetier and Vedel, 1973) and poly-U binding to chromosomes has been cytologically demonstrated (Jones et al., 1973). Further studies are required before the nature of amino acid incorporation inhibition by poly-U on tissue sections can be assessed.

The reduction in amino acid incorporation in tissues incubated

with excess unlabelled amino acids is of special interest. It remains to be determined if the labelling observed in cytological sections is enzymatic binding of amino acids to transfer RNA species by aminoacyl t RNA synthetases. Variations in iso-accepting t RNA populations on their cognate synthetases occur in cells during development and such changes may reflect the involvement of t RNA in regulatory processes (Sueoka and Kano-Sueoka, 1970; Littauer and Inouye, 1973). Although t RNA metabolism in relation to growth and development has received little attention in plants, some interest has been recently generated in this area of cell regulation (Vanderhoef et al.,1970; Norris et al.,1973; Melera et al., 1974; Bick and Strehler, 1972).

Observations on cytoplasmic contents of ungerminated embryos

Although attempts to cytologically demonstrate in vitro protein synthesis were unsuccessful, since a reduction of amino acid incorporation occurred in dry seed tissues in the presence of unlabelled amino acids and also with poly-U, ultrastructural studies were carried out on cytoplasmic contents to check for structures that might be involved in the enzymatic retention of amino acids. If the incorporation on frozen sections represents amino acid charging of transfer RNA, such activity should be associated with cells or cell regions containing 'groundplasm' and structures as ribosomes, mitochondria, or proplastids,

where such RNA species would normally be found. Functional amino acyl synthetase or transfer RNA would not be expected to be 'embedded' in storage bodies often found filling storage cells of dry seeds. At least it would be difficult to reconcile a physiological function. Any speculation for the presence of such protein synthesis components in densely packed storage cells is only tenable if RNA containing structures are associated with such storage bodies.

Exploratory histologic studies on frozen or paraffin sections demonstrated lipid, phospholipid, and protein staining particulate masses throughout the embryonic axis. Carbohydrate containing substances were only reliably seen to be associated with cell walls. Besides nuclear DNA and nucleolar RNA, no other nucleic acid staining was seen. The cytoplasm often displayed a pink tint with the RNA stain, but color was not decreased after RNA hydrolysis. On thick Epon sections cut from various embryo regions, only a few cell types were readily distinguished on the basis of cytoplasmic contents. These included (a) cells with few storage bodies found in epicotyl and provascular tissue (b) cells with an abundance of particles staining intensely with basic dyes and displaying a red metachromasia or (c) cells with an abundance of small bodies staining less intensely and not displaying metachromasia. No serial sectioning was done, however, and the differing cytoplasmic contents may merely reflect different regions of the same cell.

Organelles identified by electron microscopy as protein bodies (Swift and Buttrose, 1972; Jones, 1974; Lott et al., 1971; Jacobsen et al., 1971; Briarty et al., 1969) were particularly abundant in all regions (Figure 27). (Terms as 'aleurone grain', 'protein bodies' or 'protein granules' are often used synonymously (Briarty et al., 1969)). The protein bodies conform to the more intensely staining structures on thick sections. Membrane bound protein bodies were either electron dense or relatively transparent; transparent types contained a more flocculent material. Characteristic of the electron dense bodies are one or more cavities free of any electron dense substance. These cavities conform to globoid containing phytin storage regions (Swift and Buttrose, 1972; Lott et al., 1971; Jacobsen et al., 1971). Protein bodies are formed as a result of protein accumulation in vacuoles or filling of the endoplasmic reticulum (Bain and Mercer, 1966; Englemann, 1968) after an increase in the ribosome content of the cytoplasm. In appropriate sections of the onion seed, vacuolar regions containing small electron dense peripheral deposits were often observed (Figure 27-28) and they are interpreted as initial maturational steps in protein body formation (Simola, 1971; Briarty et al., 1969; Bain and Mercer, 1966; Opik, 1968). Free or membrane bound ribosomes were not associated with 'matured' protein bodies.

Also profusely distributed in the onion seed were electron transparent organelles known as lipid bodies (Figure 29) (Swift and Buttrose, 1972; Lott et al., 1971) or spherosomes (Briarty et al., 1969;

Figure 27. Electron Micrograph Showing Storage Body Contents in Onion Seed Embryo Cotyledon Tissues.

Electron micrograph demonstrating storage body contents in cotyledonary cells. Note (a) the electron dense protein bodies (pb), often containing electron transparent cavities (b) the relatively transparent gobular (lb) and sheet (lbs) like lipid bodies and (c) vacuolar regions containing electron dense substances conforming to maturing protein bodies (mpb). Similar cytoplasmic contents were found in both vascular cylinder and cotyledon tissues throughout the embryo axis, but type of particle and density of packing often varied. x6000.

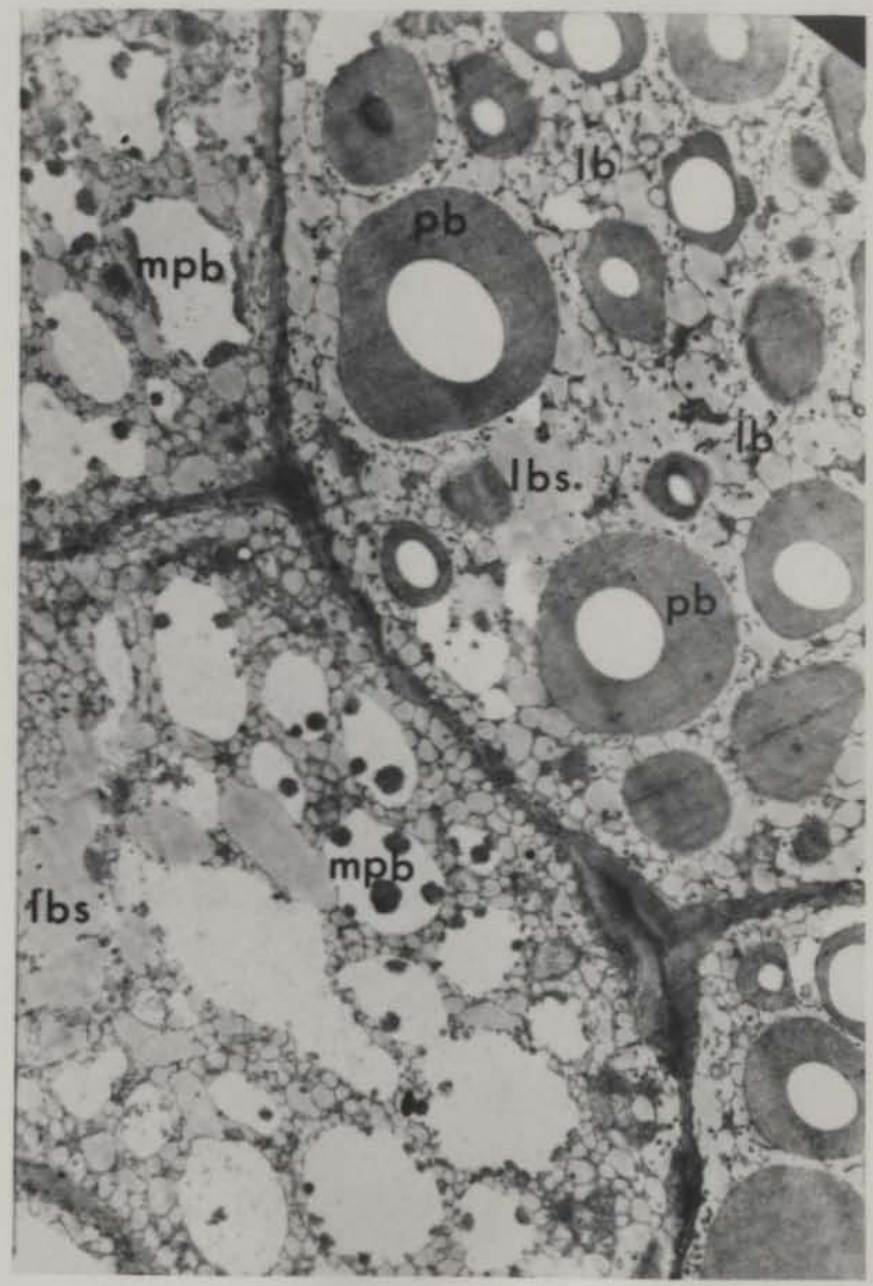

lb
pb
mpb
lb
lbs.
pb
mpb
lbs

Figure 28. Electron Micrograph Showing Storage Body Contents in Onion Seed Embryo Cotyledon Tissue.

Some vacuolar regions have a flocculent material while others contain electron dense particles (or patches) of various sizes. These are believed to be maturational steps of protein body formation(mpb). (pb, protein body; lbs, lipid body sheets). x6000.

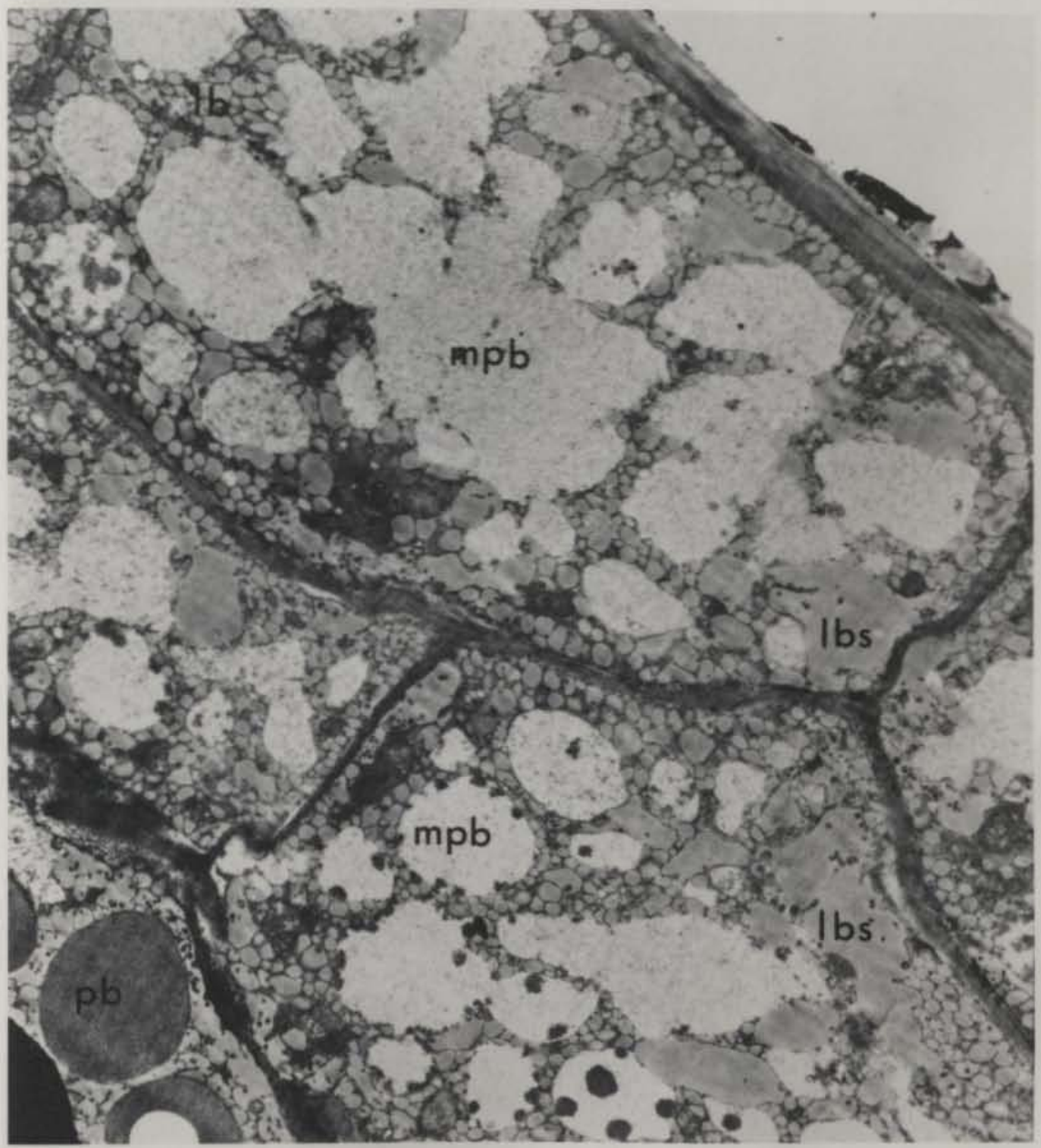
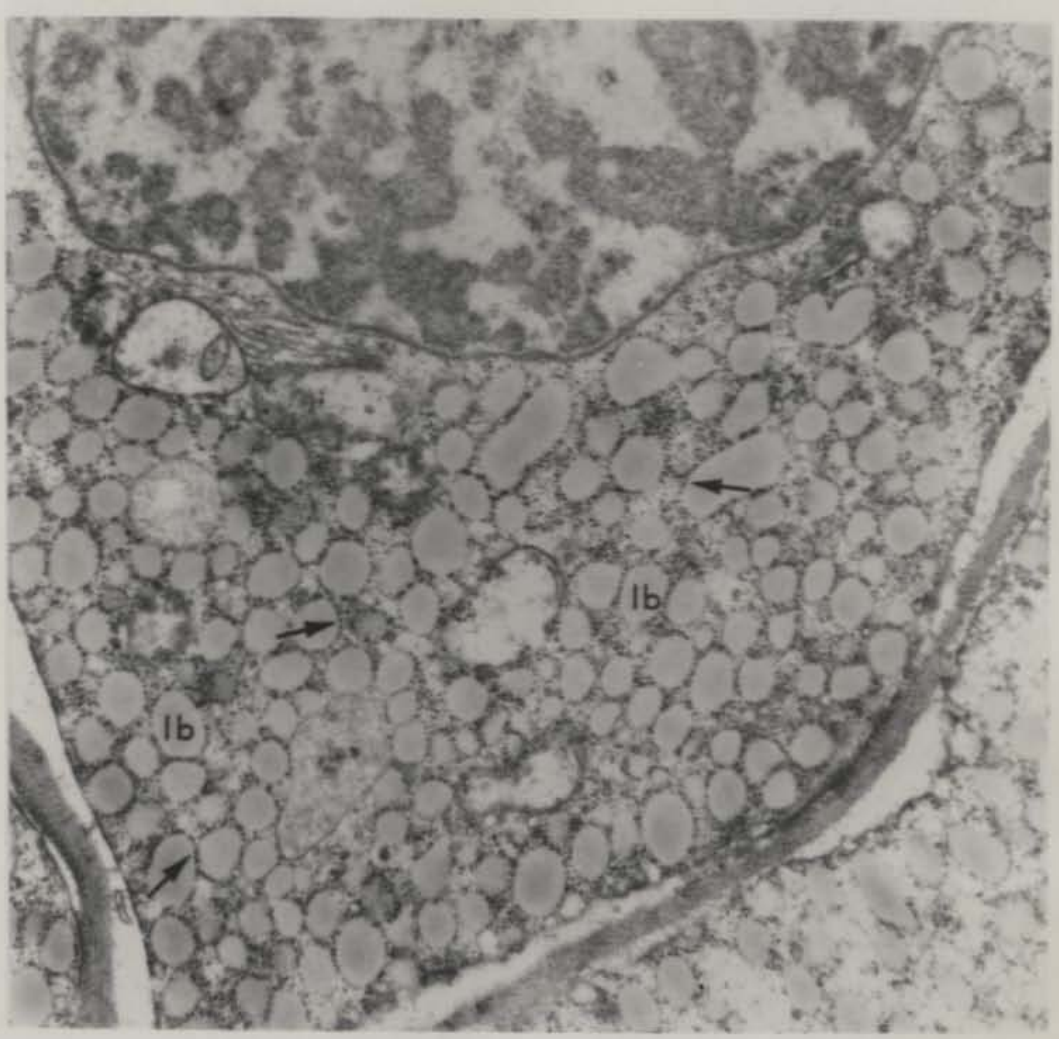

lb
mpb
lbs
mpb
lbs
pb

Figure 29. Electron Micrograph Showing Storage Body Contents in Onion Seed Embryo Epicotyl Tissue.

Note the ribosome studding (arrow) of lipid bodies (1b). Such ribosome studding was common for lipid bodies in all tissues of the embryo axis. The cell section shown here has an abundance of lipid bodies, but this is not meant to typify cells in this region, for other epicotyl cells contained cytoplasmic contents similar to Figures 27 and 28. x27,600.


Ib
Ib

Jones, 1969; Jacobsen, 1971; Jones, 1974). They appeared characteristically globular but were also found in cytoplasmic sheets. The bodies were not restricted to the cell periphery but dispersed throughout, often surrounding protein granules. Limiting membranes were not distinguishable but ribosomal studding was a characteristic feature. There is some evidence that lipids are elaborated by portions of the E.R. (Stein and Stein, 1967; Stein and Stein, 1968) and it has been suggested (Frey-Wyssling and Muhlethaler, 1965) that spherosomes are formed from E.R. in an immature state.

In comparison to the abundance of storage bodies, there was a scarcity of smaller definable organelles as mitochondria or plastids in onion seed embryos. Peroxidase and phosphatase rich bodies were detected by light microscopy, but have not been resolved at the E.M. level. Besides surrounding the lipid bodies, ribosomes were often seen in cell regions where storage particles were not densely packed. Structures conforming to rough E.R. or Golgi were only seen infrequently.

If in vitro amino acid labelling in dry embryo tissues is due to charging of endogenous transfer RNA, activity should be preferentially found in cell regions (or types) where 'groundplasm' is present. It was tenable to speculate that activity would be lower in cotyledon tissues densely packed with storage bodies, but autoradiographs displayed little or no differences in incorporation between different embryo regions. It is not known if the ribosomes

surrounding the spherosomes are indicative of a potential for protein synthesis (or a residual ontogenetic characteristic) and have associated amino-acyl synthetase activity. Scanning E.M. autoradiography could be useful for grain visualization on large cell organelles such as storage bodies which retain an adequate three dimensional structure after freeze drying and coating procedures. Preliminary investigations demonstrated that in thick sections of onion seeds, various storage bodies retained good structural integrity after air or freeze drying procedures, with or without fixation.

Methylases

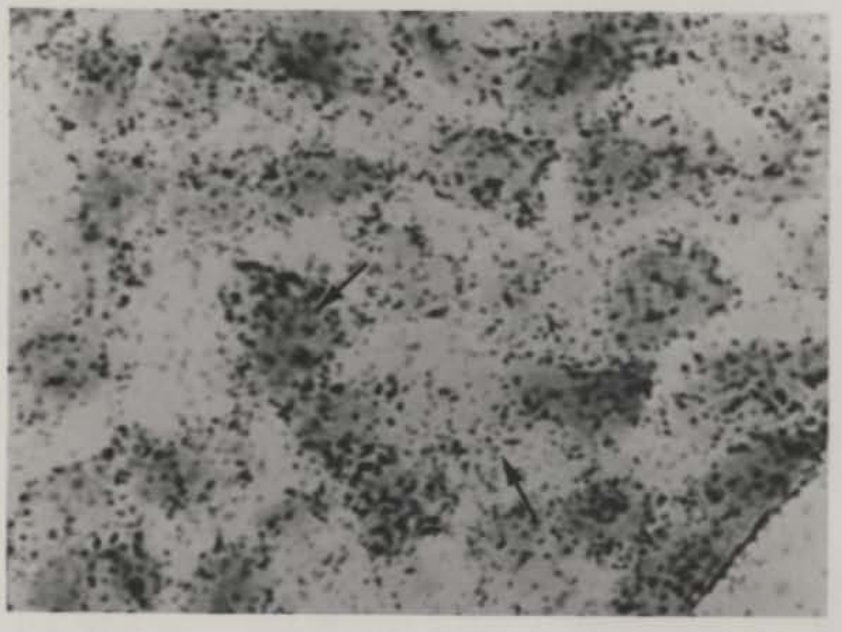
Experiments with methionine as a methyl donor failed to demonstrate reliable levels of any methylase activity. With both methionine and RNA nucleotides in the enzyme assay mixture, incorporation was distributed over nuclei and cytoplasm, (Figure 30) but substantial amounts of activity remained in both meristematic and ungerminated embryo tissues after DNAse or RNAse digestion. The same result was obtained with both thick razor slices or frozen sections. Where nucleotides were included in the incubation mixture, it was hoped to increase RNA synthesis; the synthesis of new RNA polymers may be important if methylation proceeds rapidly. In experiments with ungerminated embryos, the rationale was to incubate under conditions

Figure 30. Incorporation from ^{3}H-Methionine into Onion Seed Root Tip Nuclei.

Autoradiograph displaying

In Vivo incorporation from ^{3}H-methionine into epicotyl cells of a thick slice of tissue taken from a seed germinated for 36 hrs. Note label over nuclei and cytoplasm (arrows).

The assay mixture contained 0.05M Tris-Cl buffer pH 7.4, 10mM $MgCl_2$, 150mM KCl, 1.0mM dTT, 0.5mM each of GTP, ATP, UTP 50uCi ml^{-1} ^{3}H-methyl-methionine (s.a. 190m Ci mM^{-1}). Autoradiographs were exposed for 14 days.



of minimal or no protein synthesis, so that label entering nucleic acids could be more easily resolved from incorporation into peptide polymers (or other compounds). Preferential nuclease sensitive incorporation into nuclear, nucleolar, or cytoplasmic regions was not obtained in any experiments. Activity is possibly due to incorporation (or non specific adsorption) into a number of tissue substances, but incorporation into protein, the most obvious candidate for an amino acid as methionine or charging of transfer RNA residues, was not investigated. It is tenable that methyl groups from methionine are incorporated into protein and (or) nucleic acid polymers (and other compounds) but at such low levels that different activities are not distinguishable.

RNA methylase activity only was detected when SAM was substituted for methionine as methyl donor (Figure 31). In meristematic tissues incubated with SAM, nuclei displayed a reliable level of activity and most incorporation was sensitive to RNAse but not DNAse. Activity was distributed over cell nuclei with no preferential nucleolar incorporation. Attempts to 'activate' DNA methylation with exogenous trypsin were unsuccessful. When trypsin was included in the reaction mixture, tissue digestion was extensive and no meaningful interpretation of activity was possible. Thus, in plant meristematic cells, SAM can be used as methyl donor to demonstrate satisfactory levels of cytological RNA methylase, in contrast to DNA or protein polymer methylation reactions.

Figure 31. *In Vitro* Incorporation from ^{3}H-S-adenosyl-methyl-methionine into Onion Seed or Bulb Root Tip Meristematic Nuclei.

Autoradiographs displaying

A. Incorporation from SAM into cortical cells of an onion seed root tip.

B. Incorporation from SAM into cortical cells of an onion bulb root tip.

C. Incorporation from SAM into cortical cells of a bulb root tip after RNAse digestion.

Frozen sections were prepared from onion seed or bulb root tips (2-4 mm long). The assay mixture contained 0.05M Tris-Cl buffer pH 8.0, 5MM KCl, 0.02mM dTT, 50uCi ml^{-1} ^{3}H-S-adenosyl-methyl-methionine (s.a. 7.3 Ci mM^{-1}). In C. tissues were post treated with RNAse. Autoradiographs were exposed for 21 days. x800.


A
B
C
D

The type of RNA cytologically methylated is of interest. It was once believed that t RNA methylases in nucleated cells were localized in the nucleolus (Birnstiel et al.,1963; Sirlin et al., 1963) but it has since been established that most t RNA methylase activity is found in the cytoplasm (Tsutsui et al.,1966; Simon et al.,1967; Baguley and Staehelin, 1968). Studies have also suggested that methyl groups of r RNA are incorporated into nucleolar 45 s RNA (Greenberg and Penman, 1966; Zimmerman and Holler, 1967); in this study, however, there appeared to be no preferential nucleolar labelling.

Messenger RNA (mRNA) is believed to be derived from a heterogenous class of RNA (hnRNA). This hnRNA, in contrast to nucleolar rRNA, is characterized by heterogeneity in size with no detectable discrete species (Weinberg, 1973) and it is believed to be synthesized solely in the nucleoplasm (Penman, 1966). Although it is metabolically labile, it is known to be synthesized at such a rate, that after a short labelling period, it represents most of the acid precipitable radioactivity incorporated into cellular RNA (Darnell, 1968). It is tenable that a large proportion of RNA retained in the nucleoplasm of tissue sections is similar to this hnRNA, but hnRNA is one of the few species of RNA that is believed not to be methylated*(Weinberg, 1973).

In addition to the ribosomal RNA of the nucleolus and the hnRNA of the nucleoplasm, work in the early 1960's (Muramatsu and Busch, 1965; Muramatsu and Hodnett, 1966), presented evidence for the presence in

* I have been informed (Dr. B. Sells) that methylated m-RNA has been reported. Cell 1: 37-41 (1974).

eukaryotic cell nuclei of a class of RNA (several species) now known as 'small molecular weight stable nuclear RNA (SnRNA).' Although SnRNA's have been found in a number of different cell types, they have not been investigated in plants. Most SnRna species are found outside the nucleolus while others are believed to be nucleolar (Weinberg, 1973) and this RNA is methylated post transcriptionally. It is believed that nucleoplasmic SnRNA is associated with protein after synthesis (Walters et al.,1970) but it is easily dissociated from chromatin by low molar salt and even isotonic buffers (Weinberg and Penman, 1969; Rein, 1971). It is not known if this RNA species is retained on tissue sections under the present experimental method.

Another species of RNA, chromosomal RNA (cRNA) was described in 1965 (Huang and Bonner, 1965) and its properties and function in different organisms have been reviewed (Holmes et al.,1972). Chromosomal RNA is believed to be covalently bound to chromatin histone (Huang and Bonner, 1965; Jacobsen and Bonner, 1971) but there is evidence that c RNA resembles r RNA (Dahmus and McConnell, 1969). If the activity observed in cytologic studies conforms to methylated c RNA, it is interesting that it is not localized preferentially in the nucleolus.

If there were a low level of methyl group transfer to protein polymers on cytologic sections, it is doubtful that it could be easily detected assuming plant protein methylase products compare with those characterized biochemically from animal tissues. Methylase I transfers methyl groups to arginine particularly in added histone (Paik and Kim, 1968;

Paik and Kim, 1969) and is mainly found outside the nucleus; Methylase II transfers methyl groups to amino acid carboxyl groups and is similarly found outside the nucleus; Methylase III is found in cell nuclei (Allfrey et al.,1964; Kim and Paik, 1965; Sekeris et al.,1967) and transfers methyl groups to lysine residues in added histone. It is not known, if incorporation into histone by methylases I and III would be substantially removed by the acetic acid used in fixation (Dick and Johns, 1968). Also since methyl groups transferred to carboxylic acid residues by methylase II, form ester linkages, they may be unstable to acid hydrolysis (Kim and Paik, 1970; Kim and Paik, 1971b) and lost from tissue sections by acetic acid and TCA treatment.

Although RNA methylases have not been well characterized in plants, their existence was established in the early 1960's (Birnstiel et al.,1962; Srinivasan and Borek, 1964). Of specific interest to plant cell physiology is the observation that the methylation of purine residues of both t RNA and r RNA is enhanced by the plant hormone gibberellic acid (Chandra and Duynstee, 1971). Evidence questioning in vivo work on plant RNA methylation, has demonstrated that plants incorporate methyl groups much more rapidly into pectin or its precursors than into RNA, and pectin also contaminates RNA fractions on acrylamide gels. Another potential source of contamination in plants could also be hemicellulosic residues (Bonner and Varner, 1965). The present cytologic work supports the presence of active RNA methylases

in plants. Information from several sources has led to acceptance of SAM as the immediate methyl donor in RNA although evidence has not shown that methionine is the major endogenous source for RNA methylation in plants. Other substrates besides nucleic acid and protein polymers possibly compete for methionine; SAM is the methyl donor for methylation of phosphatidylethanolamine to phosphatidylcholine (Wilson et al., 1960), a ubiquitous component of membranes of higher organisms (Rothfield and Finkelstein, 1968). Spermidine and putrescine polyamines, which are common in higher plants (Smith, 1971) have high synthetic rates in rapidly growing tissue (Tabor and Tabor, 1972) and plant seedling cell free extracts are known to decarboxylate SAM (Baxter and Coscia, 1973) in the synthesis of polyamines. Thus, although protein and DNA polymers, as well as many other compounds, can potentially compete for SAM, an efficient level of RNA methylase can be cytologically demonstrated in plant root meristematic cells.

Since RNA and protein synthesis does not begin in onion seed embryos until after several hours hydration, it would be interesting to determine if RNA methylase exists in ungerminated seeds. Information on the ability of condensed chromatin in different 'resting' cells to effect in situ methylation would be informative. Lack of activity could be due to absence of functioning enzymes or the proper template requirements. This could be established by biochemical assay with 'artificial' under-methylated RNA polymers.

Poly(ADP-Ribose)Polymerase

A high level of incorporation from ^{3}H-NAD was cytologically detected in both meristematic and ungerminated onion embryo tissues (Figure 32). In the growing roots at least 30% of the cells were 'heavily' labelled (Table 10). Labelling was intensely distributed over the nuclei with no preferential nucleolar activity; distinctly labelled cells were from meristematic tissues, the pattern resembling in vitro DNA polymerase activity. It is interesting that in the ungerminated embryo, an intense incorporation was observed in all tissues from root cap epidermis to the cotyledon tip and at least 90% of the cells in the embryonic axis were labelled. Endosperm tissue, however, contained little or no activity.

When reaction products from enzyme digests were chromatographed on thin layer plates, radioactive regions with mobilities similar to ADP-ribose and 5-AMP were localized on chromatograms (Figure 33). Thus, at least part of the activity cytologically demonstrable is tentatively identified as incorporation of NAD into a poly(ADP-ribose)polymer.

A great deal of interest has centered around observations on several mammalian tissues that ADP-ribosylation of nuclear proteins causes an inhibition of endogenous DNA polymerase activity in isolated nuclei (Burzio and Koide, 1970; Hilz and Kittler, 1971; Nagao et al.,1972; Lehmann and Shall, 1972). Poly(ADP-ribose)polymerase activity from a number of sources has also been reported to be low in S phase (Smulson

Figure 32. In Vitro Incorporation from ^{3}H-Nicotinamide Adenine Dinucleotide into Onion Seed Root Tip Meristematic Nuclei and Ungerminated Seed Tissues.

Autoradiographs displaying

A. Incorporation from ^{3}H-NAD into cortical cells of an onion seed root tip. x800.

B. Incorporation from ^{3}H-NAD into cells of the root cap apex of an ungerminated onion embryo. x150.

Frozen sections were prepared from germinated onion seed root tips (2-4 mm long) or ungerminated embryos. The assay mixture contained 0.05M Tris-Cl buffer pH 8.3, 5mM $Mg(CH_3COO)_2$, 3.3mM NaF, 50mM KCl, 3.0mM dTT, 50uCi ml^{-1} ^{3}H-nicotinamide adenine dinucleotide (s.a. 3.46 Ci mM^{-1}). Autoradiographs were exposed for 7 days.

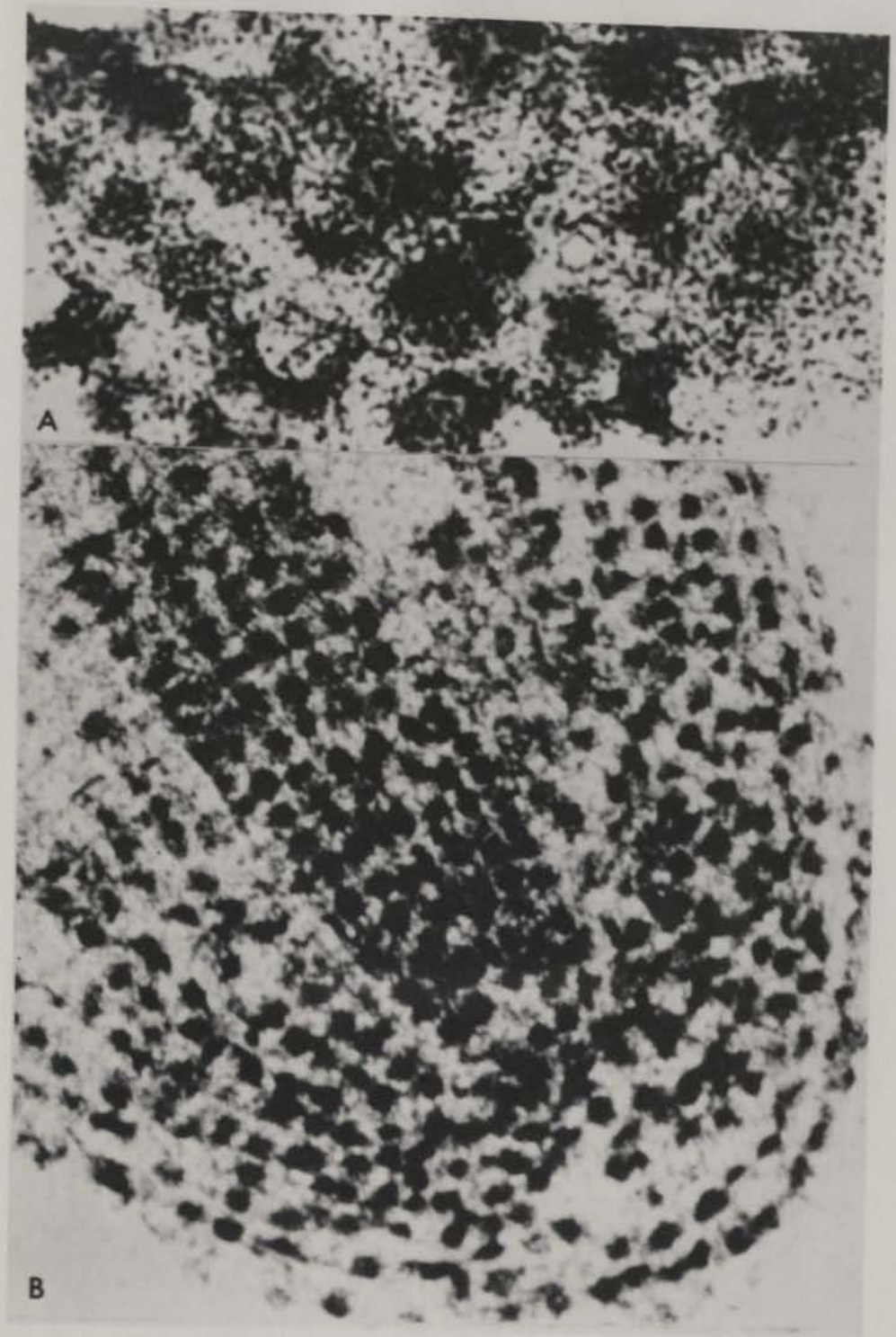

A
B

Table 10. Percentage Onion Seed Root Tip Meristematic Nuclei Displaying Intense *In Vitro* Incorporation from ^{3}H-NAD.

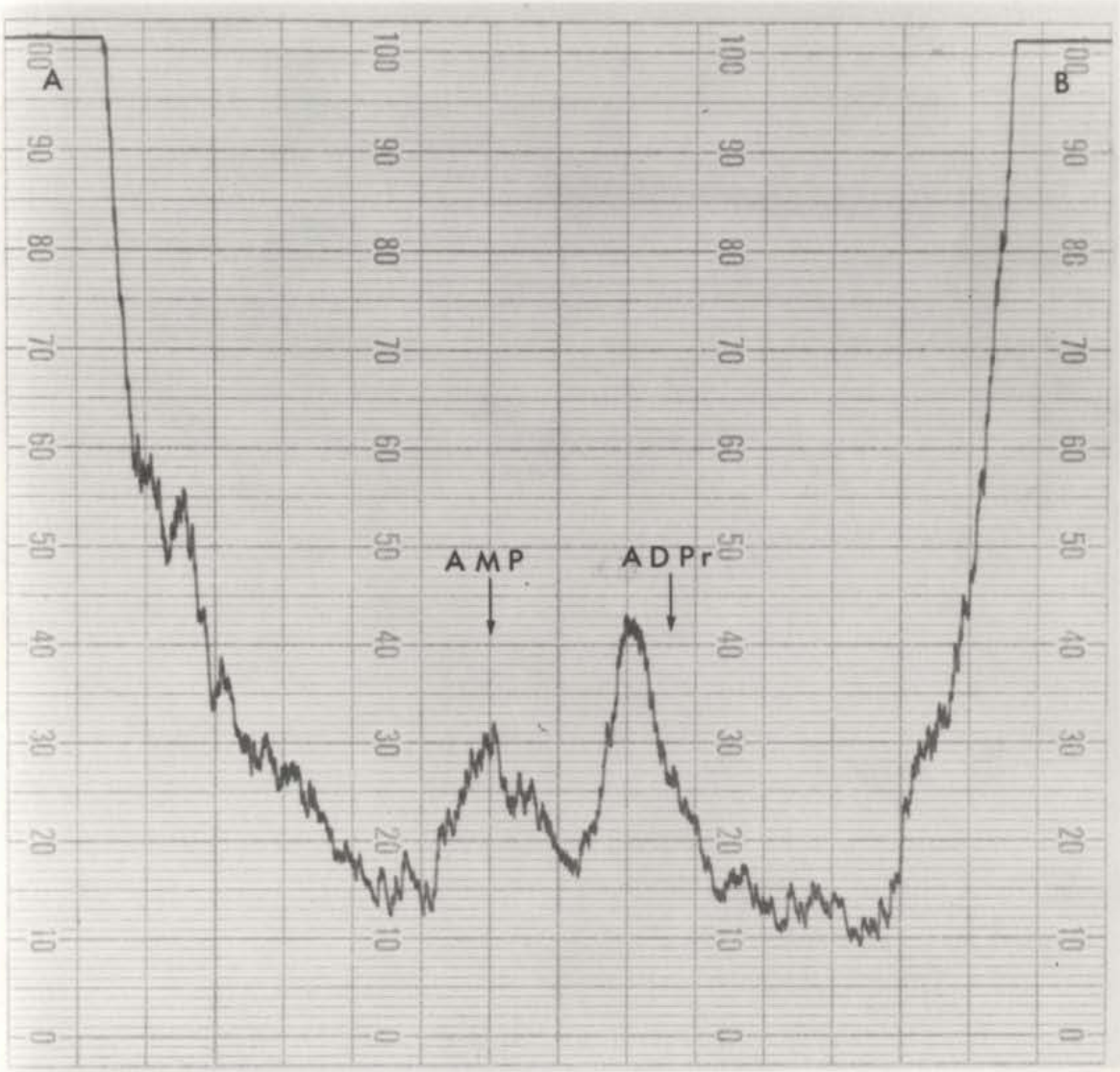
Number of nuclei section^{-1} counted	% nuclei displaying intense* Incorporation from ^{3}H-NAD
115.0±50.52	33.18±7.50

Frozen sections were prepared from germinated onion seed root tips (2-4 mm long). The assay mixture contained 0.05M Tris-Cl buffer pH 8.3, 5mM $MgCl_2$, 3.3mM NaF, 50mM KCl, 3.0mM dTT, 50µCi ^{3}H-NAD (s.a. 3.46 Ci mM^{-1}). Autoradiographs were exposed for 7 days. 16 sections were analyzed for activity.

* Nuclei displaying intense incorporation had grain densities beyond the resolution of individual grains.

Figure 33. Radiochromatogram Detection of AMP-ribose and AMP as possible Products of a Poly(ADP-ribose)Polymer.

The assay mixture contained 0.05M Tris-Cl buffer pH 8.3, 5mM $Mg(CH_3COO)_2$, 3.3mM NaF, 50mM KCl, 3.0mM dTT, 50uCi ml^{-1} ^3H-NAD (s.a. 3.46Ci mM^{-1}). Embryo homogenates were incubated, washed with TCA, hydrolyzed with NaOH, neutralized, incubated with phosphodiesterase followed by pronase incubation. Ethanol dissolved digests were run on polyethyleneimine cellulose plates. Scans were at a linear range of 100 c.p.m., time constant 100 sec., and a chart speed of 0.1 cm hr^{-1}. 5-AMP and ADP r standards were detected under UV light. The two major activity peaks (A) and (B) are C^{14} marker spots used for calculating R_f values.


A
B
AMP
ADPr

et al.,1971; Sugimura et al., 1973; Haines et al., 1969).

A role for the polymer in DNA synthesis has come from observations that it can release DNA polymerase from chromatin and block the activity of certain endonucleases (Burzio and Koide, 1973; Yoshihara and Koide, 1973; Yoshihara et al.,1973; Yoshihara et al.,1975), which possess the ability to activate the template for DNA synthesis (Burzio and Koide, 1973; Hewish and Burgoyne, 1973; Ishida et al.,1974). It has also been reported that the polymer can inhibit liver DNAse (Yamada et al.,1974).

Other evidence has not upheld a negative correlation between DNA replication and poly(ADP-ribose) activity (Lehmann and Shall, 1972; Lehmann et al.,1973; Burzio and Koide, 1971; Burzio and Koide, 1972; Muller et al.,1974). Recent work further suggests a role for the polymer in template activation and DNA synthesis stimulation. It was shown that in He La cells ribosylation of nuclear proteins is related to template activation for DNA synthesis, as demonstrated by bacterial polymerase probes (Roberts et al.,1973). Stimulation of DNA synthesis by ribosylation was detected throughout the cell cycle of He La cells, with maximal enhancement in G_2 . Binding studies with DNA polymerase (Roberts et al.,1974) indicate exposure or generation of additional 3-OH primer sites due to ribosylation reactions.

The reason for these different effects on DNA synthesis are not known. There is some evidence, however, that the presence or absence of poly(ADP-ribose) inhibitable nucleases, which may have primer function, can

explain the contradictory evidence about the role of the polymer in DNA replication (Roberts et al.,1974).

An early report suggested that cells having high levels of RNA synthesis also have high specific poly (ADP-ribose) polymerase activity (Haines et al.,1969). It has been similarly demonstrated with actinomycin D that continuing RNA synthesis is necessary for maintaining the activity of the polymerase (Smulson and Rideau, 1972). Other workers have, however, detected no such relationship, and some (Lehmann et al.,1974) have cautioned against the concentrations of actinomycin inhibitor used in comparing RNA synthesis and poly(ADP-ribose) polymerase kinetics. It has also been demonstrated that purified preparations of poly(ADP-ribose)polymerase are inhibited by actinomycin D (Yoshihara, 1972). Besides earlier in vitro work (Burzio and Koide, 1971) showing that incubation of chromatin with NAD did not affect its capacity for RNA synthesis, it is noted that transcription, as probed by bacterial RNA polymerases (in contrast to DNA polymerases) is not affected by poly(ADP-ribose) formation during the cell cycle (Roberts et al.,1973).

It has been suggested from in vitro studies that poly(ADP-ribose) is covalently bound to nuclear proteins, principally histones (Nishizuka et al.,1968; Nishizuka et al.,1969; Otake et al.,1969) and ADP-ribose is attached to histones in vivo both as a monomer and a polymer (Ueda et al.,1975). Since the polymer seems primarily bound to histone, which is non covalently linked to DNA and RNA, it is encouraging that a high level of activity can be retained cytologically

after fixation in acetic-acid: alcohol and after repeated washes in TCA and distilled water.

Optimum conditions for enzyme activity were not determined. Glycohydrolases which degrade the polymer are known to be present in a number of mammalian tissues (Miwa and Sugimura, 1971; Ueda et al.,1972; Miyakawa et al.,1972; Stone and Shall, 1973) and polymer degradation has been noted in several cell systems after varying periods of incubation. Enzyme activity would also be influenced by enzymes effecting NAD breakdown as NADase, NAD pyrophosphorylase, and phosphodiesterase. It is not known what role polymer or precursor degradative enzymes play in influencing enzyme activity in either the dry seed or the actively growing meristem.

In the germinating seed meristem approximately 30% (Table 10) of the cells demonstrated an intense activity similar to results for in vitro DNA polymerase on frozen sections. It is thus tenable to speculate a positive correlation with active S-phase nuclei, supporting the most recent hypothesis for poly(ADP-ribose) polymerase function. In the ungerminated seed, however, intense activity was found in tissues other than presumptive meristems. Although little if any thymidine (at high specific activity) was found to be incorporated in vivo in storage cotyledon cells, an intense poly(ADP-ribose) polymerase was found there, as in all tissues of the ungerminated embryo. The pattern of label in the ungerminated seed, excepting the endosperm, is reminescent of RNA

polymerase activity in the early hours of hydration, before nucleolar labelling becomes distinct. Future hypothesis on the role of this enzyme in the eukaryotic cell will have to consider its presence in plants as well as the pattern of enzyme activity in various seed tissues.

It is difficult at this time to assess a biological role for poly(ADP-ribose). Studies reported here add further novel characteristics. Although much evidence has centered around involvement of the polymer in DNA synthesis, its role in RNA transcription and chromosome structure remain to be elucidated. It is possible that it may be involved in cell cycle chromatin modifications similar to phosphorylation acetylation, and methylation reactions. ADP-ribosylation may be a general mechanism for regulation of enzymic activities. In addition to chromatin ribosylation reactions, other compounds such as diphtheria toxin, catalyze the transfer of the ADP-ribose moiety of NAD to peptide elongation and transfer factors (Honjo et al, 1968; Robinson and Maxwell, 1972). After infection of E coli with bacteriophage, the DNA-dependent RNA polymerase is modified by ADP-ribosylation of the sub units (Goff, 1974; Zilling et al.,1974). Many 'non specific' functions cannot be ruled out for the enzyme, supporting the hypothesis that ADP-ribosylation by poly (ADP-ribose) polymerase may play a dynamic role in the cell in regulating enzyme activities.

SUMMARY AND CONCLUSIONS

Autoradiography has been used to cytologically demonstrate nuclear RNA polymerase, DNA polymerase, RNA methylase and poly(ADP-ribose)polymerase in frozen sections of plant tissue. DNA polymerase, poly(ADP-ribose)polymerase and RNA methylase have been cytologically established for the first time (from any tissue source) and poly(ADP-ribose)polymerase had been recorded for the first time from a higher plant. Attempts to demonstrate in vitro protein synthesis on tissue sections were unsuccessful.

Both nucleolar and nucleoplasmic RNA polymerases were demonstrated in endosperm tissues of ungerminated onion seed and in unsprouted bulb roots when incubated in an assay mixture containing ^{3}H-UTP. Throughout the tissues of the embryonic axis, general nucleoplasmic labelling was observed before any label was found to be distinctly associated with nucleoli. It is not known if nucleolar fusion in the endosperm tissue effects a net concentration of RNA polymerase enzyme, thus permitting its earlier detection, or if nucleolar polymerases are activated more readily in the endosperm than in the embryo axis. In the unsprouted onion bulb, both nucleolar and nucleoplasmic polymerases could be readily detected. The method permits the distinction between nucleolar and nucleoplasmic polymerases and it would be interesting to establish if they are the same enzymes as the RNA polymerases I and II,

which have been fractionated from plant chromatin.

The nucleoli in both onion bulb root and seed tissues are predominantly fibrillar, characteristic of tissues with minimal or no ribosomal RNA synthesis. In appropriate sections of the embryo, nucleolar chromatin is continuous with extranucleolar chromosomal material and one or more rows of condensed chromatin masses are found throughout the nucleolar organizing region. The condensed masses are probably representative of a repressed state of chromatin microfibrils, 'normally' active in the transcription of ribosomal RNA synthesis. The presence of extensively vacuolated nucleoli in the seed cotyledon tip supports the hypothesis that vacuoles need not be dynamically associated with ribosomal RNA synthesis.

In any physiological studies (in plant tissue) on the initiation of cell activity after a period of 'rest' it is important to establish any characteristic suggestive of growth or degenerative changes. Analysis of onion seed and bulb roots for G_2 cells revealed the presence of a distinct number of G_2 cells in the bulb roots. Further studies demonstrated that the unsprouted roots are capable of a low level of growth and cell division. Ultrastructural work also demonstrated possible growth and (or) degenerative changes under storage. These included extensive cell wall degradation, vesicle formation from stacks of endoplasmic reticulum, and presence of various types of membrane modifications, some resembling autophagic vacuoles.

A significant level of nuclear DNA polymerase activity can be detected in frozen sections of root meristem cells when incubated in an assay mixture with ^{3}H-TTP. Much lower levels of activity are obtained when tissue slices or intact roots are pretreated with toluene or freeze-thawed at -20°C, -60°C or -200°C. Such procedures, however, permit composite pictures of activity to be more easily obtained. Incorporation is optimal in the presence of all four deoxyribonucleoside triphosphates and displays sulfhydryl group dependence. The nuclei displaying *in vitro* DNA polymerase are believed to be those in S phase at the time of tissue preparation and are not nuclei displaying DNA repair activity mediated by the experimental method.

A low level of DNA polymerase activity can be detected in the ungerminated onion seed embryo. This activity is found in the presence of cycloheximide or ribonuclease and thus appears not to be dependent on *de novo* protein synthesis. It needs to be established whether the activity in the dry seed is involved in DNA repair mechanisms in seed storage.

When ungerminated onion seed embryo tissues were incubated in the presence of saturating levels of micrococcal DNA polymerase, incorporation from ^{3}H-TTP is higher in presumptive meristematic tissues as the epicotyl, provascular and epidermal cells than in storage cotyledonary tissues. This indicates retention of a more efficient template and (or) primer in these tissues for the enzyme. It should be established if DNA strand scission occurs in aging seed tissues.

Ultrastructural studies demonstrated extensive condensation of the chromatin in all regions of the embryonic axis. Although qualitative differences in the condensation state are apparent between some nuclei of presumptive meristematic tissues, such as the epicotyl, as compared to storage cotyledon cells, extensive serial sectioning is required before any association between nuclear ultrastructure and template activity of nuclear chromatin can be made. These studies should be supplemented with E. M. cytochemical probes for DNA templates.

Nuclear or cytoplasmic *in vitro* protein synthesis could not be established on cytologic sections. A high level of amino acid incorporation was retained over both nuclei and cytoplasm, but was not decreased with cycloheximide or a combination of cycloheximide, chloramphenicol and puromycin. Activity was decreased in ungerminated embryos in the presence of either an excess of unlabelled amino acids or with poly-U and it should be established if amino-acyl-synthetases are responsible for the incorporation.

Ultrastructural studies of the cytoplasmic contents of various regions of the embryo demonstrated possible areas for retention of activity by charged transfer RNA. Although densely packed protein and lipid bodies are found in all regions, cells often display regions of groundplasm containing ribosomes, endoplasmic reticulum and mitochondria. Although protein bodies appear not to be associated with ribosomes, most lipid bodies are characteristically studded with such organelles.

Attempts to establish a cytological method for either protein, DNA or RNA methylases, were successful only for RNA methylase activity. With methionine as substrate in an *in vitro* assay mixture, RNA methylase could not be reliably demonstrated in either germinated or ungerminated seeds. A suitable level of RNA methylase activity was demonstrated in meristematic root tips of either germinated seeds or sprouted bulbs, when S-adenosyl-L-methionine was substituted for methionine. Activity was generally distributed over the whole nucleus with no preferential nucleolar incorporation. Approximately 30% of the meristematic cells displayed RNA methylase activity in both bulb and seed root tips. It needs to be established which species of RNA are being methylated while still bound to chromatin. No attempt was made to investigate activity in ungerminated seeds using 'activated' methionine.

An enzyme tentatively identified as poly(ADP-ribose)polymerase has been recorded for the first time in a higher plant. When cell extracts were incubated in an assay mixture with ^{3}H-NAD, an acid insoluble polymer was formed. Labelled 5-AMP and ADPr were detected after phosphodiesterase and protease digestion. The enzyme was also demonstrated cytologically. When root meristem cells were incubated, approximately 30% of the cells displayed an intense incorporation. The activity was distributed over the whole nucleus with no preferential nucleolar incorporation. When tissues of the ungerminated seed were incubated with NAD, intense activity

occurred in all tissues from root cap epidermal cells to the tip of the cotyledon with more than 90% of the cells displaying activity. Reliable activity could not be established in endosperm tissues of ungerminated or germinating seeds. Most work to date suggests a role for poly(ADP-ribose) polymerase in template modification for DNA synthesis. The demonstration of similar numbers of cells displaying DNA polymerase and poly(ADP-ribose)polymerase in root meristems support this hypothesis. A physiological role for the high activity retained in tissues of the ungerminated seed will have to be considered in future hypotheses on the function of this enzyme.

The *in vitro* procedures established in these studies for the formation of insoluble isotope containing products by chromatin bound enzymes, should be useful in further studies of cell or tissue differentiation. To extend the method it will be of interest to see how reliably reaction products can be retained in chromatin of various animal tissues.

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APPENDIX I

Additional Studies

Work at establishing a role in the plant cell cycle for cyclic nucleotides as well as investigations into possible syntheses of the physiologically related prostaglandins, were carried out under tenure of the graduate fellowship, but are not reported here.

APPENDIX II

List of Primary Publications

Payne, J. F. and A. K. Bal (1972). Rapid activation of RNA polymerases in quiescent root meristem cells of Allium cepa L. bulbs. Z. für Pflanzenphysiol. 67: 464-467.

Bal, A. K. and J. F. Payne (1972). Endoplasmic reticulum activity and cell wall breakdown in quiescent root meristems of Allium cepa. Z. für Pflanzenphysiol. 66: 265-272.

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