INITIATION AND MAINTENANCE OF CELL DIVISION IN ARTICHOKE TISSUE

BY

DARA LEE MELANSON

DOCTOR OF PHILOSOPHY

UNIVERSITY OF EDINBURGH.

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You have the freedom to be

yourself.... here and now

Jonathan Livingston Seagull.

I hereby declare that this thesis has been composed by myself and that all the work presented herein is my own.

November, 1977.

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ABBREVIATIONS

A_{260}	-	Absorbance log(Io/I) at 260 nm wave length.
ADP	-	Adenosine diphosphate.
AMP	-	Adenosine monophosphate.
A.R.	-	Analytical reagent.
ATP	-	Adenosine 5' -triphosphate.
bis-acrylamide	-	N,N' - methylene bisacrylamide.
BSA	-	Bovine Serum Albumin.
^{o}C	-	Degrees centigrade.
Ci	-	Curie(s)
cm	-	centimetre(s)
Coomassie Blue	-	Coomassie Brilliant Blue R, (Trisodium 4'-anilino-8-hydroxy-1, 1'-azonaphthalene - 3,65'-trisulfonate)
cpm	-	counts per minute, also counts/minute
2,4-D	-	2,4-Dichlorophenoxyacetic acid
2-D	-	2 Dimensional
dATP	-	Deoxyadenosine triphosphate
DNA	-	Deoxyribonucleic acid
EDTA	-	Ethylenediaminetetra - acetic acid
ft-c	-	Foot-candle (measurement of light)
g	-	gram(s), or unit of gravitational force
GMP	-	Guanosine monophosphate
GTP	-	Guanosine triphosphate
^{3}H	-	Tritium (radioactive isotope of hydrogen)
K	-	1×10^{3} rpm
ln	-	Natural logarithm
M	-	Molar, (Molarity)

mA	- milliamp(s)
Me_2SO	- Dimethylsulphoxide
mg	- milligram(s)
ml	- millilitre(s)
mm	- millimetre(s)
mM	- millimolar
mol. wt.	- Molecular weight
N	- Normal, (Normality)
nm	- nanometre(s)
OD	- Optical density
pg	- picogram(s) = 1×10^{-12} grams
pmoles	- picomoles = 1×10^{-12} moles
PAS	- sodium 4-aminosalicylate
PCA	- Perchloric acid
psi	- pounds per square inch
$^{32}P_i$	- radioactive isotope of phosphorous (orthophosphate)
(γ -^{32}P) ATP	- gamma labelled ATP
rpm	- revolutions per minute
RNA	- Ribonucleic acid
rRNA	- ribosomal ribonucleic acid
S	- Svedberg unit
SLS	- Sodium lauryl sulphate
TCA	- Trichloroacetic acid
TEMED	- N,N,N',N' tetramethylethylenediamine
TNS	- tri-isopropylnaphthalene sulphonate
Tris	- Tris (hydroxymethyl) aminomethane
μCi	- microcurie(s)

µg	-	microgram(s)
µl	-	microlitre(s)
µM	-	micromolar
UV	-	Ultra-violet
V	-	Volt(s)
var	-	variety
w/v	-	weight per volume (as percent)

SPECIAL TERMINOLOGY

Other than the aforesaid abbreviations the following terms have been used throughout the text:

1. 'cultural state(s)' refers to auxin treated and non-treated tissue.

2. Non-treated and control tissue are used synonomously.

3. Where there is a plus (+) sign, either preceding 2,4-D or before a number, i.e., +18 hours, this refers to auxin treated tissue in general or at a specific cultural age. The minus (-) sign, has similarly been used for the non-treated tissue.

ABSTRACT

The initiation and maintenance of cell division in Jerusalem Artichoke tuber tissue has been investigated in 2 different ways:

1. A kinetic analysis of ribosomal RNA synthesis and maturation was undertaken to determine the points where accumulation of this RNA is regulated. The evidence presented indicates that the massive increase in the level of rRNA in response to auxin is in part due to an enhancement in transcription of the precursor rRNA and in part due to post-transcriptional control involving an increase in the rates and degree of processing of the precursor to the mature rRNA.

2. The acidic proteins of the nucleus and the cytoplasm were investigated during the early stages of culture, prior to the onset of cell division in the auxin treated tissue. Both the synthesis of these proteins and their modification by phosphorylation were monitored by the highly discriminating 2-dimensional gel electrophoresis technique. Significant changes in the acidic proteins of the nucleus were found early in culture, with approximately 6 additional proteins being synthesized and accumulated in this organelle. As G-1 phase progressed, the number was increased to 20 and then to 40 at the start of DNA replication. Alterations in the phosphorylation pattern occurred only at S, with the specific modification of 6 proteins. No changes in the cytoplasmic proteins were detected.

PART I

INTRODUCTION

It is generally accepted that all parts of a mature plant contain cells which are totipotent. That is, they contain cells, which under the right conditions, may be cultured to a callus and then reverted to a mature plant. This phenomenon, an aspect of a unique regeneration potential, has one interesting consequence. It suggests that mature differentiated cells in a plant contain the same genetic information. Since it is common observation that differentiated cells perform different functions, they must utilize this information in characteristic ways by only allowing the expression of certain genes.

It is now believed that the genetic material of most living systems is DNA (deoxyribonucleic acid). In order for the individuality of each cell type to be expressed the following process normally takes place. Nucleotide sequences on the DNA are transcribed to RNA (ribonucleic acid), which is transported to the cytoplasm where it is translated into protein. To the best of our knowledge all major differences between cells involve proteins, and it is one specific group, the enzymes, that are responsible for the complex regulatory mechanisms of the cell. This ultimately manifests itself in the highly integrated and fully functional multicellular organism.

But, what controls the expression of particular genes? Specific regulatory mechanisms must be available for the activation and expression of particular genes depending on the needs of the cell. There are 3 major possibilities.

1. Control of gene expression may take place at the transcriptional level, the original model of which was described by Jacob and Monod, (1961), for the bacterial systems. Here, a specific 'effector' probably a protein, switches on a gene resulting in the production of mRNA (messenger RNA) for a particular protein product. The levels of this product could then be controlled either by feedback repression or a specific 'repressor'. Although in prokaryotes, transcription can be controlled by the interaction of effectors and repressors, eukaryotic cells appear too large for efficient regulation by this mechanism. If this is the case, the production of regulatory molecules, may, themselves, be rigidly controlled.

2. The regulation of specific protein synthesis may occur at the post-transcriptional level. This theory requires that mRNA precursors are transcribed continuously. Control is exerted by the conservation and selection of specific RNA sequences for transport to the cytoplasm where they are translated into protein. The remainder is degraded without leaving the nucleus. In order to account for the discontinuous synthesis of specific enzymes such as thymidine kinase (Hotta and Stern, 1965) the transcription of mRNA may occur in a sequential fashion.

3. The synthesis of proteins may also be under ~~transcriptional~~ translational control. The few examples on report involve RNA viruses, whereby the interaction of the RNA with protein initiation factors and the availability of ribosomes themselves are just two of the possibilities which appear to regulate the types and numbers of proteins made (Lodish, 1976).

The contents of this thesis deal in particular with the transcriptional and post-transcriptional control of gene expression in Jerusalem Artichoke cells. Since this tissue can be induced to undergo cell division with a specific chemical effector, it has been used as a

model system with which to investigate the biochemical events involved in this response.

TRANSCRIPTIONAL CONTROL

In eukaryotes the principal genetic material is mainly located in the cell nucleus. This organelle consists of a soluble disperse nucleoplasm which contains various proteins and RNA, and a dense insoluble fraction termed chromatin. It is the chromatin of a cell which contains the primary genetic informational molecule, DNA. In addition, there is an equal amount by weight of the basic histones (Bonner et al, 1968), varying proportions of acidic proteins, and a small amount of RNA. Cytological studies have established that chromatin is heterogeneous in structure; the more diffuse regions being called euchromatin and the denser regions, heterochromatin. It is with the euchromatin that the synthesis of RNA is associated (Frenster et al, 1963 ; Frenster, 1969).

It was suggested years ago that gene function could be regulated by DNA-associated proteins (Stedman and Stedman, 1951), this role being originally assigned to the histones (Allfrey et al, 1963). There are, however, with few exceptions just 5 types of histone in all eukaryotic cells (Hnilica et al, 1962 ; Fambrough et al, 1968 ; Towill and Noodén, 1973). Furthermore, histones are, in the main, not tissue specific being uniform in distribution, relative proportions, and composition in the different cell types. It became apparent, then, that the number and complexity of the histones was far too limited to function as specific regulators of gene transcription for the multitude of genetic loci on the chromosomes. There are, however, reports of varying proportions of histone to DNA in different tissues (Bonner et al, 1968 ; Fambrough et al, 1968) which might contest this

opinion. Subsequent investigations by Paul and Gilmour, (1968) in which chromatin was reconstituted suggest that the histones repress the transcription from DNA in a non-specific manner.

Since the evidence pointed to chromosomal components other than the histones as specific gene regulators, attention was subsequently focused on the acidic proteins. First of all, this protein fraction is of quantitative importance, comprising greater than 50% of the chromosomal mass in many cases (see review ed. by Cameron and Jeter, 1974). They are also extremely heterogeneous and include, in all probability, proteins functioning from structural to the highly metabolically active, like DNA polymerase. Our present knowledge about non-histone proteins comes from various isolation and analytical procedures. It was found that dissociation of the chromatin with salt-urea (MacGillivray et al, 1972) resulted in a more quantitative recovery than did other procedures which involved the prior removal of histones. In view of the capacity of the acidic proteins to self-aggregate and to bind to other cellular components they have, in the past, been difficult to isolate and the best solution has been resolution in denaturing systems such as SLS and SLS/urea electrophoresis. MacGillivray and Rickwood, (1974) and Trewavas, (1976a) have demonstrated that the two-dimensional method involving iso-electric focusing in polyacrylamide gels in the first dimension and then SLS electrophoresis in the second dimension has greatly improved separation. With the advent of this technique the discrepancies which have arisen with other less discriminating techniques should be resolved. Despite these criticisms, the acidic proteins of the nucleus appear to be extremely complex and for the most part are of high molecular weight. To date, they have been found in association with the chromosomes (either

bound to DNA or histones), the nuclear envelope, nucleolus, nucleoplasm and the cytoplasm for those proteins in common with both the cytoplasm and nucleus (Goldstein, 1974).

One approach in the investigation of the role of the acidic proteins in the nucleus has involved a search for tissue specificity. It might be reasoned that if this protein fraction controlled the specificity of DNA transcription then marked differences should be observed between different tissue types and species. Thus far, this has not been found to be the case (Shaw and Huang, 1970 for pig ; Shelton and Neelin, 1971 for chicken ; and MacGillivray et al, 1972 for bovine and mouse). The methods employed, however, were not sensitive enough to detect minute changes in the proteins. For instance, in the last example (MacGillivray et al, 1972) the gel scans of proteins that were separated on a molecular weight basis showed a number of peaks, any one of which could contain several proteins. As a result the interpretation of their data may be erroneous and the use of improved methods such as the two-dimensional gel system where the proteins are evident as discrete spots should help to answer this question of tissue specificity.

An alternative approach to the identification of specific acidic proteins has been to study biological systems undergoing developmental change or gene activation. The sea urchin embryo and the rat testis have proven to be two developmental systems well suited to the study of the role of these proteins in differentiation (Platz et al, 1975 ; Seale and Aronson, 1975). In addition, there have been a number of reports of changes in the non-histone chromosomal proteins of target organs after treatment with hormones. For example, Teng and Hamilton, (1970) found that estrogen caused the appearance of new acidic proteins

in uterine tissue.

PHOSPHORYLATION OF THE ACIDIC PROTEINS

Because the nuclear acidic proteins display extensive phosphorylation, there has been considerable interest in this particular event as a possible regulator of gene activity (see review edited by Huijing and Lee, 1973). The proteins are phosphorylated in the nucleus with protein kinases (Kleinsmith and Allfrey, 1969 ; Shelton et al, 1972) and endogenous phosphatases catalyze the reverse reaction (Kleinsmith and Allfrey, 1969 ; Langan, 1967). Greater than 50% of the total cellular protein phosphate is localized in the nucleus, and of this 90% is associated with the acidic proteins and the remainder with the histones. The function of phosphorylation is purported by some researchers to be concerned with the specific regulation of individual genes (Stein et al, 1974). They base their hypothesis on results obtained from various acrylamide gel analyses of the non-histone phosphoproteins. For instance, Platz et al, (1970) ; Teng et al, (1971) and Rickwood et al, (1973) have not only demonstrated that this fraction is highly heterogeneous but that the pattern of phosphoprotein appears to be tissue specific. This opinion has recently been challenged by MacGillivray and Rickwood, (1974) who have used the highly discriminating two-dimensional gel electrophoresis method to resolve the proteins. They claim that many of the phosphorylated and also non-phosphorylated proteins of mouse liver, kidney and brain chromatin are identical. Previously, the same workers had concluded that they were different (Rickwood et al, 1973) and certainly their later experiment still displayed tissue differences in the labelling pattern of the proteins on the gel. Additional experiments must be carried out to settle this dispute.

The pattern of phosphorylated acidic proteins has been observed to vary during cell division and tissue development (Karn et al, 1974 ; Platz et al, 1975 ; Maller et al, 1977). It is still feasible, therefore that this protein fraction may function as a specific gene regulator. For instance, Kleinsmith et al, (1975) claimed that when HeLa chromatin was reconstituted with dephosphorylated non-histone proteins, a 50% reduction in template activity resulted. In addition, Schmidt and Goodman, (1976) have suggested that the cortisone enhanced phosphorylation on non-histone chromosomal proteins in the salivary gland cells of the larval dipteran Sciara coprophila may be partially responsible for the observed increase in RNA synthesis following hormone treatment. Trewavas and Stratton (1977) however, noted that many of the phosphorylated proteins of the barley embryo were lost with germination.

In addition to a possible regulatory role, there is good evidence to show that various enzymes in the nucleus may be activated by phosphorylation. DNA-dependent RNA polymerase activity is apparently increased 3 fold (Martelo et al, 1974) and similarly the phosphorylation of avian virus DNA polymerase increased the rate of DNA synthesis 10 fold. (Tsiapalis, 1977) Although phosphorylation may not specifically regulate gene activity, the possibility exists that it may function to maintain the structural integrity of chromatin. One striking example is the phosphorylation of histone f1 (H.1) just prior to the onset of mitosis. Bradbury et al, (1974a) and Marks et al, (1973) have demonstrated that the phosphorylation of histone f1 in Physarum and HeLa cells in early G-2 apparently functions to condense the chromatin thus allowing mitosis to proceed. In conclusion, there is no doubt that the phosphorylation of nuclear proteins is important but, in the absence of any definitive

evidence towards it being a method of specific gene regulation, it more likely functions in other ways. These could possibly entail structural, enzymatic or even determinates of the selection and processing of RNA (Trewavas, 1976b ; Blanchard et al, 1977).

CELL CYCLE

Considerable attention has been paid of late, to the appearance and turnover of the acidic proteins during the cell cycle. Since the onset of DNA replication and mitosis are both essential events requiring the synthesis of RNA, it offers an opportunity of studying the mechanisms by which the transient expression of genes is regulated. In continuously dividing cells it has been shown that the composition of the non-histone proteins varies throughout the cell cycle (Bhorjee and Pederson, 1972 ; Stein and Borun, 1972 ; Karn et al, 1974). In particular, they demonstrated an increased rate in the synthesis and accumulation of these proteins prior to the onset of DNA synthesis. These results suggest that the acidic proteins may regulate chromosome structure and function and thus determine the synthesis of specific protein products. For instance, Stein et al, (1975) found that histone gene expression in HeLa cells is regulated at the transcriptional level. They showed that RNA transcripts from chromatin reconstituted with S-phase non-histone chromosomal proteins hybridized with histone complementary DNA while those with G-1 non-histone proteins did not to a significant degree. In addition, there are, apparently, subtle changes in chromatin structure during G-1 and S phases as well as the more obvious mitotic and meiotic changes. Pederson, (1972) found that the actinomycin D-binding capacity of HeLa chromatin rises to a peak at this time, indicating a structural change, allowing easier access. As stated previously, it is with euchromatin that the

synthesis of RNA is associated. Euchromatin displays a similar content of histones as the condensed heterochromatin, but has, as well, an excess of nuclear polyanions such as RNA, residual proteins and phosphoproteins (Frenster, 1974). Although the involvement of the acidic proteins in the regulation of transcription has only been shown to be of a correlative nature in these experiments, it does imply that one role of the acidic proteins is the control of cell cycle stage-specific gene readout. The finding that different acidic proteins appear in response to changes in the growth pattern of a cell could be partially explained as the induction of enzyme synthesis. It appears, however, from the results above that certainly some regulatory macromolecules are present in the general increase of the acidic proteins concomitant with S-phase.

PROOF OF REGULATORY ROLE FOR THE NUCLEAR ACIDIC PROTEINS

In order to affirm that the acidic proteins function as regulators, a specific test for their biological importance must be devised. Recently, Gilmour and Paul, (1975) have attacked this problem by directly determining whether globin messenger RNA sequences can be detected in the RNA transcribed *in vitro* by *Escherichia coli* RNA polymerase from the chromatin of hemopoietic and non-hemopoietic tissues. Using reverse transcriptase, complementary DNA (cDNA) was synthesized *in vitro* from globin messenger RNA. Chromosomal proteins from both mouse foetal liver and brain were fractionated on CsCl gradients to get rid of endogenous RNA sequences. Following, the histones and non-histones from each were separated by chromatography on hydroxyapatite. Samples of non-histone protein from foetal liver or brain were then reconstituted with purified mouse DNA and pooled histones, and transcribed with *E. coli* RNA polymerase. When the RNA

transcripts were titrated against the globin cDNA they found that chromatin reconstituted with brain non-histones failed to hybridize while liver reconstituted chromatin hybridized to 40%. A similar level was attained for native foetal liver chromatin. Therefore, the globin gene is available for transcription only when foetal liver non-histone proteins are present. This represents the most conclusive proof thus far that the acidic proteins of chromatin restrict DNA transcription in a very specific manner. It also shows that the isolation of chromatin does not result in the loss of these determinant structures.

ASSESSMENT OF THE MECHANISM OF ACTION

While there is considerable evidence which suggests that the acidic proteins are involved in the genetic regulation of eukaryotic cells not much is known about their mechanism of action. There is, in animal systems, the well documented estrogen story. This hormone is required for the cytodifferentiation of the chick oviduct, and one of the more striking features is the synthesis of egg-white proteins, of which ovalbumin comprises 60% while progesterone, another steroid hormone, specifically controls the synthesis of egg-white protein, avidin (O'Malley and Means, 1976). Since the net level of cellular mRNA for ovalbumin rises following hormone stimulation (Harris et al, 1975) it did not appear that the synthesis of this protein was under post-transcriptional control. To affirm this, Harris et al, (1976) prepared chromatin from hormonally withdrawn chicks and found that following in vitro transcription few mRNAs for ovalbumin were detected. When estrogen was added, however, the appropriate mRNA was synthesized. It was concluded, therefore, that the steroid hormone altered the chromatin template so as to make the ovalbumin gene available to be

transcribed. A model envisaged by O'Malley and Means, (1976) is as follows : The steroids enter the cell, bind to a cytoplasmic receptor and this unit is translocated to the nucleus. Here, the receptor binds to a specific site on the chromatin and allows certain genes to be expressed. This mechanism for the regulation of gene expression probably represents an isolated case and does not necessarily apply to normal cell maintenance in most tissues. For instance, a similar mode of action has not been found for plant growth substances (see review by Trewavas, 1976c) and, therefore, other methods of control must be searched for.

It has been suggested (Kleinsmith et al, 1975) that the phosphorylation and dephosphorylation of the acidic proteins may provide the switching mechanism by which the genetic sequences are made available for transcription. This hypothesis is based on the observations of pronounced variations in the synthesis and turnover of the phosphoproteins during the various stages of the cell cycle (Platz et al, 1973 ; Karn et al, 1974). As stated previously, however, control by this mechanism does not seem likely. If it were the case, the genome would have to code for a specific protein kinase for a single acidic protein and then carry out the appropriate phosphorylation reaction. Subsequently, a specific phosphatase would have to be synthesized since natural turnover of the phosphate is not a reliable on/off switch. This expenditure of energy, the length of time involved, and the risk of mistakes does not make phosphorylation a particularly good control mechanism. It is more likely that a certain regulatory acidic protein binds to a specific site on the chromatin and causes local unwinding, with the level of synthesis being controlled either by a specific repressor or by the polymerase itself.

RNA POLYMERASE

Despite the redundancy of the rRNA genes and the relative stability and ease of measuring the final mature product, the possible involvement of the acidic proteins in the regulation of ribosomal RNA synthesis has not been studied. Many of the previous investigations of the control of transcription have, in fact, concentrated on the polymerase enzyme itself. For instance, there are marked changes in RNA metabolism, especially ribosomal RNA, associated with auxin-induced growth transitions in plant tissue (Trewavas, 1968 ; Key, 1969). There are, from the literature, at least 3 ways in which the control of rRNA synthesis is regulated. They are as follows:

1. The availability of the chromatin template for transcription.
2. Increased levels of the polymerase. Polymerase I is situated predominently in the nucleolus and is associated with the synthesis of ribosomal RNA, while the nucleoplasm contains mainly polymerase II, the enzyme which is assumed to be involved in heterogeneous RNA synthesis (Roeder and Rutter, 1970 ; Strain et al, 1971 ; Polya and Jagendorf, 1971 ; Duda and cherry, 1971).
3. Activation of pre-existing polymerase by a specific protein factor which would increase the association of the polymerase to the chromatin.

In the presence of excess endogenous RNA polymerase, it has been suggested that more of the genome may be unmasked resulting in an enhancement in the synthesis of RNA in auxin treated tissue. In order to distinguish between a greater availability of the genome and an increased activity of the polymerase itself, saturating levels of exogenous RNA polymerase, such as from E. coli may be added to the chromatin from non-treated tissue. If the presence of the polymerase

has no effect on the rate of RNA synthesis, then auxin must cause an unmasking of the gene template. Conversely, if the presence of excess polymerase brings the rate of RNA synthesis in control tissue up to the level demonstrated by auxin treated tissue then auxin must enhance the activity of RNA polymerase itself. When such experiments were performed on soya bean hypocotyl (O'Brien et al, 1968 ; Holm and Key, 1971) the results suggested that while there appeared to be some selective unmasking of the template, the major effect was on the activity of the RNA polymerase. O'Brien et al, (1968) interpreted their results as an indication of greater amounts of the enzyme in association with the chromatin. Holm and Key, (1971), however, proposed that there was increased template availability following auxin treatment. They reasoned that the difference in the nearest neighbour analyses of the RNA synthesized by control and auxin chromatin obtained by Holm et al, (1970) was suggestive of an alteration in the sites of transcription. In the experiments using excess levels of exogenous polymerase the enhancement of total RNA synthesis made interpretation difficult. In addition, this work was done when it was thought that removal of the histones was required for increased template activity.

Further research has demonstrated that auxin may enhance the RNA synthetic ability of soya bean hypocotyl some 8 to 10 fold (Holm et al, 1970). More recently, Chen et al, (1975) ; Guilfoyle et al, (1975) and Lin et al, (1976), have shown that treatment of soya bean hypocotyl with 2,4-D (2,4-dichlorophenoxyacetic acid) selectively enhances the activity of polymerase I compared to polymerase II.

Some workers have queried whether the enhancement of polymerase I activity is actually due to increased amounts of the enzyme or due,

instead, to an auxin-induced increase in the association of the polymerase to the chromatin. For instance, Gore and Ingle, (1974) correlated the increase in chromatin-bound polymerase activity with the accumulation of rRNA in auxin treated Jerusalem artichoke tissue. It has been suggested that the auxin effect is conveyed by a mediator protein which changes the molecular properties and thus activates the pre-existing RNA polymerase. There are, however, conflicting opinions as to the mechanism of this response.

In one of the earlier investigations, Matthysse and Phillips, (1969) found that, in the presence of a protein mediator, auxin increased the rate of RNA synthesis in both isolated nuclei and isolated chromatin. Since isolated nuclei also responded to hormone treatment this implied that the mediator was in the nucleus. Venis, (1971) prepared crude extracts from pea and corn shoots and passed them through a Sepharose-2,4-D column, and found that certain protein fractions were retained. Elution yielded a protein factor that stimulated DNA-dependent RNA synthesis supported by E. coli polymerase. It was suggested that the factor may be influencing the initiation of RNA chains. When Rizzo et al, (1977) repeated the experiment with soya bean, only RNA polymerase I appeared to be stimulated. Mondal et al, (1972) similarly claim to have isolated RNA polymerase I and its respective initiation factor from coconut endosperm nuclei. While the preceding workers have suggested that auxin enhances the synthesis of RNA by increasing the frequency of the initiation of transcription, Guilfoyle and Hanson, (1974) have proposed that the polymerase-mediator association merely results in the production of longer transcripts and does not serve to initiate the process. They base their conclusion on a thin-layer chromatographic separation of

the RNA hydrolysis products and on a polyacrylamide gel analysis of the RNA itself. Since they made their measurements on RNA that was synthesized in vitro, on isolated soya bean hypocotyl chromatin, it cannot be certain whether the results are representative of the true in vivo state. In an attempt to find a specific regulatory factor for RNA polymerase, Hardin et al, (1972) incubated several membrane fractions from the soya bean in the presence and absence of 2,4-D. They found that auxin specifically released a factor which stimulated the activity of RNA polymerase. It was found, however, that it was the α-amanitin sensitive polymerase which was effected and they concluded that it is messenger RNA and not ribosomal RNA synthesis that is regulated in this fashion. Since, to my knowledge, these experiments have not been repeated and since in vivo tests involving the release of the factor from the membrane and the subsequent purification of a polymerase-factor complex has not been demonstrated, the validity of such a mechanism is questionable.

In the experiments quoted above, it has been assumed that the stimulating factor was already present in the tissue. Teissere et al, (1975) have, however, suggested that auxin must initially induce the synthesis of a mediator protein and this is followed by the subsequent modification of polymerase I activity. Although they claimed that this factor (factor γ) was doubled in auxin treated lentil roots I could not detect such a change from their data. Previously, Teissere et al, (1973) had proposed that there was first an increase in messenger RNA production followed by an enhancement of polymerase I activity. As this implies that proteins must be made prior to the synthesis of new rRNA they may not have been looking at the production of a stimulator factor, but at the synthesis of the polymerase itself. Moreover, since

the use of crude fractions of transcription stimulators in a cell free system may cause artifactual RNA synthesis, it is my opinion that the methods so far employed in these investigations are not likely to yield any definitive results regarding a mediator factor.

REGULATION OF TRANSCRIPTION

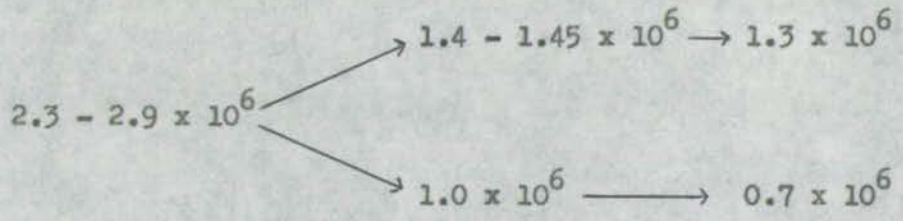
The function of protein factors in association with the RNA polymerase is not known, but according to Chambon, (1975) in animal systems they appear to act primarily by stimulating chain elongation. Recently, Grummt and Grummt, (1977) working with Ehrlich ascites cell cultures, have disputed the existence of a mediator protein at all. They suggest that the rate of precursor rRNA transcription may be controlled by the actual nucleoside-triphosphoate levels of the cells, rather than by short-lived proteins which act as a position regulator of the RNA polymerase. Specific proteins have also been implied in the activation of DNA polymerase (Gefter, 1975 ; Weissbach, 1977) but there is again, no evidence that they are required for the initial response. In fact, Bryant (1976) suggested that DNA synthesis may also be regulated by the availability of deoxyribo-nucleoside triphosphates rather than by the control of their polymerization into DNA. Furthermore, in a recent review, Trewavas, (1976c) has pointed out that the kinetics of the activation of RNA polymerase is not consistent with the concept of a stimulator protein. Since there is a lag period of up to 3 hours before the induction of RNA synthesis by auxin, this implies that the relevant polymerases may be synthesized and this may occur on previously formed messenger RNAs. Since Teissere et al, (1973) have suggested that chromatin transcriptional activity is increased prior to the enhancement of polymerase activity, the proposal of a stable messenger is questionable and additional experiments must

be done to establish this point.

In conclusion, although an auxin-mediator protein, specific for RNA polymerase I, has been sought after, in no case has the purification and activity of such a factor been demonstrated and therefore, it does not seem likely that one even exists. Furthermore, it is the opinion of Yomo and Varner, (1971) who have used density labelling to study the germination events in barley, that many enzyme changes are probably caused by de novo synthesis and are not activated by some factor.

POST-TRANSCRIPTIONAL REGULATION OF RIBOSOMAL RNA

In most eukaryotic cells ribosomal RNA is transcribed from a polycistronic set of genes (Birnstiel et al, 1968 ; Trendelenburg et al, 1974) around which the nucleolus forms (Brady and Clutter, 1972 ; Perry, 1976). The Jerusalem artichoke is no exception with 1600 copies/genome (Ingle et al, 1976), although much larger amounts have been recorded for other plant and animal species (Reeder, 1974 ; Ingle et al, 1975 ; Nagl, 1976). In view of its redundancy and the stability of the product, its synthesis and maturation and transport to the cytoplasm have been followed in great detail (Attardi and Amaldi, 1970 ; Perry, 1976). Most species of eukaryotic rRNA are synthesized as larger precursor molecules (Darnell, 1968) and these precursors undergo specific processing steps before being transported to the cytoplasm. Since the 2 mature rRNAs compete with the precursor sites on the DNA, this indicates that the initial transcript contains sequences of both types of rRNA (Grierson and Loening, 1974). The pathway of maturation of ribosomal RNA in plant cells (Leaver and Key, 1970 ; Rogers et al, 1970 ; Cox and Turncock, 1973 ; Grierson and Loening, 1974 ; Chapman and Ingle, 1976) is as follows :



The measurements of molecular weight and the stages of processing have been obtained by polyacrylamide gel electrophoresis of the RNA. Within certain gel concentrations and ionic strengths this system displays a linear relationship between the log of the molecular weight and the electrophoretic mobility (Loening, 1969). It has, therefore been used routinely in the estimation of size.

It is a common observation that prior to the induction of cell division in many tissues, there is a massive accumulation of ribosomal RNA. In addition, it has been noted that although the rate of transcription can vary, the appearance of mature ribosomes does so to an even greater degree. This implies that post-transcriptional regulation might be an important feature in eukaryotes and a study of the maturation of rRNA may provide information on the control points in cellular metabolism. For instance, it could, in all probability represent a model system by which the level of the more unique and relatively unstable messenger RNAs are regulated. Grierson et al, (1970) have postulated that different growth conditions yield differences in the processing of rRNA and they correlated the rate of ribosome synthesis with the half-lives of the precursor. Furthermore, in rapidly growing cells the overall rate of processing seems geared to the cell generation time. For example, Taber and Vincent, (1969) found that in yeast cells with a 3 hour generation time, the processing rate is 5 to 10 fold faster than in mammalian cells with a generation time of 12 to 15 hours. There is also considerable

variation among cell types with regard to processing efficiency : quiescent or slow growing cells exhibiting substantial 'wastage' in which a fraction of the rRNA precursor molecules are fully degraded without giving rise to mature rRNA components. This has been demonstrated in resting lymphocytes (Cooper and Gibson, 1971 ; Cooper, 1973) in unfertilized Urechis eggs (Das et al, 1970) and in differentiating yolk sac erythroid cells (Fantoni et al, 1972). Leaver and Lovett, (1974) concluded that the limitation in processing of the precursor rRNA in Blastocladiella zoospores may be due to a deficit of proteins in the nucleolus. A similar conclusion was reached by Rogers et al, (1970) when they noticed an increased life, or delay in the processing of the precursor rRNA in pea root tips. More direct measurements of the dependence of RNA synthesis on protein availability have been done by Roth and Dampier, (1972) in yeast. They found that RNA synthesis ceased when the culture medium was lacking in a single amino acid or when a protein inhibitor, cycloheximide, was used. A similar lowering of rRNA synthesis by the intracellular absence of an amino acid has been noted in animal cells (Hershko et al, 1971 ; Pardee, 1974). There is, in fact, a large amount of evidence to suggest that the supply of ribosomal proteins limits the synthesis and processing of precursor ribosomal RNA and the final assembly of ribosomes (see reviews by Attardi and Amaldi, 1970 ; Warner, 1974 ; Perry, 1976). Most of the ribosomal structural proteins as well as several additional protein species are associated with the initial rRNA precursor molecules (Kumar and Warner, 1972 ; Shepherd and Maden, 1972). These proteins are synthesized on cytoplasmic polyribosomes, transported to the nucleolus, and assembled with the rRNA precursor and 5S RNA into a large ribonucleoprotein

structure (Warner, 1974). Whether the proteins have any positive role in determining cleavage specificity is uncertain, but as with prokaryotes, the processing of rRNA cannot proceed very far in the absence of a continuous protein supply (Williams et al, 1969 ; Craig and Perry, 1970).

PROBLEMS IN THE STUDY OF RNA SYNTHESIS AND PROCESSING IN PLANT TISSUE

The study of rRNA processing does have its particular problems. For instance, in all plants the initial rRNA precursor appears to demonstrate a certain degree of heterogeneity. Since, in a gel analysis of rRNA maturation, the shape of the precursor peak or the ratio of label does not change with pulse time it has been suggested that the transcribed lengths may not all be identical(Grierson et al, 1970). An objection was raised by Cecchini et al, (1972) who queried the study of the processing of precursor rRNA in pieces of tissue on the grounds that the life-times are too short compared to the times required to increase the specific activity of the nucleotide pools appreciably. A similar conclusion was reached by Chapman and Ingle, (1976) who have done one of the most comprehensive studies of the processing of plant nucleolar RNAs to date. Although they estimated the half-life of the 2.5×10^6 precursor rRNA of artichoke cells to be approximately 24 minutes they realized that due to the time required for the equilibration of the nucleotide precursor pools, this value could be as small as 10 minutes. Also, changes in RNA metabolism have normally been monitored by using radioactive precursors. The results, then, are difficult to interpret since the turnover rate of individual RNA species must be taken into account (Trewavas, 1976c). This is especially critical when assessing the effect of growth substances on the synthesis of particular RNAs. For instance, there may be different precursor pools for different RNA

species as shown for HeLa cells by Weigers et al, (1976).

POST-TRANSCRIPTIONAL REGULATION OF MESSENGER RNA

Due to the high turnover rate of the more unstable messenger RNAs, the effect of auxin on this population has virtually gone undetected. There is, nevertheless, evidence that auxin can increase mRNA levels. For instance, both Fraser, (1975) and Byrne and Setterfield, (1977) have proposed that mRNA is the predominant species synthesized shortly after the addition of 2,4-D to artichoke cultures. More importantly, Verma et al, (1975) used a cell-free translation technique to demonstrate that auxin increased the level of a specific mRNA, that of cellulase in pea epicotyl. But did it arise by enhanced synthesis, a change in the processing, a greater stability, or all three?

The production of messenger RNA may involve post-transcriptional modifications similar to that demonstrated in the maturation of ribosomal RNA. For instance, it has been suggested (Néissing and Sekeris, 1973 ; Pederson, 1974 : Levner et al, 1975 ; Malcolm and Sommerville, 1977) that the supposed precursor of mRNA, heterogeneous RNA (hnRNA), associates with a heterogeneous size range of non-basic proteins which may be critical to the stabilization of the mRNA and assist in the processing and transport to the cytoplasm. Some are apparently enzymes involved in the cleavage and modification of the RNA (Neissing and Sekeris, 1973). The presence of poly A tracts in the mRNAs could have a similar function (Brawerman, 1974 ; Lewin, 1975 ; Perry, 1976) and/or behave to select certain sequences for transport. The initiation of a poly A tract and the elongation of the chain would also require specific enzymes and co-factors. Therefore, the activity of the processing enzymes and the transport proteins

could determine the pattern of mRNA directed to the cytoplasm and subsequently, the production of protein.

JUSTIFICATION FOR RESEARCH

When intact plants, like soya bean, are treated with auxin, massive increases in ribosomal RNA are normally observed. Similarly when excised pieces of tissue, such as explants from Jerusalem artichoke tubers are cultured in the presence of auxin ribosomal RNA accumulates in vast amounts. Although it is a commonly observed event and numerous experiments have been carried out on the induction and processing of rRNA, the majority of researchers have failed to ask the critical question: Why is ribosomal RNA synthesis enhanced at all? The most logical explanation as suggested by Trewavas, (1976c) would be the requirement by the cell for the synthesis of greater amounts of protein. This could conceivably take place by the attachment of more ribosomes to the messenger. The number of protein molecules that are synthesized from a particular mRNA depends on the frequency with which ribosomes initiate and complete translation of the template. In auxin treated tissue, part of these proteins would be required for the induction of DNA replication and subsequent cell division. In intact plants, such as the soya bean, a study of the activation by auxin of RNA polymerase is a challenging prospectus as it may lead to a better understanding of how the expression of particular genes are regulated. Systems like the artichoke are, however, not suited for such a study, since the initial activation of RNA polymerase is not, apparently, under the control of the investigator. This is because the excision of all tissues causes an increase in ribosomes and hence, both treated and non-treated artichoke explants initially display a similar stimulation of RNA and protein synthesis, independent of the

presence of auxin (Mitchell, 1969). It is possible, nevertheless, to investigate the pathway of maturation of ribosomal RNA in the proliferating tissue compared to the more quiescent cells. Any differences observed may then be related to the metabolic state of the tissue, which ultimately depends on the synthesis of proteins, both structural and enzymatic. I have, therefore, undertaken a study involving the processing of ribosomal RNA in auxin treated and non-treated Jerusalem artichoke tuber cells in the hope of gaining a better understanding of the maintenance of cell division.

Protein synthesis is an integral part of the growth of a cell, and is, therefore, not only essential for its normal functioning but is apparently involved in the control of specific phases of the cell cycle. For example, Kovacs and Van't Hof, (1970) have shown that by inhibiting protein synthesis in Vicia roots by carbohydrate starvation, the cells are prevented from entering S-phase. It was suggested that the lag phase represents a time when proteins necessary for DNA replication and mitosis are synthesized. Similarly, in synchronous cultures of Chlorella cells, the progress of S-phase is halted by cycloheximide an inhibitor of protein synthesis, which implies that there was either rapid enzyme turnover or the synthesis of new proteins was required (Wanka and Moors, 1970). In addition, Yasuda et al, (1974) noted that DNA replication was prevented in artichoke cultures when cells in G-1 were treated with cycloheximide. These examples suggest that de novo synthesis of the various enzymes and other proteins is of critical importance to the induction and maintenance of S-phase. In particular, although total protein is synthesized throughout the cell cycle, the synthesis of many shows a discontinuous pattern which is related to a certain stage in the cycle. Many of the enzymes

involved in the synthesis of deoxyribonucleotides display a peak of activity in late G-1 or S-phase. In Acer cells the activity of thymidine kinase peaks at S (King et al, 1974) and in Jerusalem artichoke a similar increase in the activity of TdR kinase, thymidine monophosphate kinase, and DNA polymerase was associated with the onset of DNA replication (Harland et al, 1973). This phenomenon of discontinuous synthesis must, in some way, be regulated, and this could occur either at the level of transcription, in the post- transcriptional processing of the mRNAs, or during the translation of the mRNA into protein. I concentrated on the possibility that the regulation of protein synthesis in preparation for the onset of DNA replication in the artichoke occurs at the transcriptional level. To do this I have assumed that the acidic proteins are the regulators of genomic expression and have accordingly separated both phosphorylated and non-phosphorylated acidic proteins of the nucleus and the cytoplasm by the highly discriminate 2-dimensional gel electrophoresis technique. Both auxin treated and non-treated artichoke tuber cells were assayed in this matter from the time of excision to the onset of the first S-phase in the treated tissue.

PART II

MATERIALS AND METHODS

SECTION A. PLANT MATERIAL

Mature tubers of a single clone of Jerusalem Artichoke, Helianthus tuberosus, L. (var. Bunyards Round), were obtained from plants grown in the garden of the Botany Department, King's Buildings, Edinburgh. Tubers were planted in March and the crop harvested in late December. The tubers from each individual plant were stored in damp sand in polythene bags at 4°C. Under these conditions of storage the tubers remained dormant approximately 4 to 5 months.

SECTION B. RADIOCHEMICALS

These were all obtained from The Radiochemical Centre, Amersham.

(G-^{3}H) Adenosine; specific activity 6.4 Ci/mmole, was obtained as an aqueous solution and stored at -20°C.

Adenosine 5'-(γ -^{32}P) triphosphate, ammonium salt; (γ -^{32}P) ATP specific activity 16 Ci/mmole, was obtained as a freeze dried solid and stored at -100°C until use, when it was taken up in cold distilled water.

^{35}S-methionine; approximate specific activity 500 Ci/mmole, obtained as an aqueous solution and stored in liquid nitrogen (-195°C).

Phosphorus-32; approximate specific activity 100 Ci/mg P, was obtained as the orthophosphate in dilute hydrochloric acid, pH 2-3, and stored at room temperature.

(CH_3-^{3}H) Thymidine; specific activity 23 Ci/mmole, was obtained as an aqueous solution and stored at -20°C.

(G-^{3}H) Uridine; specific activity 6.5 Ci/mmole, was obtained as an aqueous solution and stored at -20°C.

SECTION C. ENZYMES.

Luciferin-luciferase from 250 mg dried firefly lanterns, FLE-250. Sigma Chemical Company.

SECTION D. CHEMICALS

Acrylamide, specially purified for electrophoresis was used for the isoelectric focusing and SLS slab gels. BDH Chemicals Ltd.

Agarose, for gel electrophoresis type H.S.C. Litex, Denmark.

Ampholines, pH 3.5 - 10 and pH 5 - 7, obtained as a 40% sterile stock solution. LKB.

FF-ATP; 1 mg adenosine-5'-triphosphate disodium salt plus 40 mg $MgSO_4$ in preweighed vials. Sigma Chemical Company.

Sodium lauryl sulphate (SLS), specially purified for biochemical work. BDH Chemicals Ltd.

Thin-layer chromatogram sheets, plastic backed, No 6064 Eastman.

The remaining chemicals used were of analytical reagent, (A.R.), grade.

Acrylamide and N,N'-methylene bisacrylamide were recrystallized from chloroform and acetone respectively when required for RNA gels (Loening, 1967).

Glass distilled water was used throughout the experiments.

SECTION A. DECONTAMINATION AND CLEANING OF EQUIPMENT

1. Cleaning of glassware

All glassware was washed in hot water containing detergent then rinsed well with tap water, followed by glass distilled water, and dried in an oven.

2. Decontamination of apparatus used for radioisotope work

After pouring away the scintillation fluid, glass bottles and tops were soaked in methylated spirits, washed in warm water containing detergent, followed by thorough rinsing with tap water. After a rinse in distilled water, they were dried in an oven.

Other contaminated pieces of equipment were soaked overnight in 2% Decon-75, (Decon Laboratories Ltd.), then washed as in (1), above.

3. Siliconization of glass gel tubes

Approximately 3 g potassium dichromate was dissolved in 7 ml distilled water and made to 100 ml with concentrated sulphuric acid to make a concentrated solution of chromic acid. The gel tubes were immersed in the chromic acid for 24 hours then washed thoroughly with tap water, followed by glass distilled water. They were dried in an oven, allowed to cool, and immersed in a 2% solution of 2,4-dimethyl-dichlorosilane in carbon tetrachloride. After 30 minutes the liquid was poured away and the tubes left to dry. The tubes were given a final rinse with glass distilled water and again dried.

4. Sterilization methods

Preparation of tissue for culture was carried out in a sterile room which was maintained by two UV lamps (Philips, tubular 15W),

left on unless the room was in use. A small positive pressure of filtered air reduced the inflow of contaminated air when the room was entered. Instruments, wrapped in aluminium foil, and glassware were sterilized in sealed tin boxes at 150°C for 4 hours. Conventional flaming techniques were used for routine sterilization of instruments during use.

SECTION B. ANALYTICAL METHODS

1. Cell number determination

The number of cells in artichoke tuber explants or discs was determined by the method of Brown and Rickless, (1949). Samples of 5 explants or 1 disc were placed in 3.0 ml of 5% chromium trioxide. These were left for 48 hours at 4°C and the cells counted. The tissue was macerated by rapidly hauling in and out of a pasteur pipette. Aliquots of the suspension were applied to a haemocytometer slide and the cells counted.

2. Protein estimation by the Folin and Ciocalteus phenol reagent

The method of Lowry et al, (1951), was used whereby production of a blue colour relies on the reduction of the folin reagent by the amino acids, tyrosine, tryptophan and cysteine. The following solutions were prepared.

1. Fresh alkaline copper tartrate composed of 2% $CuSO_4.5H_2O$, 4% Na-K tartrate, 2% Na_2CO_3 in 0.1 N NaOH in a 1:1:200 ratio.
2. Commercial Folin and Ciocalteus phenol reagent diluted 1:1 with water.
3. 0.1 N NaOH.
4. A standard solution of Bovine Serum Albumen, (BSA), in 0.1 N NaOH, containing 200 µg/ml.

A standard curve of 0, 25, 50, 100, and 200 µg BSA was prepared for each assay. Suitable samples of tissue extracts in 0.1 N NaOH were taken and made to 1.0 ml with 0.1 N NaOH. 5.0 ml of the alkaline copper tartrate was added to each tube, mixed well, and allowed to sit 10 minutes for the complete formation of the protein copper complex. A volume of 0.5 ml of the diluted Folin reagent was added to each tube

FIGURE 1.

Lowry standard curve for protein determination

(Method as in Lowry *et al*, 1951)

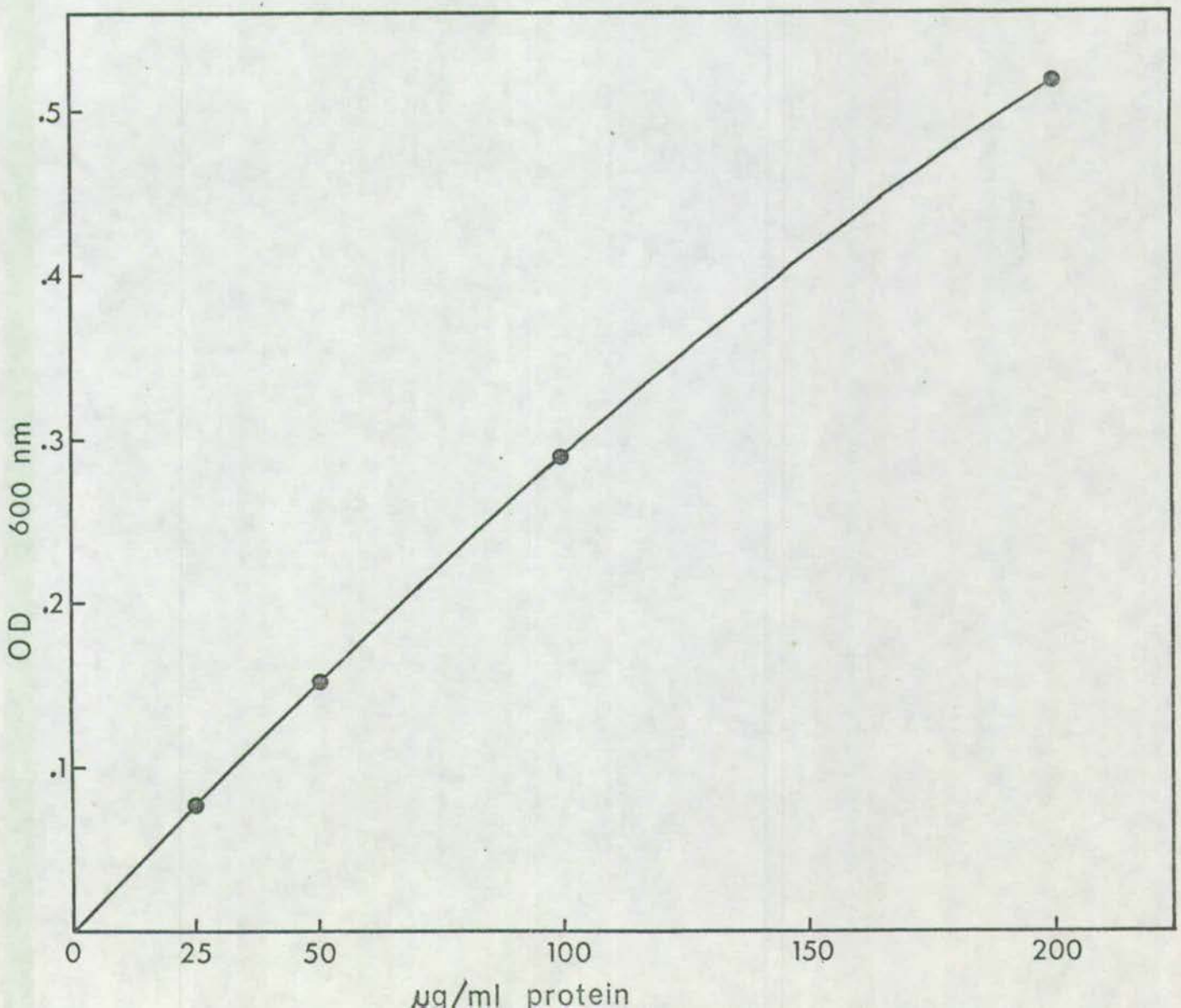


FIG. 1

and the contents thoroughly mixed. After 30 minutes the blue colour which had developed was measured with a Corning Colorimeter 252 using a red, (600 nm), filter and protein content determined relative to a calibration curve containing 25 to 200 µg BSA. A typical standard curve is shown in Figure (1.)

3. DNA determination by the Diphenylamine reagent

The method of Burton, (1956) was followed and depends on the reaction of the deoxyribose component of the DNA with diphenylamine to give a blue colour. Although the diphenylamine reaction will produce a colour with other sugars the ribose of RNA does not interfere (Fraser, 1968).

Diphenylamine reagent: 1.0 g diphenylamine in 66.0 ml glacial acetic acid and 1 ml concentrated H_2SO_4 with 0.33 ml of acetaldehyde at 16 mg/ml added just prior to use.

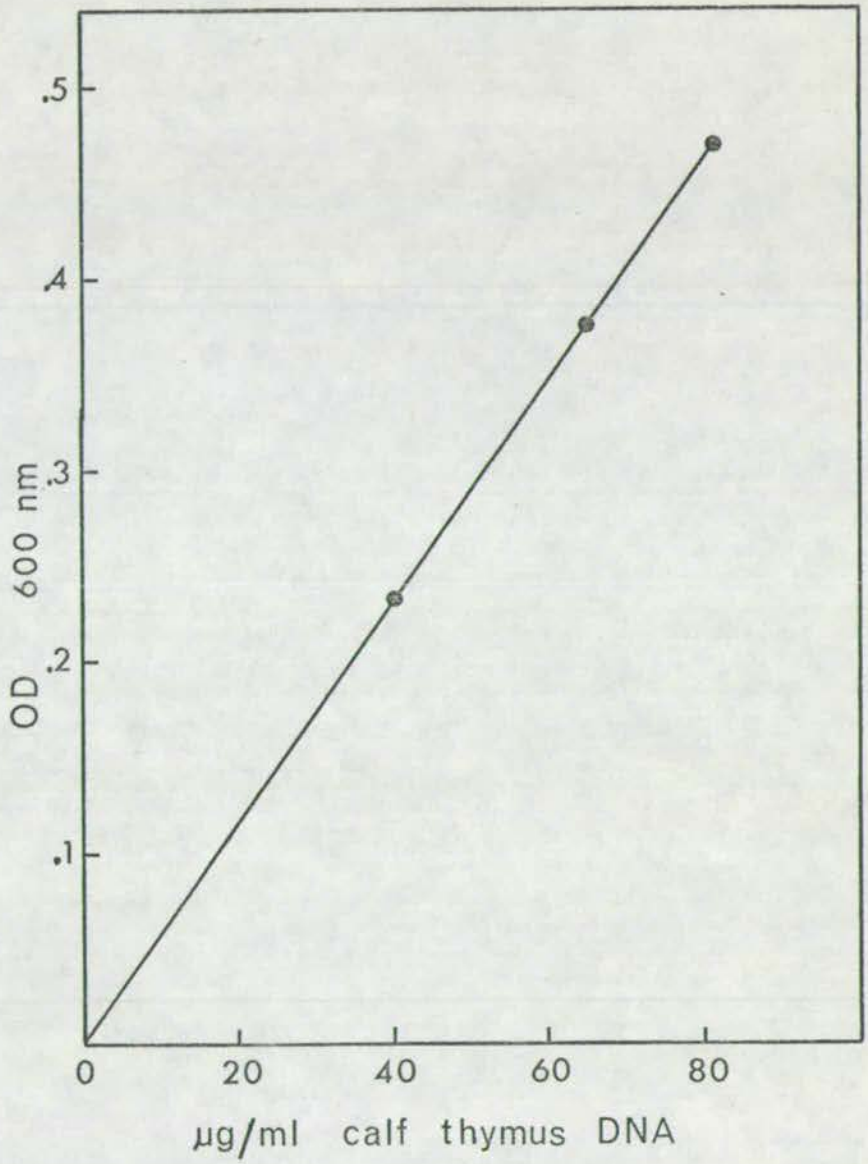
The tissue homogenate was hydrolized in 0.5 N perchloric acid (PCA) for 20 minutes at 70°C. Since no definite end product is obtained, a standard solution of calf thymus DNA at 200 µg/ml was acidified with an equal volume of 1.0 N PCA and hydrolyzed with each set of samples. After hydrolysis the tubes were cooled in water and the samples centrifuged at 2000 rpm for 10 minutes. 2 volumes of diphenylamine reagent was added to 1 volume of hydrolysate and the tubes stoppered with marbles and incubated overnight at 25°C. The blue colour was measured with a Corning Colorimeter 252 using a red (600 nm) filter and DNA content determined from the standard calibration curve of the calf thymus DNA shown in Figure (2.)

FIGURE 2.

Diphenylamine standard curve for DNA determination

(Method as in Burton, 1956)

FIG. 2



4. Radioactive techniques

(a) Preparation of scintillant

A standard toluene-based scintillation fluid containing 0.4% (w/v) butyl-PBD was used. 2500 ml of toluene was filtered and 10.0 g butyl-PBD dissolved into it. A 1:2 mixture of Triton-toluene was prepared by dissolving an additional 2.0 g of butyl-PBD into 1000 ml of the standard scintillant and slowly adding 500 ml Triton X-100 with continuous stirring.

(b) Determination of radioactivity

(i) Liquid scintillation

Approximately 6.5 ml of standard scintillant was added to glass scintillation vials each containing either a radioactive sample dried onto a GF/C filter, or a 5.0 mm x 10.0 mm strip of plastic backed cellulose emulsion for those samples subjected to thin-layer chromatography.

Liquid samples were added to individual vials and made up to a volume of 1.0 ml with water. To this solution 10.0 ml of Triton-toluene was added and the mixture vigorously shaken until a clear, stable emulsion was obtained.

Radioactivity was determined in an Intertechnique SL 30 liquid scintillation counter.

(ii) Geiger tube detection of $^{32}P_i$

Polyacrylamide gels containing ^{32}P-orthophosphate labelled nucleic acids were monitored in the following way. The gels were frozen in aluminium boats in solid CO_2 and cut into 1.0 mm or 0.5 mm (acrylamide-formamide gels) slices using a Mickle gel slicer, (The Mickle Laboratory Engineering Co.). The slices were dried onto the emulsion side of 16 mm cine film and counted in a controlled transport system under a Geiger

tube coupled to a programmed scaler unit (J & P Engineering, Reading, Ltd.). The Geiger tube had approximately 30% the efficiency of the liquid scintillation system.

(iii) Autoradiography

Detection of radioactivity in proteins resolved on SLS-discontinuous slab gels was accomplished by autoradiography. The gel (dried onto filter paper) and a sheet of X-ray film (Kodax Blue Brand) were sandwiched and fixed with tape between thick glass plates and left to expose in the dark for a minimum of 3 days and up to 5 weeks.

(iv) Fluorography

When activity was low, the intensity of the spots was enhanced by fluorography (Bonner and Laskey, 1974 ; Laskey and Mills, 1975). Following electrophoresis the slab gels were soaked in 30% methanol : 10% glacial acetic acid to remove the SLS. The gels were dehydrated by shaking in 20 volumes dimethylsulphoxide (Me_2SO) for 2 changes, 20 minutes each, followed by immersion in 4 volumes 22.2% (w/v) PPO-Me_2SO for 3 hours with shaking. The gels were rehydrated by washing in H_2O for at least 1 hour, then dried onto filter paper. Although the rehydration step causes precipitation of the PPO inside the gel matrix, which results in the gel becoming opaque, the fluorographic efficiency does not appear to be reduced (Bonner and Laskey, 1974). To improve the sensitivity of fluorography, Kodax X-omatic H X-ray film was flashed from a height of $8\frac{1}{2}$" with a Sun-Pak GT 32 flash gun (Flash duration 1/1000 second) through an Ilford S 902 filter covered with a piece of Whatman No 1 filter paper. The gel was placed against the sensitized side of the film, sandwiched as before and left at -100°C for 5 weeks. This procedure sensitizes the film by the production of a silver atom from a silver ion. The low temperature decreases the

rate of thermal reversion of the silver atom before it is stabilized by the formation of a second atom from the emission of β-particles from the radioactive material.

SECTION C. EXPERIMENTAL TECHNIQUES

1. Preparation of culture media

The composition of the mineral salts medium for the culture of artichoke tuber tissue was as follows, slightly modified from Yeoman et al, (1965) by the absence of phosphate. This nutrient was omitted to enhance the uptake and incorporation of ^{32}P-orthophosphate in some experiments.

Solution	Component	Final Concentration
A	$MgSO_4 \cdot 7H_2O$	0.146 mM
	KNO_3	0.800 mM
	KCl	0.880 mM
B	$Ca(NO_3)_2 \cdot 4H_2O$	1.0 mM
	$FeCl_3 \cdot 6H_2O$	0.004 mM

A and B solutions were stored at 100 times final concentration and diluted accordingly. Sucrose was added to 4% (0.117 M) final concentration Two conditions of culture were employed in this study; one in the presence of the auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), which promoted cell division and one without, where division was essentially absent. 2,4-D was dissolved in ethanol, then made to 10^{-3} M with distilled water. This stock solution was diluted 1000 fold to give a final concentration of 10^{-6} M.

Culture medium and distilled water were autoclaved at 15 psi for 15 minutes.

2. Sterile culture of the tissue

Tubers, showing no signs of surface damage or infection were scrubbed

to remove adhering soil and surface-sterilized by immersion in a solution of sodium hypochlorite containing 2% (w/v) available chlorine for 30 minutes. The tubers were transferred to the sterile room and washed thoroughly with sterile distilled water. The tissue was prepared under normal fluorescent lighting conditions, but transfer to the culture media was carried out in low intensity green light (Ilford bright green safe light < 1 ft-c, Filter No 909) since the inhibitory effect of light on cell division (Fraser, 1968) is only observed after the addition of 2,4-D and during the first 12 hours of growth, (Davidson, 1971).

Two methods of culture were used during this study. Tissue prepared as explants was used in the study of ribosomal RNA maturation in auxin treated and non-treated tissue. Tissue in the form of discs was used in the experiments on protein changes associated with the initiation of cell division in the artichoke tuber. The two will be dealt with separately as Methods I and II, respectively.

3. Method I: Experiments on rRNA maturation

(a) Preparation of explants

The ends of the artichoke tubers were cut off leaving a segment about 3 cm long from which cylindrical explants (2 mm diameter x 2.4 mm long) were removed as described in detail by Gore, (1972) and Hepburn, (1974). The tissue explants were randomized and approximately 150 explants were cultured in 15 ml of sterile sucrose-mineral salts medium with or without 2,4-D. The tissue was continuously revolved at 2.5 rpm in 600 ml bottles in the dark at 25°C.

(b) Labelling of explants with radioisotopes

Sterile ^{32}P-orthophosphate was injected into culture bottles to a final concentration of 50 µCi/ml in 0.1 µM phosphate, pH7.0. The same

technique was followed for pulse labelled cultures except the isotope was added in the absence of carrier and the chase was effected by the aseptic removal of the explants to sterile medium containing 1 mM phosphate, pH 7.0. In those experiments dealing with nucleotide pool sizes, (G-^{3}H) adenosine was used at a final concentration of 20 uCi/ml in the absence of carrier.

(c) Preparation of nucleic acid

The following solutions were prepared.

1. Kirbys grinding buffer: 1% sodium tri-isopropylnaphthalene sulphonate (TNS), 6% sodium 4-aminosalicylate (PAS), 50 mM Tris-HCl, pH 8.0 with 50 mM NaCl with enough phenol mixture to dissolve the PAS (Parish and Kirby, 1966 ; Jackson and Ingle, 1973).

2. Phenol mixture: phenol containing 10% redistilled m-cresol and 0.1% 8-hydroxyquinoline, saturated with 50 mM Tris-HCl, pH 8.0 (Parish and Kirby, 1966 ; Jackson and Ingle, 1973).

To facilitate the accurate timing of radioactive labelling and the processing of many samples, the ^{32}P-orthophosphate labelled tissue was frozen in solid CO_2 at -80°C. Tissue was ground to a powder in a pestle and mortar and homogenized in 3 to 5 ml/g fresh weight of Kirby's. An equal volume of phenol mixture was added and shaken vigorously to effect deproteinization. The phenol and aqueous phases were separated by centrifugation at 1200 x g for 10 minutes. After the phenol layer was removed the aqueous phase together with the interface were made 0.5 M NaCl to maintain ionic strength and to depress the solubility of phenol in the aqueous phase; and this was re-extracted with an equal volume of phenol mixture. After centrifugation a third extraction of the aqueous phase was accomplished and the nucleic acids were recovered from the aqueous phase by precipitation with two volumes of ethanol for

several hours at 0°C. The precipitated nucleic acids were collected by centrifugation as before, washed 2 x with 8 ml 80% ethanol containing 0.2% sodium lauryl sulphate (SLS) and dissolved in 0.15 M sodium acetate, 0.5% SLS (Jackson and Ingle, 1973). This was followed by reprecipitation in 2 volumes of ethanol for several hours at 0°C.

In some experiments purification of ribosomal RNA was attempted by salt precipitation from the total nucleic acid preparations (Parish and Kirby, 1966). The washed sample was dissolved in 0.1 M sodium acetate to an estimated 1 mg/ml and solid NaCl added to 3.0 M final concentration. After precipitation for 24 hours at 0°C the rRNA was collected by centrifugation at 1200 x g for 20 minutes. The pellet was washed 2 x with 3.0 M sodium acetate, pH 6.0 to remove NaCl and residual DNA. This procedure specifically removes the highly polymerized DNA, but in addition the soluble RNAs and inulin, a low molecular weight storage compound found in the artichoke, are also reduced. Inulin co-precipitates with rRNA and hinders entry of the RNA into polyacrylamide gels by precipitating on top of the gel.

(d) Quantitative measurement of RNA synthesis

In the previous experiments the synthesis of rRNA was measured by the incorporation of ^{32}P-orthosphosphate. It is important to realize that the rate of incorporation of label into macromolecules represents rates of flow of the radioactive precursor through various pools of intermediates into the macromolecules and are not equal to rates of synthesis. The exogenous label is diluted depending on cell permeability, size of precursor pools, the rate of turnover of the pool, expandibility of the pools, etc. The assumption that these parameters are similar in treated tissue and non-treated tissue and even between different ages in the same culture might not be valid. Therefore, an alternative technique, which is not complicated by such variables was used and

compared to the results obtained from the $^{32}P_i$ experiments. In this method, the specific activity of ATP, a direct precursor to the macromolecules was determined and related to the amount of label incorporated. Measurements were carried out according to Emerson and Humphreys, (1971) and as outlined by Humphreys, (1973).

(i) Kinetics

In this method the metabolic conversion of labelled adenine to the macromolecules is simplified in the following way. The rate of change of labelled adenine in RNA is dependent upon two factors; firstly, the rate of incorporation of labelled material into RNA and secondly, the rate of loss of labelled adenine from RNA. Adenine is incorporated from ATP into RNA and the rate of incorporation of labelled material = rate of synthesis of RNA x the specific activity of ATP. Likewise, the rate of loss of label from RNA is equal to the rate of degradation of RNA x the specific activity of adenine in RNA. Therefore the following symbols may be used:

R = amount of RNA

R^* = radioactivity as cpm in RNA

A = amount of ATP

A^* = radioactive ATP

$S_R = (R^*/R)$ = specific activity of adenine in RNA

$S_A = (A^*/A)$ = specific activity of ATP

V_S = rate of synthesis of adenine into RNA from ATP

V_D = rate of degradation of RNA or loss of adenine from RNA

dt = small time interval

and the rate of movement of labelled molecules represented by :

$$\frac{dR^*}{dt} = S_A \times V_S = S_R \times V_D$$

The Humphreys method relies on the assumption that at short time intervals the specific activity of ATP (S_A) is much greater than the specific activity of RNA (S_R) and that the rate of synthesis of RNA (V_S) is much greater than the rate of degradation (V_D). This would then eliminate part of the equation and the rate of change of labelled RNA is only dependent on the rate of synthesis and the specific activity of the direct precursor to the macromolecule, ATP.

Therefore, $\frac{dR^*}{dt} = S_A \times V_S$

By definition: $S_R = R^*/R$; and $R^* = S_R \times R$

By differentiating the equation:

$$\frac{dR^*}{dt} = S_R \times \frac{dR}{dt} + R \times \frac{dS_R}{dt} = S_A \times V_S$$

But $S_R \times \frac{dR}{dt} = 0$ over a short time interval

Therefore, $\frac{dS_R}{dt} = S_A \times V_S/R$ ($V_S/R = K_S$, the rate constant of synthesis

Therefore, $\frac{dS_R}{dt} = S_A \times K_S$

To obtain the rate constant of synthesis at a single point requires the tangent or the slope of the $S_R \times t$ curve (change in the specific activity of RNA with labelling time) and the specific activity of ATP at the time in question. Then, by multiplying the rate constant, K_S by the amount of RNA a measure of the synthesis of RNA is obtained.

Therefore, $K_S \times R$ = rate of synthesis of RNA

A detailed study of metabolic turnover rate using isotopes may be found in Reiner, (1953).

(ii) Estimation of the nucleotide triphosphate precursor pool

The following solutions were prepared.

1. Acid washed activated charcoal: activated charcoal was washed 3 times in 0.1 N HCl, 3 times for 30 minutes at 37°C in 0.1 N NH_4OH in 50% ethanol and 3 times in water, then made to a concentration of 100 mg/ml.

2. Two sets of standards: 250 µg/ml each of GTP, ATP, dATP and 250 µg/ml each of GMP and ADP in H_2O.

3. Chromatography solvent: H_2O : isobutyric acid : NH_4OH (33 : 66 : 1) prepared fresh each time.

Tissue labelled with (G-^{3}H) adenosine was stored frozen in liquid nitrogen at -195°C pending collection of all the samples in a time course. A perchloric acid (PCA) soluble nucleotide sample from 20 explants was prepared by homogenization in a pestle and mortar with 2 ml 0.5 N PCA at 0°C. A volume of 0.25 ml of an acid washed activated charcoal suspension was added to each nucleotide extract and to the standards. Nucleotides bind strongly to activated charcoal at neutral or acid pH while many other compounds, including polynucleotides and salts do not (Tsuboi and Price, 1959). The tubes were allowed to sit on ice 5 minutes, swirling periodically. The charcoal and adsorbed nucleotides were sedimented by centrifugation at 2100 x *g* for 5 minutes at 0°C. The supernatant was removed and the charcoal washed a total of 5 times with cold distilled water, especially to ensure removal of the salts which would interfere with chromatography. The nucleotides were eluted from the charcoal during a 30 minute incubation in 0.5 ml of 0.1 N NH_4OH in 50% ethanol at 37°C. Since the nucleotides are only partially removed from the charcoal by alkali a yield of 50% is considered maximal. The charcoal was removed by an initial centrifugation at 2100 x *g* followed by a 10 minute centrifugation at 25,000 x *g*. The washed nucleotide sample was then air dried and finally suspended in 10 µl of 0.1 N NH_4OH for

application on thin-layer. The chromatograms were developed with the solvent and after the plate was dry the standard spots were located by UV light and marked. The midpoint of the ATP spot was noted on the samples and the slower half used for the ATP assay. The sample ATP was eluted from the plate by vigorous shaking of the spot in 0.5 ml of cold 0.4 M glycylglycine buffer, pH 7.4. The plastic strips were removed and the suspension centrifuged to remove the cellulose.

The supernatant was used for the ATP assay. For this assay the highly specific and sensitive luciferin-luciferase method was used which relies on the emission of one photon of light for each ATP molecule present (Emerson and Humphreys, 1971 ; Stanley and Williams, 1969). The amount of ATP in each sample was calibrated against a set of standards containing 1 to 50 pmoles of ATP (Figure 3). Approximately 10 to 30 µl of each sample was added to a scintillation vial containing 0.9 ml glycylglycine buffer, and 0.1 ml of firefly lantern extract was pipetted in and swirled quickly to mix. The vial was immediately placed in the scintillation counter and six consecutive 0.1 minute counts were taken. A model 526 Packard Tri-Carb liquid scintillation counter was set for optimum tritium efficiency and used out-of-coincidence since the events to be measured were simply single photons (Stanley and Williams, 1969). Radioactivity measurements of the ATP samples were made in Triton-toluene scintillant as described in Section (B-4-b-i).

(iii) Radioactive precursor (ATP) incorporation into nucleic acid

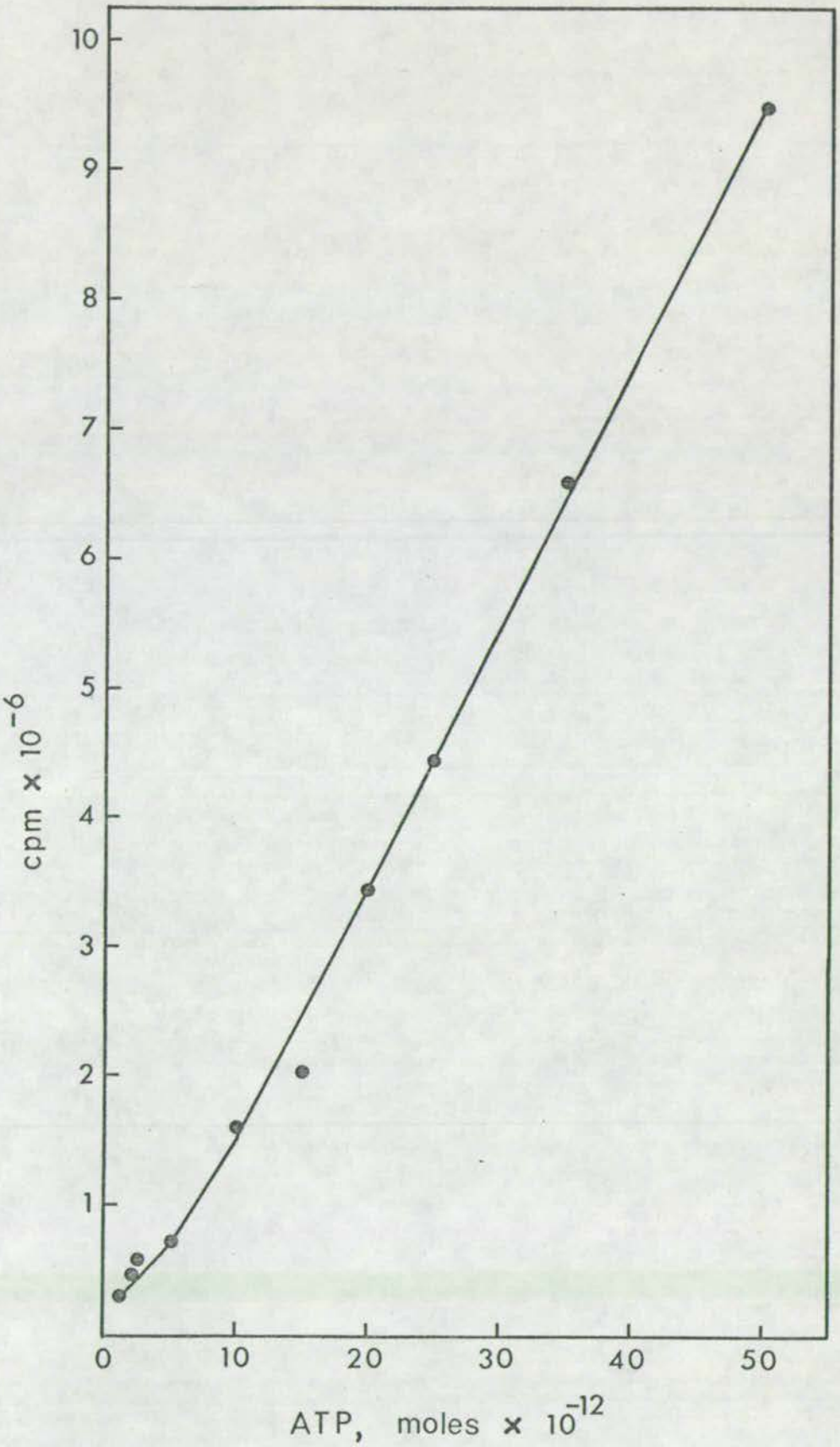
To determine the amount of radioactive AMP in RNA, the pellet from the PCA extraction, which contains the nucleic acids, was washed 2 times with PCA to remove all free nucleotides and hydrolyzed in 2 ml of 0.3 N NaOH at 37°C. Under these conditions all of the RNA in the tissue should be extracted after 1 hour but not degraded quantitatively to

FIGURE 3.

Luciferin-luciferase standard curve for ATP determination

(Method as in Humphreys, 1973)

FIG. 3



mononucleotides (Hutchison and Monro, 1961). Therefore, after 90 minutes a 1.0 ml sample was removed at which time too, the solubilization of protein is minimal and does not interfere with the ultra-violet measurement of ribonucleotides. The following procedures were carried out on the short term hydrolysis sample while the remaining 1.0 ml was left a further 18 hours to ensure complete hydrolysis of the RNA.

1. A 0.1 ml aliquot was taken to determine total radioactivity in the sample.

2. An estimate of the tritium exchanged during alkaline hydrolysis was required since the tritium atom in the 8-position is alkali labile and exchanges with H_2O whereas the 2-position is more stable (Shelton and Clark, 1967). For this purpose, an anion-exchange resin, Dowex-formate was used. A 25% Dowex-1 formate (w/v) suspension was prepared by washing Dowex-1-x 8 chloride with 2.0 N formic acid until all the chloride was removed as tested by precipitation with $AgNO_3$, then washed with H_2O. Dowex formate quantitatively binds mono and polynucleotides leaving behind the nucleosides as well as the tritium which has exchanged with H_2O (Lomax and Greenberg, 1967). An aliquot of 0.2 ml was added to an equal volume of Dowex formate and the slurry was centrifuged at 800 x g for 2 minutes and the supernatant counted for soluble radioactivity.

3. An equal volume of 15% trichloroacetic acid (TCA) was added to a 0.3 ml aliquot. The alkali stable DNA was precipitated at $0^{\circ}C$ for several hours and the precipitate collected on GF/C filters.

4. The alkali solubilized radioactivity and A_{260} in RNA was determined by the removal of a final 0.3 ml sample to an equal volume of 1.5 N PCA. After cooling on ice the sample was centrifuged at 25,000 x g for 10 minutes. The supernatant was counted for PCA-soluble or counts in

RNA. After correction for the percent exchange of tritium the amount of label in RNA and DNA per explant was calculated.

The remaining 1.0 ml volume of alkali digest, left overnight at 37°C to ensure complete hydrolsis of the RNA, was used to determine the relative amounts of AMP and GMP in RNA. This value must be known since ^{3}H-adenosine is converted into guanosine which is also incorporated into RNA. As only the specific activity of the ATP pool is to be measured, only the radioactivity in adenine in RNA must be included in the calculations. Firstly, an aliquot was removed for a total radioactivity measurement. To the remaining 0.8 ml of hydrolysate 0.2 ml of 3.0 N PCA was added, cooled 10 minutes and centrifuged at 25,000 x g for 10 minutes to remove DNA and cell debris. A 0.2 ml volume of acid washed activated charcoal suspension was added to the supernatant, let stand 5 minutes then the charcoal and the adsorbed nucleotides sedimented by centrifugation at 800 x g for 5 minutes. Before aspirating off the supernatant a 0.1 ml volume was removed for counting to determine the fraction of tritium exchanged. The charcoal was then washed 4 times in cold H_2O and the nucleotides eluted in 0.5 ml of 0.1 N NH_4OH in 50% ethanol. The charcoal was removed as before and the supernatant air dried. The nucleotide sample was taken up in 10 µl of 0.1 N NH_4OH, spotted on a cellulose thin-layer chromatogram and developed as previously described. The chromatogram was dried and the spots viewed and marked under UV light. The AMP and GMP spots were cut out and counted to determine the percentage radioactivity in RNA that was due to AMP.

In all cases, the extra acid in 15% TCA, 1.5 N PCA, or 3.0 N PCA was to neutralize the base and bring the final concentration of acid to about 0.5 N. All radioactivity measurements were made as described in Section (B-4-b-i). Correction for efficiency of counting was made

using an internal standard.

(iv) Calculations

To obtain a direct measure of the amount of RNA accumulated from the time when radioactive adenosine was added to the explants the following calculations were made (Humphreys, 1973).

1. The specific activity of ATP in each time sample was expressed as counts/minute/mole x 10^{-12}, which was subsequently converted to counts/minute/µg by dividing by 330, the molecular weight of adenine nucleotide.

2. Total cmp = 10 x 0.1 ml of 90 minute hydrolysis.

3. ^{3}H exchange = 10 x Dowex-soluble cpm.

4. $$\text{DNA} = 3.3 \times \text{TCA ppt cpm} + \left(^{3}H \text{ exchange} \times \frac{3.3 \times \text{TCA ppt cpm}}{\text{total cpm} - {}^{3}H \text{ exchange}}\right)$$

5. $$\text{RNA} = 20 \times \text{PCA-soluble cpm} - \left(^{3}H \text{ exchange} \times \frac{3.3 \times \text{TCA ppt cpm}}{\text{total cpm} - {}^{3}H \text{ exchange}}\right)$$

6. $$\mu g \text{ RNA/explant} = \frac{A_{260} \times \text{volume} \times \text{dilution factor} \times 37\ \mu g/OD \times 13.32}{\text{No. of explants}}$$

7. $$\text{Fraction of cpm in AMP} = \frac{\text{AMP cpm}}{\text{GMP cpm} + \text{AMP cpm}}$$

The counts/minute in DNA and the counts/minute in AMP in RNA were then calculated by dividing the radioactivity in DNA and RNA by the number of explants and multiplying the latter by the fraction which was in AMP. By dividing the radioactivity in RNA by the µg amount of RNA in an explant at that time, the specific activity of AMP in RNA was found and expressed as counts/minute/µg. A specific activity curve for RNA was drawn up from these values and the change in the specific activity at each sampling time was calculated by drawing the tangent to the curve at that point. This rate of change, expressed as counts/minute/hour was divided by the appropriate specific activity of ATP in counts/minute/µg, to give the rate constant of synthesis, K_S, in hours $^{-1}$. This value was then multiplied by the amount of RNA in µg, to give the change in

RNA in µg/hour for the period of time in question.

4. Method II: Investigation of the initiation of cell division

(a) Preparation of discs

The ends of the artichoke tubers were cut off and using a sterilized 7.0 mm diameter cork borer, cylinders were cut out and placed in sterile distilled water. The cylinders were then sliced into 1.1 mm segments with a metal instrument which held a series of razor blades and the discs were placed in a small volume of sterile media lacking 2,4-D while the rest were collected. The discs, measuring 1.0 mm thick x 6.9 mm diameter were randomized and approximately 10 discs/ml media were cultured in the presence or absence of the auxin, 2,4-D, also in the dark. Here, however, phosphate (KH_2PO_4) was included in the medium to a final concentration of 9.0×10^{-7} M. An additional modification was to increase the concentration of 2,4-D to 10^{-5} M which Yasuda et al, (1974) considered as optimal for the disc system.

(b) Labelling of discs with isotopes

Discs were aseptically removed to sterile petri dishes containing fresh media plus the particular isotopes. ^{32}P-orthophosphate was used at a final concentration of 100 µCi/ml without additional carrier phosphate Labelling with carrier free ^{35}S-methionine was effected at a concentration of 80 µCi/ml, and 200 or 100 µCi/ml when chase conditions were to be employed. For the chase, the discs were aseptically removed from the radioactive methionine and washed thoroughly with sterile distilled water followed by sterile media. The discs were then removed to a sterile media containing 10^{-5} M methionine, 30 or 60 times the concentration of the isotope. In the majority of experiments, DNA synthesis was monitored by the inclusion of sterile (CH_3-^{3}H) thymidine at 10 µCi/ml in

1 µM carrier. RNA synthesis was followed by labelling the tissue with 50 µCi/ml carrier free (G-^{3}H) uridine.

(c) TCA extraction of DNA and total protein

Consequent with each labelling experiment, between 5 and 10 discs were removed and frozen for TCA extraction of proteins and nucleic acids (Schmidt and Thannhauser, 1945 ; Hutchison and Monro, 1961). This was done specifically to determine the onset of S phase by the measurement of the incorporation of tritiated thymidine into DNA. The discs were ground in a pestle and mortar in 3 to 5 ml cold 5% TCA and made up to 10 ml. The homogenate was left on ice overnight to precipitate the macromolecules and must be kept cold since DNA could be attacked by the acid with loss of purine bases. The resulting apurinic acid remains acid-insoluble but is alkali labile so would appear with the RNA fraction. The precipitated macromolecules, along with the cell debris, were then sedimented by centrifugation at 2500 x g for 15 minutes and the pellet was washed 2 times with cold ethanol : ether (3:1) to dissolve the lipids. Approximately 2 to 3 ml 0.1 N NaOH was added to the washed pellet and left overnight at room temperature to dissolve the protein and nucleic acids. As RNA is alkali labile it is hydrolyzed but, as a word of caution, prolonged incubation could also extract protein and deaminate cytidylic acid.

Cell debris was removed by centrifugation as before and an aliquot of the supernatant removed for the Lowry estimation of protein content, (Lowry et al, 1951), as described in Section (B-2). DNA was precipitated from the remaining supernatant with cold 10% TCA overnight at 0°C and the amount of DNA estimated by Burtons diphenylamine reaction (Burton, 1956) outlined in Section (B-3). The TCA washed homogenate, prior to alkali treatment was not used

directly for DNA estimation since the bulk of the cell debris hindered hydrolysis and resulted in low readings for DNA.

(d) Isolation of nuclei; several methods

Various methods of preparing nuclei were assessed in order to obtain a high yield with minimal loss of nuclear components and minimal contamination with cytoplasmic ribonucleoprotein.

(i) Three methods with large amounts of tissue

1. 10g of frozen tissue, prepared as 1 mm slices was ground to a powder in a pestle and mortar and transferred to a conical glass-in-glass homogenizer. The powder was suspended in 100 ml sucrose buffer (0.3 M sucrose, 50 mM KCl, 10 mM MgCl, 50 mM Tris-HCl, pH 8.0) containing 1% 2-mercaptoethanol, by a single plunge of the pestle. The homogenate was filtered through two layers of nylon mesh (20 micron over 40 micron) and the debris washed with an equal volume of buffer. The nuclei were sedimented by centrifugation at 500 x g for 5 minutes. The nuclear pellet was washed once with cold buffer and the supernatant from the initial centrifugation further centrifuged at 2500 x g for 10 minutes to obtain an estimate of yield from the low speed separation.

2. Sucrose buffer plus 1% 2-mercaptoethanol was made 40% glycerol (modified from Chen et al, 1975). Although the presence of glycerol would stabilize enzymes there is the risk of nuclear damage due to osmotic shock as the viscosity at this concentration is equivalent to 1.0 M sucrose. As a preliminary experiment, tuber tissue was sliced to 1 mm thickness and 10.0 g each was homogenized for $\frac{1}{2}$ minute and 1 minute in 100 ml glycerol buffer in a Polytron at setting 5 in the cold. The homogenate was filtered through nylon mesh and the debris washed with a further 100 ml of buffer. The filtrate was centrifuged at 1000 x g for 10 minutes and the pellet washed with 30% glycerol

buffer while the supernatant was re-centrifuged at 2500 x g for 20 minutes to obtain a value for yield. In a second experiment, 150 g of tuber tissue was sliced and homogenized for 2 minutes in 300 ml glycerol buffer as before. The homogenate was filtered and the debris washed with an equal volume of buffer. The filtrate was divided into two aliquots, one centrifuged at 1000 x g for 10 minutes and the other at 2500 x g for 20 minutes. The nuclear pellets from each were further separated into 4 "wash" samples: (a) resuspension in 40% glycerol, (b) resuspension in 30% glycerol, and both centrifuged at 2500 x g for 10 minutes, (c) 2 samples resuspended in sucrose buffer but one sedimented at 800 x g for 20 minutes and the other for 5 minutes.

3. Approximately 10 g of sliced tissue was frozen and powdered in dry ice in a Waring Blender for increasing lengths of time ($\frac{1}{2}$ minute, 1 minute, 2 minutes). The powder was transferred to a chilled beaker and stirred with 50 ml cold sucrose buffer containing 1% 2-mercaptoethanol. The homogenate was filtered as before and two fractions collected as described in the First Method.

Not only do all of the above methods require large amounts of tissue but the yield of nuclei as determined by DNA estimation, and presented in Table (1), was very low. The yields are based on a level of 24 pg of DNA/telophase nucleus (Ingle et al, 1976), which is equivalent to 2.4 µg/disc or approximately 40 µg/g fresh weight. Since the majority of experiments could only be done with small, 1 to 2 gram amounts of tissue these methods proved useless. Consequently, a method of chopping the tissue (Spencer and Wildman, 1964) in Honda medium (Honda et al, 1966) was developed.

i) Honda medium

4. The composition of Honda medium used was as follows: 5.0 g/100 ml

TABLE 1.

Yields of the various methods used to isolate nuclei from artichoke tuber cells

The nuclei were isolated as described in the Methods, (C-4-d-i and ii); with the essential details presented in the Table. The first 3 techniques were suitable for large (> 10 g) amounts of tissue only. They involved grinding the artichoke tissue in a pestle and mortar, polytron homogenizor, or Waring blender. The final method of chopping the tissue (Spencer and Wildman, 1964) in Honda medium (Honda et al, 1966) was particularly useful for the smaller, 1 to 2 g amounts of tissue required in subsequent experiments.

TABLE 1.

METHOD	AMOUNT OF TISSUE (GRAMS)	HOMOGENIZATION DETAILS	FRACTIONATIONS	YIELD (µg/g fwt)	TOTAL YIELD (µg/g fwt)	% YIELD
sucrose buffer	10 g	pestle & mortar	500 x g pellet 2500 x g pellet	0.45 0.45	0.9	2.25
sucrose buffer + 40% glycerol	10 g	polytron; setting 5 ½ minute	1000 x g pellet 2500 x g pellet	0.65 0.65	1.3	3.25
		polytron; setting 5 1 minute	1000 x g pellet 2500 x g pellet	0.65 1.05	1.7	4.25
	150 g	polytron; setting 5 2 minutes	1000 x g pellet washes 40% glycerol 30% glycerol (800 g x 5) .3M sucrose (800 g x 20) .3M sucrose	 3.65 2.45 2.35 3.2	2.91 (= 25.6%)	7.28
			2500 x g pellet washes 40% glycerol 30% glycerol (800 g x 5) .3M sucrose (800 g x 20) .3M sucrose	 12.59 8.96 5.55 6.61	8.43 (= 74.4%)	21.08

TABLE 1. (continued)

METHOD	AMOUNT OF TISSUE (GRAMS)	HOMOGENIZATION DETAILS	FRACTIONATIONS	YIELD (µg/g fwt)	TOTAL YIELD (µg/g fwt)	% YIELD
Waring Blender	10 g	½ minute	500 x g pellet 2500 x g pellet	6.4 5.0	11.4	28.5
		1 minute	500 x g pellet 2500 x g pellet	1.4 2.2	3.6	9.0
		2 minutes	500 x g pellet 2500 x g pellet	0.8 1.0	1.8	4.5
Honda medium	5 g	chopping	1000 x g for 10 minutes	-	15.6	39.0
		chopping	1000 x g pellet (5 min) 1000 x g super	15.2 3.2	18.4	46.0

Dextran 40, 2.5 g/100 ml 400,000 molecular weight Ficoll, 0.25 M sucrose, 0.005 M $MgCl_2$, 0.05 M Tris-HCl, pH 7.4. Approximately 10 to 30 discs or 1 to 2 g of tuber tissue was suspended in 2 to 3 ml cold Honda medium containing 2% 2-mercaptoethanol. The discs were chopped for 10 minutes with a razor blade in a petri dish in the cold as described by Spencer and Wildman, (1964). An equal volume of Honda medium was added to bring the reducing agent to 1% and the homogenate was filtered through a single layer of 20 micron nylon mesh. The cell debris was washed with 4 volumes of Honda medium made 1% 2-mercaptoethanol and the nuclei sedimented by centrifugation at 1000 x g for 5 minutes. The nuclear pellet was resuspended in 10 to 15 ml of cold Honda medium containing 1% Triton X - 100 to remove membranous material and similarly centrifuged. A final wash with Honda medium completed the procedure.

Although this technique resulted in yields of up to 40% from 5 g of tissue, yields of 25 to 30% were more common in subsequent experiments where only 2 g samples could be used. In any nuclear isolation it is important to have an estimate of the degree to which the final nuclear preparation is contaminated by the other cell components. I have used light microscopy for this analysis, although electron microscopy and chemical and enzymatic tests would perhaps have revealed more. It was felt, however, that at this level of investigation and because of the slight variations with each extraction rigorous analysis was not required. Accordingly, Figure 4 (A, B, C) shows photographs of the nuclei under phase in each of the purification steps in the isolation of artichoke nuclei by the Honda medium method. The initial 1000 x g pellet, shown in the top photograph, was highly contaminated with cellular debris. This was reduced by subsequent washings, first with

FIGURE 4.

Phases of purification of artichoke nuclei by low speed centrifugation in Honda medium

Approximately 2 grams of tuber tissue was chopped in 3 ml cold Honda medium as described in the Methods, (C-4-d-ii). Following filtration to remove cell debris, the nuclei were sedimented by centrifugation at 1000 x g for 5 minutes. The pellet was resuspended in Honda medium containing 1% Triton X-100 and similarly centrifuged. As a final purification step the nuclear pellet was resuspended in Honda medium and again centrifuged at 1000 x g for 5 minutes. All procedures were carried out in the cold. Photographs of the nuclei at each stage of the purification were taken under phase with a Zeiss microscope.

A. Initial 1000 x g nuclear pellet — Magnification 125 x

B. Nuclei after Triton wash — Magnification 125 x

C. Nuclei after final wash — Magnification 125 x

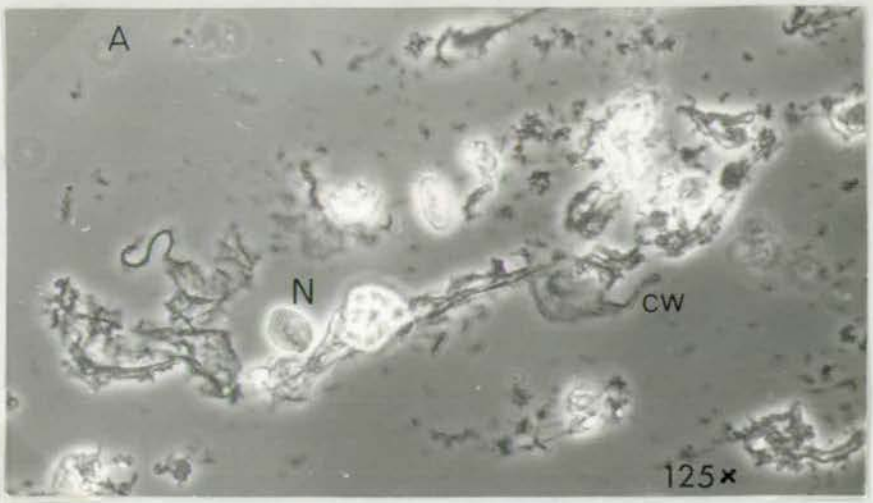
Magnification 400 x

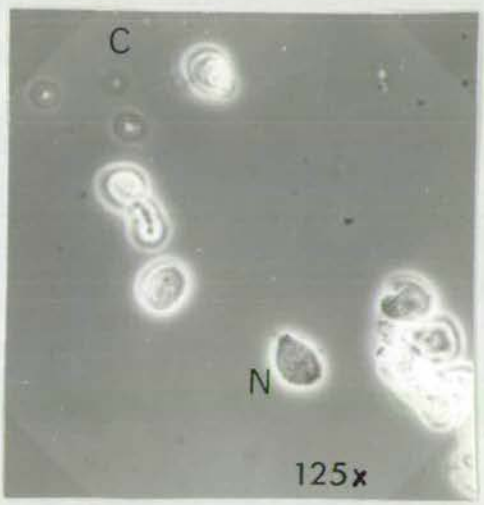
cell wall debris - cw

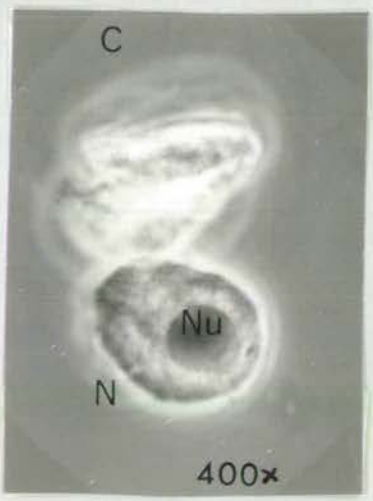
nucleus - N

nucleolus - Nu

FIGURE 4.







Honda medium containing 1% Triton (Figure 4, B) to dissolve the membranous material and finally with Honda medium alone (Figure 4, C). The final photograph shows that the nuclei were quite intact, with very distinct nucleoli and no shrinkage, resembling the in vivo state. Occasionally, extensions of the outer nuclear membrane were observed indicating that the solubilization of the membranes by Triton treatment was not absolute. Although some contamination from membrane proteins remained a possibility I felt it would be insignificant compared to the enrichment of pure nuclei. Further purification could only have resulted in a loss of yield, not only in terms of quantity of intact nuclei but also through leakage of nuclear componants. Since the extent of leakage of materials from the nuclei during extraction and subsequent purification steps is always uncertain it was important to attain a careful balance of purity, yield and intactness. I believe this state was obtained by the Honda medium method.

(e) Preparation of cytoplasmic proteins

Artichoke tuber discs were routinely frozen in tin foil in dry ice while time course samples were collected. This was in contrast to nuclear preparations, where to maintain high nuclear yield, the discs were extracted fresh. Cytoplasmic proteins from approximately 15 discs were prepared by grinding the frozen tissue to a powder in a pestle and mortar and then homogenized in 5 ml cold sucrose buffer containing 2% 2-mercaptoethanol. The homogenate was filtered through miracloth and centrifuged at 47 K for 3 hours at 4°C to pellet the ribosomes. To the supernatant was added 5 volumes of cold acetone and this was left overnight at -32°C to precipitate the proteins (Barritault et al, 1976). Following

centrifugation at 2500 x g for 15 minutes the protein pellet was washed 2 times with cold 80% acetone.

(f) Sample preparation for 2-D gel electrophoresis

Lysis buffer was prepared as follows and stored frozen in 1 ml aliquots at -32°C.

urea	10.0 M
EDTA (ethylenediaminetetra-acetic acid), Na_2	0.001 M
NaH_2PO_4 (sodium dihydrogen orthophosphate)	0.01 M
2-mercaptoethanol	2.0 %
ampholines (pH 3.5 - 10)	2.0 %

The acetone washed cytoplasmic proteins from 3 to 4 discs were solubilized in lysis buffer and approximately 120 µg of protein was applied to each gel in a volume of 50 µl. Nuclei, prepared by the Honda-chop method from 20 to 30 discs of tuber tissue were well suspended in 110 µl of lysis buffer by swirling rapidly on a Whirlimixer (Fisons Scientific Apparatus, Ltd.). The nuclei were then centrifuged at 18,000 rpm for 20 minutes at room temperature to pellet the chromatin. 50 µl of the supernatant, containing 30 to 60 µg of solubilized protein was layered on each first dimension gel.

The reliability of using 10 M urea to extract the nuclear acidic proteins was investigated. Nuclear proteins labelled with ^{35}S-methionine were prepared as above. By comparing the amount of radioactivity in the 10 M urea extract to the amount remaining in the chromatin pellet, recovery from 4 replicates was estimated at 73.5% (Table 2). Since the pellet was not washed and the histones would contain a small proportion of ^{35}S-methionine, this probably represents about 90% of the nuclear acidic proteins.

TABLE 2.

Estimation of the amount of nuclear protein soluble in 10 M urea

Artichoke tissue was cultured in media (23 discs/2 ml) with or without 2,4-D for 0 and 9 hours. At these times the discs were transferred to media containing 200 µCi/ml ^{35}S-methionine plus 10 µCi/ml (CH_3-^{3}H) thymidine in 1 µM thymidine carrier for a 3 hour pulse. The tissue was removed, washed free of excess label and placed in fresh media (23 discs/2 ml) containing 10^{-5}M methionine and incubated to a total culture time of 21 hours. The nuclei from 40 discs were then isolated as described in the Methods, (C-4-d-ii) and following suspension in 120 µl lysis buffer, the chromatin was pelleted by centrifugation, (Methods, C-4-f). The chromatin pellet was taken up in 10% TCA and collected on a GF/C filter for an estimate of radioactivity (Methods, B-4-b-i). A small volume of the supernatant was spotted on a GF/C filter and similarly assayed. The radioactivity remaining in the lysis buffer should give an estimate of the amount of protein soluble in 10 M urea.

TABLE 2.

Cultural State.	Start of 3 Hour Pulse (hours).	Radioactivity in chromatin pellet. (cpm/disc)	Radioactivity remaining in lysis buffer (cpm/disc)	Percent Yield
+ 2,4-D	0	1847.7	4484.1	70.9 %
	9	4988.7	14055.0	73.8 %
- 2,4-D	0	1120.6	3397.6	75.2 %
	9	4615.4	13143.6	74.1 %
			Average =	73.5 %

Gronow and Griffiths, (1971) estimated that two extractions of rat liver nuclei with 8 M urea removed 70% of the total nuclear proteins, but none of the DNA and only one of the histones. Similar results were obtained by Trewavas, (1976) using nuclei from the barley embryo. It may be concluded that a representative sample of the nuclear acidic proteins was obtained by this method.

(g) In-vitro phosphorylation of nuclear proteins

Nuclei were isolated from 40 discs by the Honda-chop method and incubated in 0.5 ml of Honda medium containing 50 µCi/ml (γ -^{32}P) ATP at 25°C for 10 minutes. The reaction was terminated by placing the incubation mixture on ice and adding 20 volumes cold 10^{-3} M ATP in Honda medium. The nuclei were sedimented by centrifugation at 1000 x g for 5 minutes and washed 2 times with 10 ml cold Honda medium. In the final wash, $\frac{1}{4}$ of the nuclear suspension (= 10 discs) was removed and the remainder (= 30 discs) was pelleted by centrifugation and resuspended in lysis buffer as above and layered on the first dimension iso-electric gels. The nuclear sample from 10 discs was also centrifuged and the pellet was taken up in 0.5 ml of 0.1 N NaOH to dissolve the macromolecules. A volume of 0.1 ml was removed for an estimate of radioactivity (B-4-b-i), and the rest was precipitated overnight in the cold by adding an equal volume of 20% TCA. The precipitate was washed once with cold 10% TCA and redissolved in 0.5 ml 0.1 N NaOH. The amount of protein in 0.4 ml was estimated by the Lowry method (B-2) and a second measurement of radioactivity was made.

(h) Protein kinase assay

Protein kinase activity in the nucleus was measured by the transfer of the radioactive γ-phosphate from (γ -^{32}P) ATP to

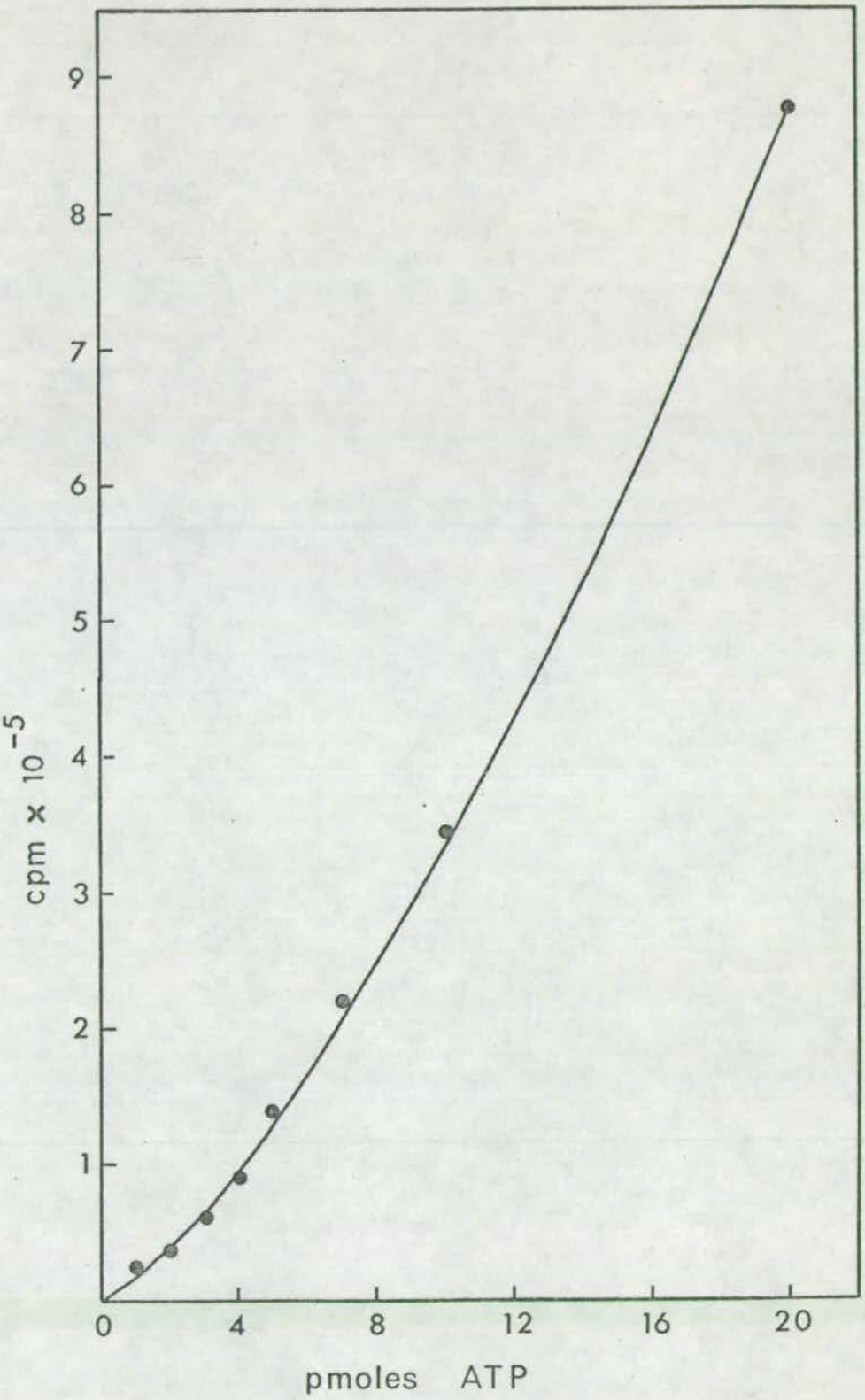
serine and threonine residues of a trichloroacetic acid-insoluble fraction. Casein was used as substrate in the assay since it is readily phosphorylated and exhibits first-order kinetics. When the concentration of casein is high and the enzyme level low the reaction rate is proportional to the enzyme concentration (Reimann et al, 1971). A solution of casein was prepared by suspending the casein in water and heating at 100°C for 10 minutes while maintaining the pH at 9.5 with NaOH. The solution was then cooled and the final concentration of protein adjusted to 30 mg/ml and pH 6.0 by slow addition of 1 N HCl (Reimann et al, 1971). (γ -^{32}P) ATP was prepared by making the freeze dried radioactive ATP to a concentration of 500 µCi/ml with cold distilled water and adding 0.05 ml containing 25 µCi to 0.4 ml of 2 x 10^{-4} M cold ATP (Keates, 1973 ; Keates and Trewavas, 1974). Nuclei were prepared as previously described in Section (C-4-d-ii) from 20 discs and suspended in 0.9 ml of buffer containing 0.3 M sucrose, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.6. A 0.375 ml volume of the nuclear suspension was added to 0.075 ml casein preincubated at 25°C and 50 µl of labelled ATP was added and mixed. After 5, 10, 20, and 30 minutes incubation 80 µl of the reaction mixture was removed to 40 µl of 25 mg/ml Bovine Serum Albumen (BSA) in 0.25 M Na_2EDTA and thoroughly mixed to terminate the reaction. Approximately 4 ml of 10% TCA containing 20 mM sodium pyrophosphate ($Na_4P_2O_7$) was then added to precipitate the macromolecules. A blank containing 0.075 ml of the nuclear suspension was added to 40 µl BSA/EDTA and mixed. 10 µl of labelled ATP and 4 ml 10% TCA/20 mM $Na_4P_2O_7$ were then added. A measure of the effect of endogenous substrate was also done by running a parallel control without added casein as substrate.

FIGURE 5.

Luciferin-luciferase standard curve for ATP determination

A large volume of 0.5 N perchloric acid containing 15% 8-hydroxyquinoline was adjusted to neutrality with 1 M Tris-HCl, pH 8.0 and 1 N KOH was added to remove the acid as the insoluble potassium perchlorate. This was used to make up an ATP standard containing 10^{-7} M ATP and 8×10^{-9} M $MgSO_4$. The standard curve was formed as described by Humphreys, (1973).

FIG. 5



(k) Nuclear ATP levels

Consequent with the measurement of protein kinase activity it was relevant to have some idea of the amount of ATP in the nucleus. 20 discs/2 ml media/plate were incubated for various times up to 24 hours. The nuclei were isolated and ground in 0.3 ml 0.5 N PCA containing 15% 8-hydroxyquinoline at 0°C (Faiz-ur-Rahman, 1969). The chelating agent, 8-hydroxyquinoline, was included to improve the yield by removing Ca^{2+} and Mg^{2+} ions which apparently adsorb phosphate esters to cell wall material (Isherwood and Barrett, 1967). The homogenate was centrifuged at 18,000 rpm for 10 minutes at 4°C to pellet the nucleic acids. After washing the pellet with cold 0.5 N PCA a measure of the content of DNA was made as described in Section (B-3). The 18 K supernatant was adjusted to neutrality with 0.05 ml 1 M Tris-HCl, pH 8.0 and 0.15 ml 1 N KOH was added to remove the acid as the insoluble potassium perchlorate. An ATP standard containing 10^{-7} M ATP and 8×10^{-9} M $MgSO_4$ was carried through the same procedure. ATP content in the samples was estimated relative to the standard curve (Figure 5) by the luciferin-luciferase assay as previously described in Section (C-3-c-ii).

5. General

(a) Polyacrylamide gel electrophoresis of nucleic acids

The preparation of solutions and electrophoretic separation of the nucleic acids were carried out according to Loening, (1967) and Bishop et al, (1967), and as described by Hepburn, (1974). A 5 fold concentration of electrophoresis buffer (E Buffer) was prepared as shown and diluted accordingly: 5 x E = 150 mM NaH_2PO_4,

180 mM Tris base, 5 mM EDTA-disodium salt, final pH 7.6 to 7.8. Stock solutions of acrylamide containing 15% (w/v) recrystallized acrylamide and 0.75% (w/v) recrystallized bis-acrylamide were stored in the dark at 4°C for a maximum of one month.

Dilution of this stock solution with distilled water and 1/5 the final volume of 5 x E Buffer gave the desired gel concentration. The mixture was degassed and 25 µl N,N,N' N' tetramethylethylene-diamine (TEMED) added for each 5 ml of stock acrylamide solution used and mixed by swirling. Polymerization was initiated by the addition of fresh 10% ammonium persulphate at a concentration of 0.25 ml for each 5 ml of stock acrylamide and swirled to mix. The solution was pipetted into perspex gel tubes to a height of 7.5 cm. The dimensions of the gel tubes were 9.0 cm long x 0.6 cm internal diameter which were closed at the bottom by a PVC support ring stoppered with a short length of 2 mm diameter glass rod. To ensure a flat top on the gel, distilled water was carefully layered on the surface. After 1½ hours the polymerization was complete and the glass plugs were removed and the tubes mounted vertically between the two tanks. Each tank contained approximately 400 ml of electrophoresis buffer containing 0.2% sodium lauryl sulphate (SLS). The gels were prerun at 50 V (6 mA/gel) for 30 minutes to remove the catalysts from the gel and to move SLS into the gel.

Drained nucleic acid precipitates were dissolved in electrophoresis buffer containing 0.2% SLS and 10% sucrose to a concentration of 1.0 mg/ml and no more that 50 µg loaded on each gel under a small voltage to counteract diffusion. The nucleic acids were normally fractionated on 2.3% (w/v) gels at 50 V (6 mA/gel) for 3½ hours. Under these conditions a good separation of the

high (25S and 18S) RNAs and their precursors was achieved at the expense of the low (4S and 5S) molecular weight RNA species which ran off the bottom of the gel.

The gels were washed 30 minutes in distilled water prior to scanning at 265 nm in a quartz cell in a Joyce Loebl polyfrac UV scanner. The light source was a medium pressure mercury vapour lamp with a sample wavelength selection (265 nm) by an interference filter and a liquid filter which consisted of a 100 µg/ml solution of p-dimethylaminobenzaldehyde in methanol. The E_{265} was recorded by a servoscribe potentiometric recorder geared for scans of 1x, 2x, and 4x the gel length. The scanning system was calibrated by fractionation of known amounts of RNA which were measured in a Pye Unican SP 800 recording spectrophotometer with an absorption of 25 OD units/mg RNA. The total peak area was determined in terms of the weight of a tracing and related to the amount of RNA fractionated.

When 7.5% (w/v) polyacrylamide gels were required dilution was made from a stock solution containing 15% (w/v) recrystallized acrylamide and 0.375% (w/v) recrystallized bis-acrylamide. The same procedure was then followed.

(b) Polyacrylamide-formamide gel electrophoresis

RNA was also fractionated under denaturing conditions on polyacrylamide-formamide gels by a method from Staynov et al, (1972) which eliminates conformational effects and separates the nucleic acids on the basis of molecular weight only. Formamide treatment is a mild, non-degrading procedure for the denaturation of nucleic acids, requiring only 10 to 15 minutes in the absence of excess heat, water, acid or base (T'so et al, 1962). As commercial

formamide contains traces of formic acid and ammonium formate which would interfere with the polymerization of acrylamide the formamide was purified by two extractions with diethyl ether and the residual ether was removed by rapid stirring overnight. The formamide was then filtered and stored for up to one week in the dark at 4°C. Buffered formamide, for dissolving the nucleic acid sample was prepared by making the purified formamide 20 mM with respect to diethylbarbituric acid and the pH adjusted to 9.0 with 1 N NaOH. 70% buffered formamide was made by dilution with water. Gels, to a final acrylamide concentration of 3.2% were prepared as follows:

recrystallized acrylamide	0.68 g
recrystallized bis-acrylamide	0.12 g
diethylbarbituric acid	0.092 g
TEMED	60 µl
purified formamide	20 ml

The pH was adjusted to 9.0 with 1 N HCl and the total volume made to 25 ml with purified formamide. Degassing of the acrylamide mixture prior to polymerization was not required. The reaction was initiated by adding 0.2 ml of fresh 18% ammonium persulphate and the mixture pipetted into perspex tubes as described in the previous section. The gels were overlaid with 70% buffered formamide to ensure a flat surface. After 30 minutes the polymerization was complete and the 70% formamide overlay was replaced with 100% buffered formamide. The stopper in the bottom of the tubes was replaced by muslin, and the tubes placed in the tank. The nucleic acid was dissolved in 100% buffered formamide containing 10% sucrose and approximately 50 µg in 50 to 80 µl was layered on top of the gel under the formamide overlay. Both buffer compartments were

filled with 20 mM NaCl which was circulated through the two compartments during the course of the run in order to prevent pH changes. The gels were run for 9 to 13 hours at 1.25 mA/gel (50 V) at room temperature. After electrophoresis the gels were washed for 2 to 3 hours in several changes of water to remove the UV absorbing formamide and diethylbarbituric acid, then scanned as before at 265 nm.

(c) Two-dimensional gel electrophoresis of proteins

Separation of cytoplasmic and nuclear proteins was achieved by two-dimensional polyacrylamide gel electrophoresis, modified slightly from O'Farrell, (1975). The high resolution and sensitivity of this technique rests on the separation of the macromolecules by two unrelated parameters. Proteins are separated according to their isoelectric points by isoelectric focusing in the first dimension, and according to molecular weight by sodium lauryl sulphate (SLS) electrophoresis in the second dimension.

(i) Solutions and buffers

The following solutions were stored frozen as 1 ml aliquots for use in the preparation of the first dimension gels.

Solution	Component	Concentration
A (gel overlay)	urea	9.5 M
	Ampholines (pH 3.5-10)	2.0 %
	2-mercaptoethanol	2.0 %
H (gel overlay)	urea	8.0 M
K (sample overlay)	urea	8.0 M
	ampholines (pH 3.5-10)	2.0 %

In addition various buffers and stock acrylamide solutions were prepared and stored at $4^{o}C$.

Solution	Component	Concentration
O (SLS Buffer)	Glycerol	10 % (w/v)
	2-mercaptoethanol	5 % (w/v)
	sodium lauryl sulphate (SLS)	2.5 % (w/v)
	Tris-HCl, pH 6.8	62.5 mM
D (30% stock acrylamide for isoelectric focusing)	acrylamide, specially purified (s.p.)	28.38 % (w/v)
	bis-acrylamide, recrystallized	1.62 % (w/v)
N (30% stock acrylamide for SLS gels)	acrylamide (s.p.)	29.2 % (w/v)
	bis-acrylamide, recrystallized	0.8 % (w/v)
L Buffer for separation gel	Tris-HCl, pH 8.8	1.5 M
	sodium lauryl sulphate (SLS)	0.4 %
M Buffer for stacking gel	Tris-HCl, pH 6.8	0.5 M
	sodium lauryl sulphate (SLS)	0.4 %

(ii) First dimension gels

Iso-electric focusing gels were formed as described by O'Farrell (1975), but with the following modifications in the gel mixture :

urea	5.5 g
D stock	1.33 ml
H_2O	4.0 ml
ampholines (pH 3.5-10)	0.5 ml

The cylindrical iso-electric focusing gels were constructed in silicon treated glass tubes (Section A-3) of the following dimensions; 2.5 mm internal diameter x 130 mm long. Normally gels with a pH gradient of 5 to 7 were formed, in which case a mixture of ampholines was used; 0.4 ml of pH 5 - 7, and 0.1 ml of pH 3.5 - 10. The final concentration of acrylamide was approximately 4%, which had sufficently large pores to offer little restriction on the electrophoretic mobility so that even the proteins of high molecular weight could reach their iso-electric point in the pH gradient formed by the ampholines. The high level of urea at 9.2 M not only denatures and solubilizes proteins, but also confers some degree of mechanical stability to an otherwise weak gel. The electrophoresis buffers were altered from those used by O'Farrell with the lower reservoir containing 0.5% ethanolamine and the upper containing 0.2% sulphuric acid (H_2SO_4). The gels were prerun especially to remove ammonium persulphate, an oxidizing agent. This was done according to the O'Farrell schedule but the entire electrophoretic procedure was carried out from the acidic end, or anode by reversing the electrodes. This prevented migration of the negatively charged nucleic acids into the gels which caused the streaking observed by O'Farrell. Figure 6, (A) demonstrates the streaking effect caused by running

FIGURE 6.

Effect of electrophoretic separation of proteins from the basic end of the iso-electric focusing gel

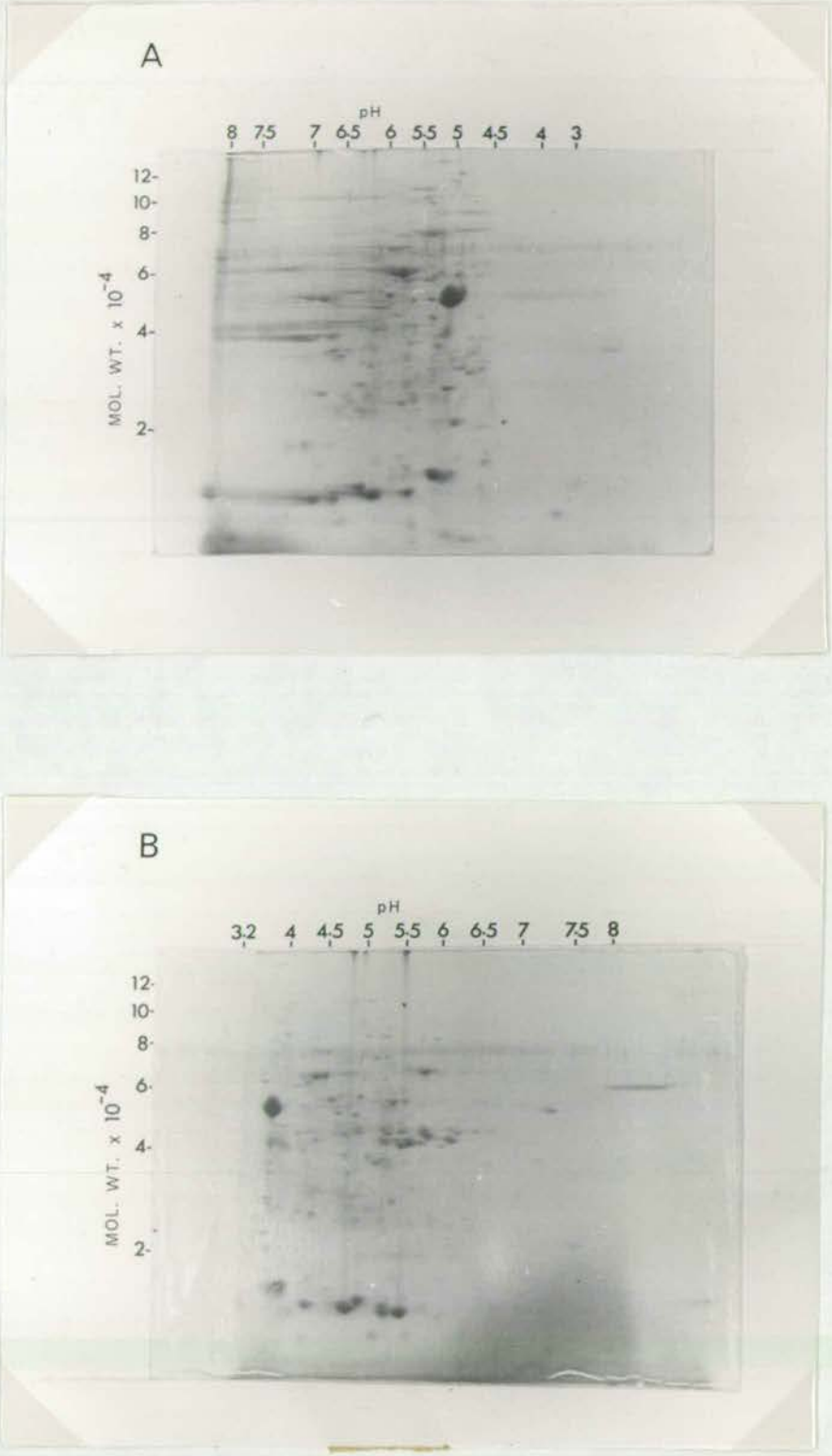
Cylindrical iso-electric focusing gels of pH 3.5 to 10 were constructed as outlined in the Methods, (C-5-c) and described in detail by O'Farrell, (1975). Approximately 150 µg total tissue soluble proteins in 50 µl lysis buffer (Methods, C-4-e) was loaded on each first dimension gel and electrophoresed overnight at 200 V (Methods, C-5-c-ii). Separation in the second dimension was achieved on an 11.25% discontinuous slab gel for $3\frac{1}{2}$ hours at 20 mA (Methods, C-5-c-iii).

These are photographs of slab gels which have been stained and dried onto filter paper (Methods, C-5-c-iv and v). Separation of the proteins on the basis of iso-electric points is in the horizontal direction, while the molecular weight distribution is in the vertical direction. The proteins are evident as stained spots.

A. The iso-electric focusing gels were run with the lower reservoir containing 0.2% H_2SO_4 (anode) and the upper containing 0.5% ethanolamine (cathode). The streaking is probably caused by the migration of the negatively charged nucleic acids through the gel.

B. The iso-electric focusing gels were run with the lower reservoir containing ethanolamine and the upper, H_2SO_4, and the electrodes were reversed. Running the gels from the acidic end prevented this migration and improved separation of the proteins.

FIGURE 6.


A
pH
8 7.5 7 6.5 6 5.5 5 4.5 4 3
12-
10-
8-
6-
4-
2-
MOL. WT. x 10^{-4}
B
pH
3.2 4 4.5 5 5.5 6 6.5 7 7.5 8
12-
10-
8-
6-
4-
2-
MOL. WT. x 10^{-4}

the first dimension gels from the basic or cathode as compared to electrophoresis from the acidic end (Figure 6, B). At the end of the run the gels were routinely equilibrated in Buffer O for $1\frac{1}{2}$ hours (O'Farrell, 1975) and stored frozen pending separation in the second dimension. This procedure removed much of the ampholines, replaced urea with another denaturing agent, SLS, and moved Cl^- ions, which are required for the second dimension, into the gel. The pH gradient for each set of gels was determined by slicing 0.5 cm sections of a blank gel each into a 2 ml volume of degassed H_2O. After standing several hours the contents were mixed and the pH read on an E.I.L. pH meter model 38A.

(iii) Second dimension gels

The focused proteins were further fractionated in the second dimension on the basis of their molecular weight by electrophoresis on a discontinuous SLS slab gel (Laemmli, 1970 ; Ornstein, 1964 ; Davis, 1964 ; Williams and Reisfeld, 1964) as described by O'Farrell, (1975).

A Bio-Rad Model 220 Dual vertical slab gel electrophoresis cell was used and the top of the glass plates were modified to contain a notch as specified by O'Farrell. This apparatus enabled two gels to be run simultaneously and in addition, provided a method of cooling by virtue of its internal water chamber. Resolution of the proteins was thereby improved by effectively slowing down the rate of separation. A 14.5 ml separation gel of dimensions, 0.8 mm x 154 mm x 105 mm, was formed between the glass plates. It was poured as a 10.5% to 15% exponential acrylamide gradient which improved resolution of those proteins in the 20,000 to 50,000 molecular weight range as compared to a 11.25% gel. The separation

gel was overlaid with a 4 ml 4.75% stacking gel to a height of 2 cm and a perspex spacer was placed carefully on the top to effect a flat surface and to allow polymerization to proceed in the absence of oxygen (approximately 30 minutes). Agarose was dissolved in buffer O to a concentration of 1% by heating to 90°C and the solution was then cooled to approximately 50°C. The first dimension gel was allowed to thaw and after removing excess liquid from the top of the stacking gel, the cooled agarose solution was poured in and the cylindrical first dimension gel quickly laid on top. The agarose normally set within 1 minute, securing the iso-electric focusing gel to the top of the slab gel. Electrophoresis was then carried out according to O'Farrell at constant current (20 mA/gel) for approximately 3 hours, or until the bromophenol blue marker dye ran off the bottom of the gel.

Cooling the agarose appeared to reduce a strongly staining double band at 60,000 and 65,000 molecular weight. Although a separate gel was run containing the following four standard markers, this double band conveniently served as an internal molecular weight marker.

lysozyme	14,000
Bovine Serum Albumen	67,000
egg albumen (ovalbumen)	44,000
Ovine Serum Albumen	60,000

(iv) Staining of two-dimensional gels

Following separation of the proteins in the second dimension the slab gels were stained for 30 minutes in 0.1% coomassie blue (Brilliant R), 50% ethanol, 7% acetic acid, with gentle shaking. The gels were destained by 3 changes in 8% glacial acetic acid,

25% ethanol; 40 minutes each, and then washed at least 15 minutes in 5% methanol, 7% glacial acetic acid to remove the ethanol.

(v) Drying of two-dimensional gels

Gels were dried onto a piece of Whatman 3 MM filter paper by a combination of pressure, exerted by a vacuum pump, and heating. A piece of polyethylene was placed onto a metal sheet covering a water bath held at 70°C. The washed gel was placed on top, followed by the filter paper, then 2 pieces of porous polythene (Vyon; Porvair Ltd.) to distribute the pressure. A rubber mat, the size of the metal sheet and joined by a length of high pressure rubber tubing to a vacuum pump, was carefully placed over the entire assembly. The pump was switched on and the pressure created by the vacuum forced the gel firmly into the filter paper. The gel was completely dry after 1½ hours.

PART III

CULTURE OF ARTICHOKE TISSUE

&

PROBLEMS FOR INVESTIGATION

CHAPTER 1. METABOLIC CHANGES ACCOMPANYING THE CULTURE OF ARTICHOKE TISSUE

SECTION A. PATTERN OF THE INCREASE IN CELL NUMBER

Introduction

The induction of DNA replication and subsequent cell division in Jerusalem Artichoke is entirely dependent on the presence of auxin (Yeoman and Mitchell, 1970). When dormant tuber tissue is sliced into a nutrient medium containing the auxin, 2,4-D, vigorous cell division is induced after a 24 hour lag period (Yeoman et al, 1965). The first cell division is almost synchronous with 63% of the cells dividing, and of the cells which do not divide, half are damaged and undergo autolysis and the rest are an inert core which shows low metabolic activity and does not accumulate RNA, DNA or protein (Mitchell, 1967 ; Yeoman and Davidson, 1971). In order to compare the response to auxin of the methods of culture used in this investigation, the explant and the disc, I made cell counts after varying periods of incubation.

Experimental design

(a) 150 explants were cultured in 15 ml of mineral salts media in the presence or absence of 10^{-6}M 2,4-D as described in the Methods, (C-3-a). At the allotted times 5 explants were removed and the cell numbers were determined in 3 ml of 5% chromium trioxide, (Methods, B-1).

(b) In the second method 10 discs were cultured in 2 ml of media, with or without 10^{-5}M 2,4-D (Methods, C-4-a), and a cell number estimate of a single disc was carried out as above. In this

experiment I also monitored DNA synthesis by the incorporation of radioactive thymidine. After 0, 6, 12, 18, 30, and 42 hours of culture the discs were transferred to fresh medium containing 10 μCi/ml (CH_3-^3H) thymidine in 1 μM thymidine carrier and a 3 hour pulse was given. The tissue was extracted with 10% TCA as described in the Methods, (C-4-c) and the acid-insoluble precipitate was assayed for DNA content (Methods, B-3) and radioactivity (Methods, B-4-b-i).

Results

(a) When the explants were sliced into 10^{-6}M 2,4-D there was a 6 to 7 fold increase in the cell number after 120 hours of culture as shown in Figure (7). The completion of the first wave of cell division is evident as a plateau between 36 and 48 hours of culture in curve (1). Synchrony, however, is not complete and only lasts for a few divisions, each division being less synchronous than the previous one (Fraser, 1968). The discrepancy between curves (1) and (2) reflects the effect of storage on the duration of the lag phase before the first wave of cell division. The majority of experiments were carried out between late December and April when the lag phase was fairly constant at 22 to 24 hours (curve 1). After this it gradually increased and became more variable until times of up to 50 hours were not uncommon (Evans, 1967 ; Yeoman, 1970). Curve (2) represents an experiment where the lag phase was nearly 48 hours long. Incubation of the tuber tissue in mineral salts media alone did not initiate mitosis in the artichoke. There was a slight decrease in the number of cells/explant from the initial 20,000 present at cutting, as damaged cells were sloughed off and for the duration of the 120 hour culture period little cell

FIGURE 7.

Increase in cell number during growth of explants in media containing or lacking the auxin, 2,4-D

A sample of 5 explants was taken at various intervals. The mean value of 6 separate counts for each time is plotted.

Media containing auxin (●, ▲)

Curve 1. (●) - experiments were carried out between late December and April when the lag phase was approximately 22 to 24 hours.

Curve 2. (▲) - experiments were carried out later in the year when the lag phase was longer and more variable.

Media lacking auxin (○)

FIG. 7

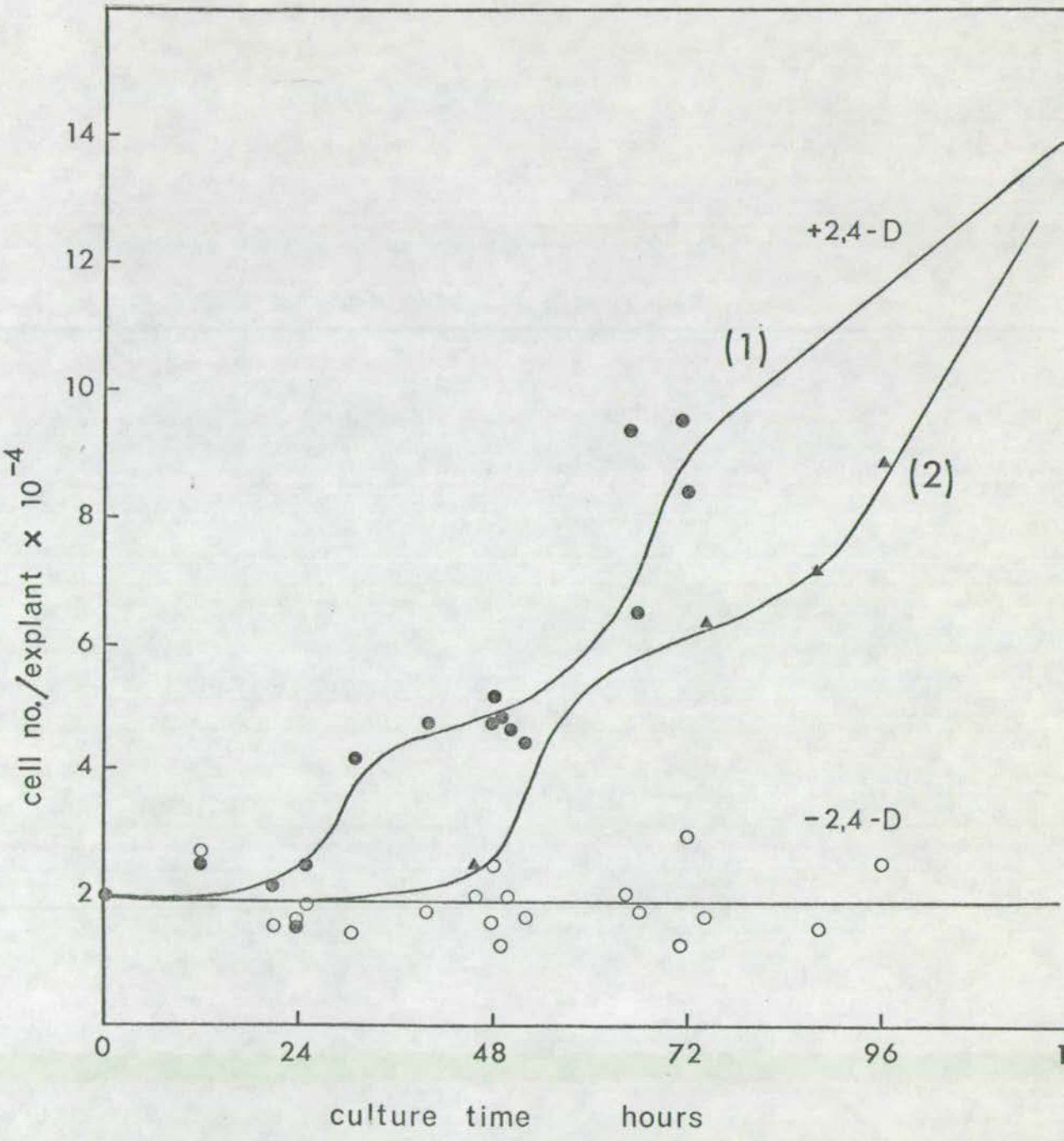

14
12
10
8
6
4
2
cell no./explant x 10^{-4}
+2,4-D
(1)
(2)
-2,4-D
0
24
48
72
96
culture time hours

FIGURE 8.

Specific activity of (CH_3-^3H) thymidine labelled DNA and changes in cell number during growth of discs in media containing or lacking 2,4-D

Duplicate samples of discs were taken after various time intervals. The plotted cell number value is the mean of 6 estimations and is shown as the (●,○) line.

For the radioactive experiments the discs were cultured in separate plates and at the allotted times a 3 hour pulse with tritiated thymidine was given (Methods, C-4-b). The value shown is at the completion of the pulse (■).

No. cells/disc x 10^{-5} ● ○
$(CH-^3H)$ thymidine incorporation cpm/µg ■
+2,4-D
-2,4-D
culture time hours
0
9
27
45
63
81
99
1
2
3
4
5
6
7

FIG. 8

division was evident.

(b) When the artichoke tissue was prepared as discs and similarly cultured, the same pattern of growth was evident (Figure 8) with an approximate 6 fold increase in cell number of auxin treated tissue after 96 hours of culture. S phase or DNA relication began after 16 hours in media containing 2,4-D and lasted approximately 14 hours when cell division occurred. There was a subsequent, partially synchronous second wave of DNA replication after 36 hours of growth and the following cell divisions were less distinct as the tissue became progressively more asynchronous. These results are similar to those of Mitchell, (1967 ; 1969) and Fraser, (1968). Few cell divisions were evident in the non-treated tissue which remained fairly stable at the initial 100,000 cells.

Discussion

Since the explant and disc demonstrated a similar response to auxin treatment in regard to cell number increase I felt that the two methods of culture could be used interchangeably. Because DNA synthesis and subsequent cell division are linked (Mitchell, 1967) and only induced by the presence of auxin, the artichoke system is particularly suited to the study of the induction of DNA replication and the maintenance of cell division. Further advantages in using the artichoke system include its uniform cell type, its ease in handling, the high percentage of cells entering synchrony and the use of a defined medium.

SECTION B. THE RESPONSE TO WOUNDING

Introduction

Excision of the tissue itself, initiates a change in the metabolism of the cells independent of auxin treatment. This so called 'wound response' is characterized in many tissues by a 4 to 5 fold increase in respiration and considerable synthesis and accumulation of protein and RNA (Adamson, 1962 ; Kahl, 1974). An acceleration in respiration occurs immediately after cutting in the artichoke (Yeoman et al, 1966) and, independent of the presence of auxin, is followed 6 hours later by a stimulation in the synthesis of RNA and protein due to the factors related to excision damage (Fraser and Loening, 1974). Because part of the research was conducted during the early times of culture I needed some measure of the various responses to wounding which included increases in fresh weight, DNA, RNA, and protein.

Experimental design

Artichoke tissue was cultured as explants or discs as described in the previous section. Ribosomal RNA was extracted from explants and measured (Methods, C-3-c and C-5-a) while protein estimates in the discs were made as described in detail in the Methods (C-4-c and B-2). DNA synthesis in the discs was monitored as described in the previous section while fresh weight measurements were made by quickly weighing a sample of approximately 10 discs which were patted dry of excess media.

Results

The results of 4 experiments shown in Table (3), provide evidence of limited DNA synthesis as a result of wounding. This

TABLE 3.

Evidence of limited DNA synthesis due to excision of the tissue

Four separate experiments are shown; 2 where the incorporation of (CH_3-^3H) thymidine into an acid-insoluble product was taken as a measure of DNA synthesis and 2 where the specific activity of the DNA itself was calculated. A 3 hour pulse with isotope was given in each case (Methods, C-4-b).

TABLE 3.

Treatment	Cultural age (hours) at completion of pulse	Incorporation of radioactivity cpm/disc		Specific activity of DNA in cpm/µg	
		1	2	3	4
2,4-D	3	382	250	456	149
	9	1035	860	383	428
	15	969	560	640	402
	21	4235	3500	2393	757
control	3	279	140	232	82
	9	1083	940	397	465
	15	265	330	305	395
	21	330	990	367	407

FIGURE 9.

Increase in fresh weight of discs cultured in media with or without auxin

A sample of 8 to 10 discs was removed after various intervals, quickly patted dry and weighed.

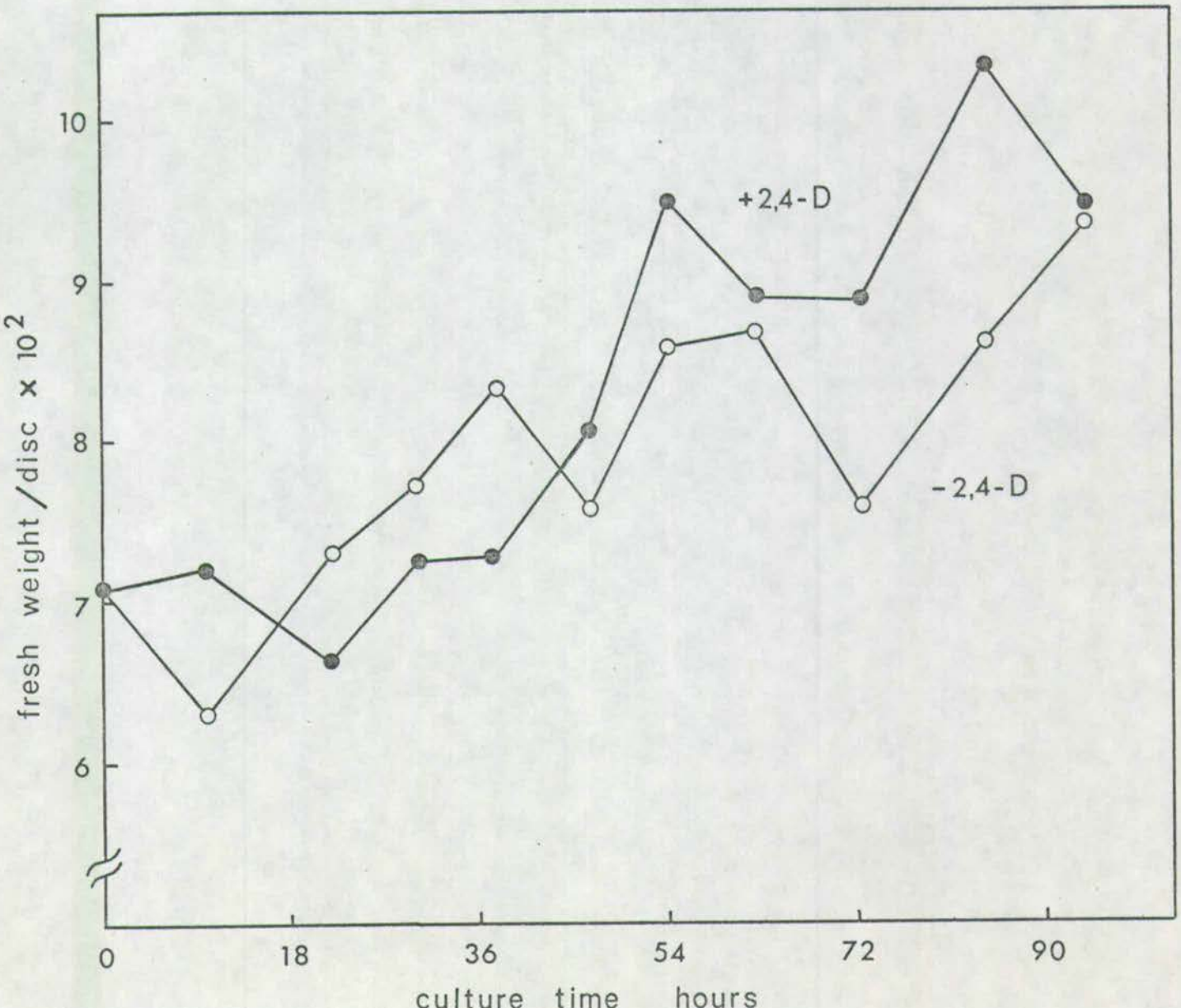

10
9
8
7
6
fresh weight /disc × 10^2
+2,4-D
-2,4-D
0
18
36
54
72
90
culture time hours

FIG. 6

typically occurred between 6 and 9 hours of culture whether auxin was present or not and is therefore, an event separate from the auxin-induced synthesis of DNA. Figure (9) shows that discs, cultured in the presence or absence of auxin, demonstrate a similar increase in fresh weight with culture. In the absence of cell division, an increase in fresh weight is a good index of increased cell volume or expansion of turgid tissue (Adamson, 1962). The results presented later in Figures (10) and (13) concerning rRNA and protein accumulation in the artichoke show that both auxin treated and non-treated tissue accumulated these macromolecules at the same rate for up to 24 hours of culture. These results are in agreement with Kahl, (1974) who remarked that the synthesis of RNA and protein in response to cutting are the greatest between 3 and 12 hours of aging.

Discussion

Both auxin treated and non-treated tissue reacted similarly to excision for the first 24 hours of culture. Thereafter, the non-treated tissue responded to the increased cellular metabolism by cell expansion, whereas the auxin treated tissue underwent growth by cell division, (Figures 7 and 8), producing small meristematic-like cells so described by Yeoman et al, (1965) and Fraser, (1968).

SECTION C. ACCUMULATION OF RIBOSOMAL RNA

Introduction

One of the most striking biochemical responses to auxin in the artichoke and in many other plant tissues is the enhancement of RNA synthesis, with the subsequent accumulation of RNA (Key and Ingle, 1968 ; Trewavas, 1968 ; Key, 1969 ; Yeoman and Mitchell, 1970 ; Jacobsen, 1977). This event was measured in both the explant and the disc as an increase in rRNA accumulation and the synthesis of total RNA respectively.

Experimental design

(a) A total of 150 explants were incubated in 15 ml of media containing or lacking the auxin, 2,4-D (Methods, C-3-a) and at various times samples were removed and assayed for rRNA content as described in the Methods (C-3-c and C-5-a).

(b) Artichoke tuber discs were incubated at a concentration of 10 discs in 2 ml of media with or without auxin for 0, 6, 12, 18, 23, 47, and 71 hours when a 3 hour pulse with 50 µCi/ml (G-^{3}H) uridine was given. Following the extraction of total nucleic acids and protein in the tissue (Methods, C-4-c) the TCA-insoluble precipitate was taken up in 3 ml 0.1 N NaOH to dissolve the macromolecules. A volume of 0.5 ml was assayed for radioactivity as described in the Methods, (B-4-b-i).

Results

(a) Figure (10) shows that for the first 24 hours of culture both auxin treated and non-treated explants accumulated rRNA at a rate of 0.029 µg/hour/explant. At subsequent times the rate was enhanced to 0.135 µg/hour/explant in the treated tissue, whereas

FIGURE 10.

The accumulation of ribosomal RNA during culture of artichoke explants in media containing or lacking 2,4-D

Total nucleic acids were prepared as described in the Methods (C-3-c), fractionated on 2.3% polyacrylamide gels (Methods, C-5-a). Ribosomal RNA was estimated from the areas of the relevant peaks from the scan of the gel at 265 nm and expressed on an explant basis.

FIG. 10

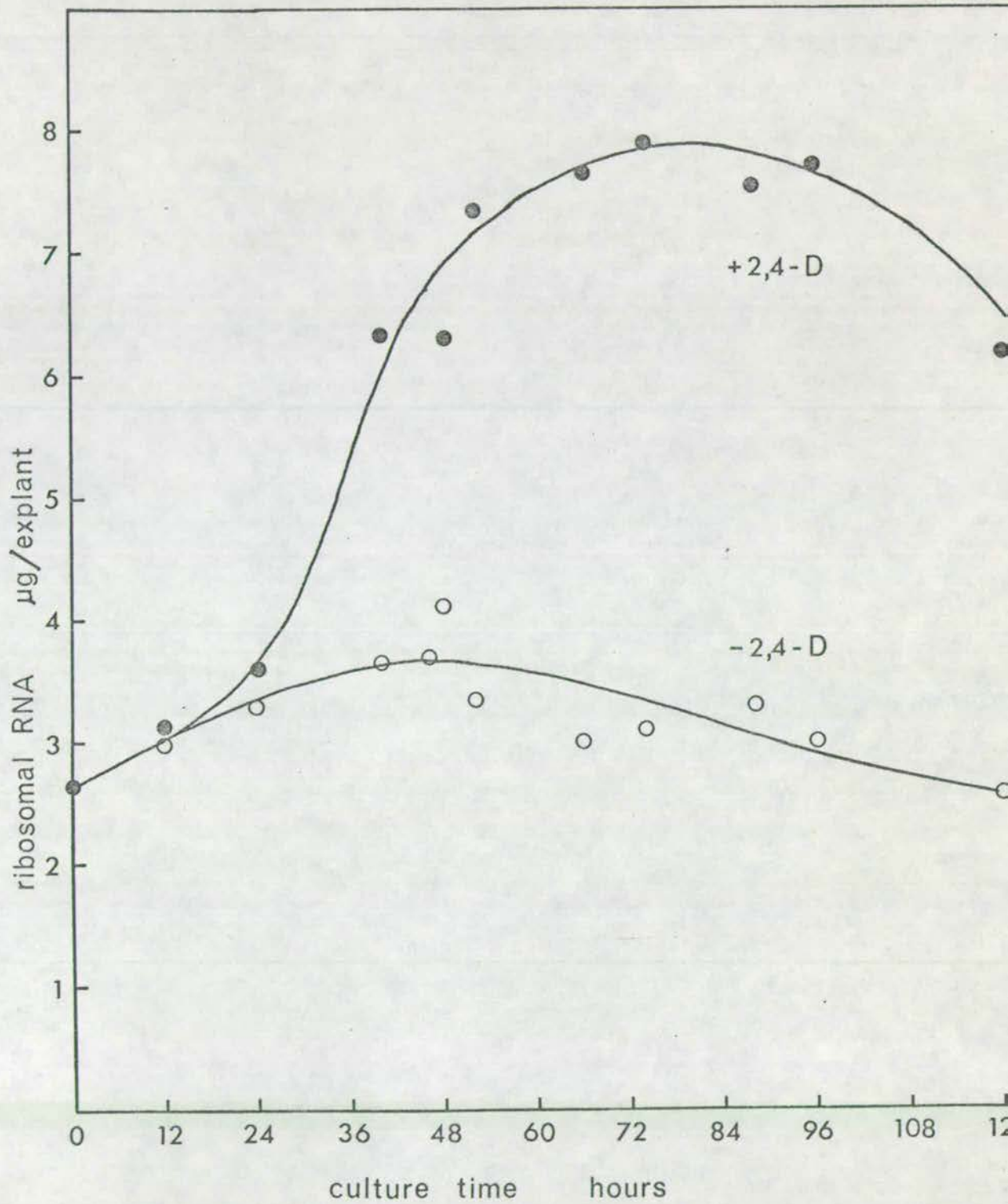

ribosomal RNA µg/explant
1
2
3
4
5
6
7
8
+2,4-D
-2,4-D
0
12
24
36
48
60
72
84
96
108
culture time hours

FIGURE 11.

Changes in ribosomal RNA/cell of explants cultured in the presence or absence of 2,4-D

The amount of rRNA accumulated in an explant after each time interval was re-expressed on a cell basis by using the results presented in Figure 7.

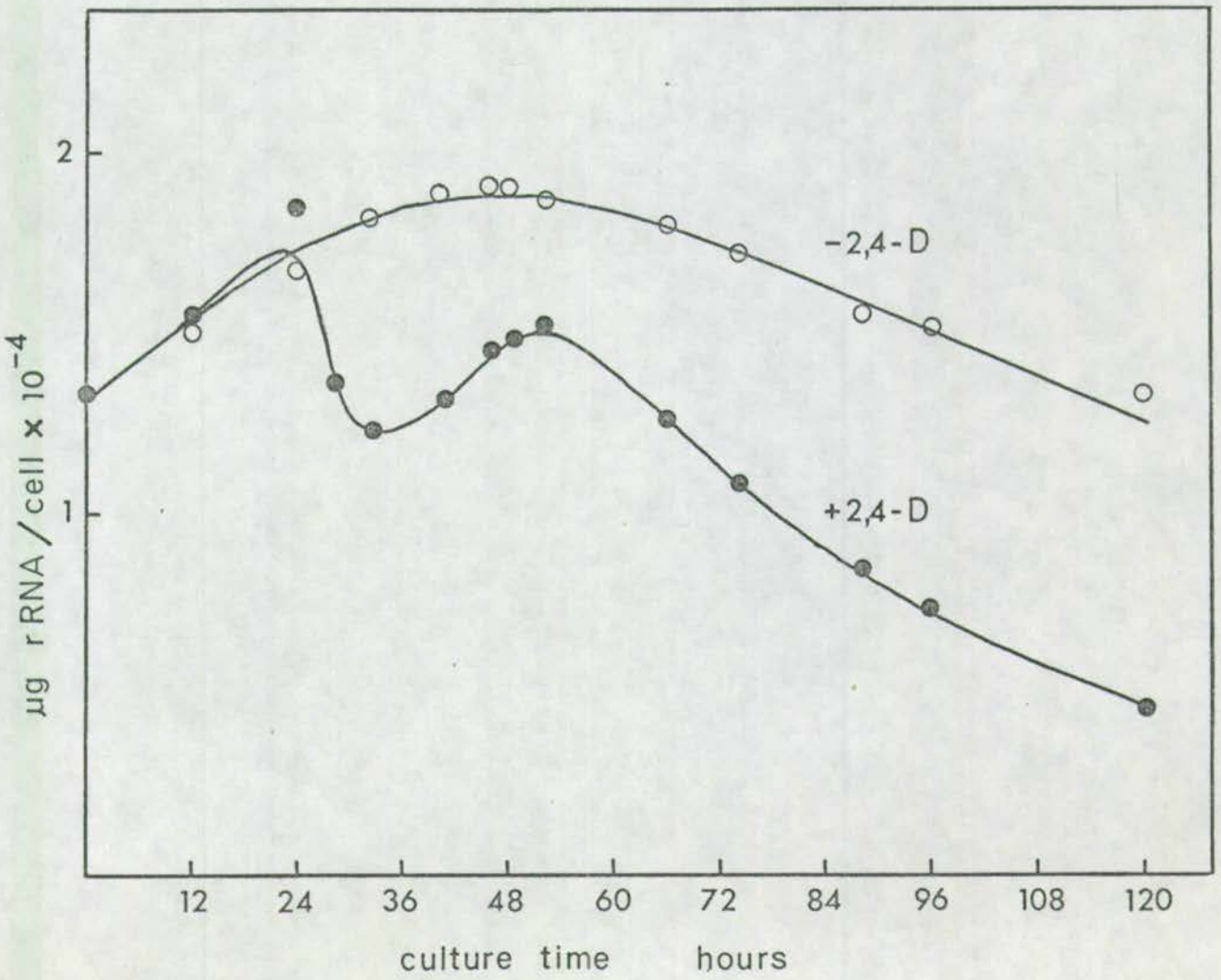

2
1
µg rRNA / cell × 10^{-4}
-2,4-D
+2,4-D
12
24
36
48
60
72
84
96
108
120
culture time hours

FIG. 11

there was little accumulation and in fact a decrease after 48 hours in the non-treated tissue. Fraser and Loening, (1974) similarly found a transitory enhancement of RNA synthesis which was further stimulated in the presence of 2,4-D. In my experiments this 2,4-D related increase in rRNA was only maintained for an additional 36 hours after which ribosomal RNA ceased to accumulate and started to decline at 84 hours, an event also observed by Gore and Ingle, (1974) and Hepburn, (1974). When rRNA accumulation was expressed on a cell basis (Figure 11) the initial cellular increase reached 1.8×10^{-4} µg rRNA/cell whether auxin was present or not. After 24 hours of culture, however, the rRNA content in auxin treated tissue rapidly declined as S phase neared completion and the cells underwent mitosis. A second, not so prominent increase in rRNA was evident between 36 and 48 hours of culture as the tissue prepared for the second division. The amount of rRNA per cell then rapidly declined as division continued in an asynchronous manner. It appears, then, that under these cultural conditions the cells overproduce rRNA as a result of both the wound response and auxin treatment and are returning to some basal level suitable to the smaller meristematic cells generated. In the non-treated tissue the rRNA, accumulated as a response to cutting, gradually declined with aging and probably reflected natural turnover.

(b) The incorporation of tritiated uridine into an acid-insoluble precipitate is presented in Figure (12). After 15 hours of culture the auxin treated tissue discs displayed a rapid decline in the synthesis of total RNA. This was apparently due to the onset of DNA replication at which time RNA synthesis ceased and then resumed approximately 12 hours later. This phase is also

FIGURE 12.

Estimate of the synthesis of total RNA in artichoke discs cultured in media with or without auxin

The tissue was given a 3 hour pulse with (G-^{3}H) uridine, (Methods, C-4-b). The incorporation of tritiated uridine into an acid-insoluble precipitate was used as a measure of synthesis.

FIG. 12

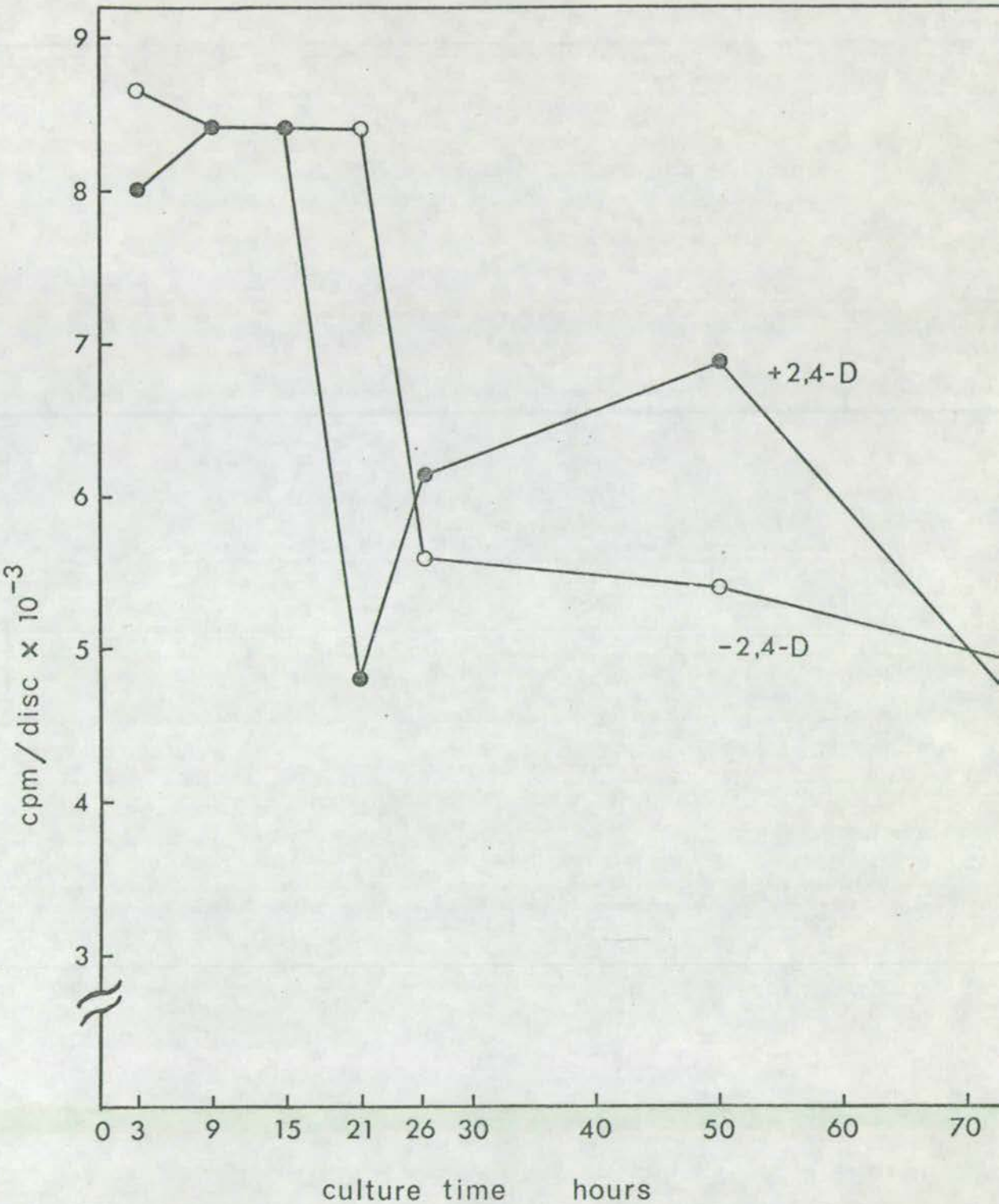

9
8
7
6
5
4
3
cpm / disc x 10^{-3}
+2,4-D
-2,4-D
0 3 9 15 21 26 30 40 50 60 70
culture time hours

TABLE 4.

Estimate of the synthesis of total RNA during growth of artichoke discs in media containing or lacking 2,4-D

The values for the previous Figure (12) are shown. These have been re-expressed on a DNA basis and an estimate of the amount of protein in each disc is given.

TABLE 4.

Treatment	Cultural age (hours) at time of pulse	cpm/disc (G-^3H) uridine corrected for uptake	Radioactivity in RNA compared to DNA cpm (RNA/µg DNA)	Amount of total cell protein µg/disc
2,4-D	3	8025	1748	266
	9	8424	1762	246
	15	8424	2090	270
	21	4792	1278	269
	26	6145	-	270
	50	6869	-	417
	74	4635	-	450
control	3	8657	1924	261
	9	8425	1911	260
	15	8425	2090	279
	21	8426	2091	294
	26	5593	-	234
	50	5412	-	263
	74	4901	-	230

when calculated on a DNA or protein basis (Table 4). Since there are a certain proportion of cells making up the inner core of the disc which do not divide (see Section A) the complete absence of RNA synthesis was not observed. Similarly, there was considerable synthesis of RNA in the non-treated and hence non-dividing tissue during the same period of time (Figure 12). As the tissue of both cultural states was aged in culture the synthesis of RNA fell.

Discussion

Auxin treatment enhanced the accumulation of rRNA in the explant and the incorporation of radioactive uridine into RNA in the disc. Since rRNA consists of approximately 80% of the total RNA of a cell (Eikhom *et al*, 1975 ; Ingle *et al*, 1976) the synthesis of RNA may be compared closely to the accumulation of rRNA. The accumulation of rRNA in µg/cell (Figure 11) displayed a similar response to the induction of DNA synthesis in auxin treated tissue as indicated by the synthesis of total RNA. Also, it would appear that the plateau in the accumulation of rRNA after 50 hours of culture may in part be due to a reduction in synthesis since the incorporation of radioactive uridine into RNA fell at this time, even though the number of cells were still increasing.

SECTION D. ACCUMULATION OF PROTEIN

Introduction

Artichoke tuber tissue also responds to auxin treatment by an increase in the rate of protein synthesis with the subsequent accumulation of protein (Yeoman and Mitchell, 1970). The accumulation of protein was monitored in auxin treated and non-treated discs up to 84 hours of culture while the synthesis of protein was only followed to the onset of S phase in the auxin treated tissue. The synthesis was measured by the incorporation of ^{35}S-methionine into protein.

Experimental design

(a) Since a measure of the accumulation of protein required long term culture a total of 10 discs were incubated in 2 ml of media, containing or lacking auxin and after approximately 45 hours of culture the discs were transferred to fresh media. Samples were removed at various times from 0 to 84 hours of culture. The discs were extracted with TCA (Methods, C-4-c) to obtain an estimate of total protein in the tissue (Methods, B-2).

(b) For short incubations, a total of 20 discs were incubated in 2 ml media in order to obtain a larger, more reliable yield. The increased concentration of tissue had no effect on the amount of protein for this short time. After 0, 6, 12, and 18 hours of culture the tissue was transferred to media containing 80 µCi/ml ^{35}S-methionine for 3 hours. The tissue was then extracted as above and the acid-insoluble precipitate was assayed for protein (Methods, B-2) and radioactivity (Methods, B-4-b-i).

Results

(a) Figure (13) shows that both auxin treated and non-treated tissue accumulated protein at a similar rate for the first 24 hours of culture. At this time a steady state level equivalent to a 60% increase in total protein was attained in the non-treated discs. In the auxin treated disc, protein continued to accumulate after 24 hours and the rate was increased from 2.5 µg/hour/disc to about 7 µg/hour/disc. This rate was maintained for at least 72 hours as the cells continued to divide. When expressed on a cell basis (Figure 14), the overall pattern of protein accumulation was similar to that of rRNA (Figure 11). There was, however, one major difference. The level of protein attained in the first division was also reached in the second division whereas the amount of rRNA/cell was reduced at this stage. This suggests that the turnover rate of ribosomes is not as great as protein. Indeed as demonstrated in Lemna (Trewavas, 1970) ribosomes apparently have a relatively long half life of at least 5 days in non-growth conditions and Setterfield, (1963) came to the same conclusion for the artichoke.

(b) Figure (15) shows the specific activity of ^{35}S-methionine labelled protein at various times up to 21 hours of culture. Both auxin treated and non-treated tissue incorporated radioactivity to nearly the same extent until the onset of S phase in the treated discs. This illustrates that the similar accumulation of protein at this time was due to synthesis and not increased degradation or turnover of the protein in the auxin treated tissue.

Discussion

It appears that the continuation of cell division in response to auxin requires the continued synthesis of protein. I do not know

FIGURE 13.

Accumulation of protein during culture of artichoke discs in media with or without 2,4-D

A sample of 5 discs were extracted with TCA (Methods, C-4-c) and the amount of protein was estimated by the Lowry procedure (Methods, B-2).

FIG. 13

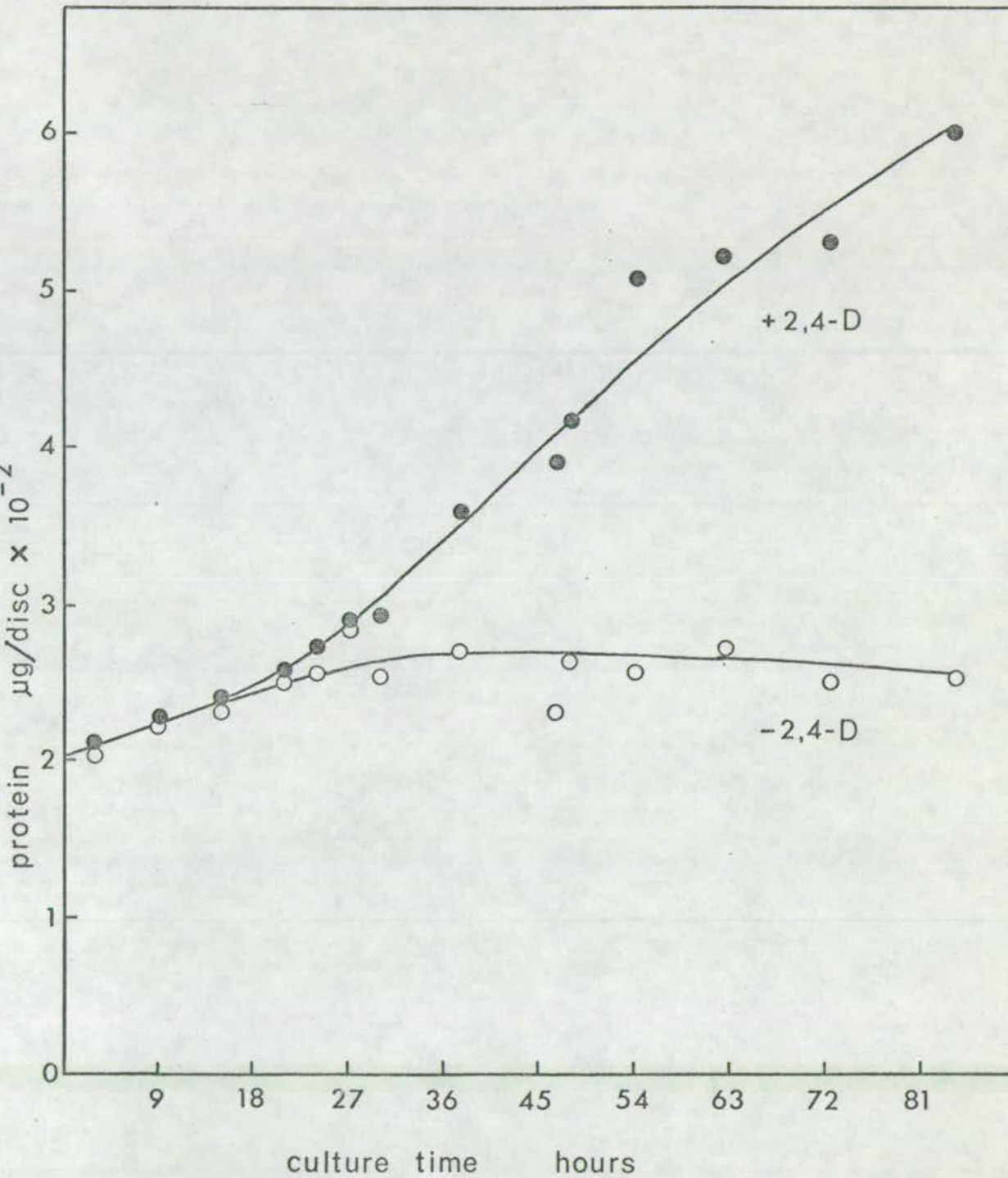

protein µg/disc × 10^{-2}
0
1
2
3
4
5
6
9
18
27
36
45
54
63
72
81
culture time hours
+2,4-D
-2,4-D

FIGURE 14.

Changes in protein/cell of discs cultured in the presence or absence of auxin

The amount of protein accumulated in a disc after each time interval was re-expressed on a cell basis by using the results presented in Figure 8.

FIG. 14

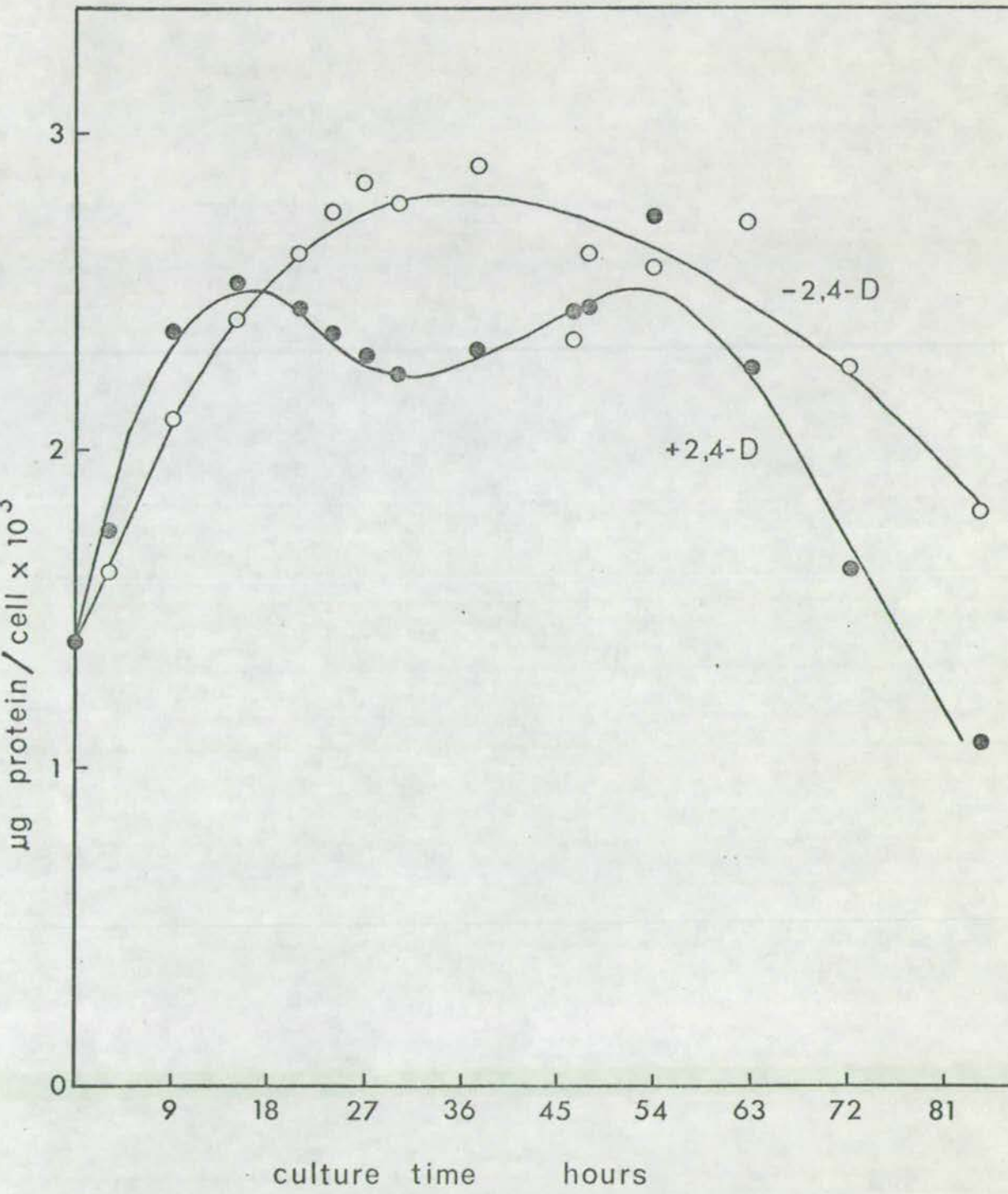
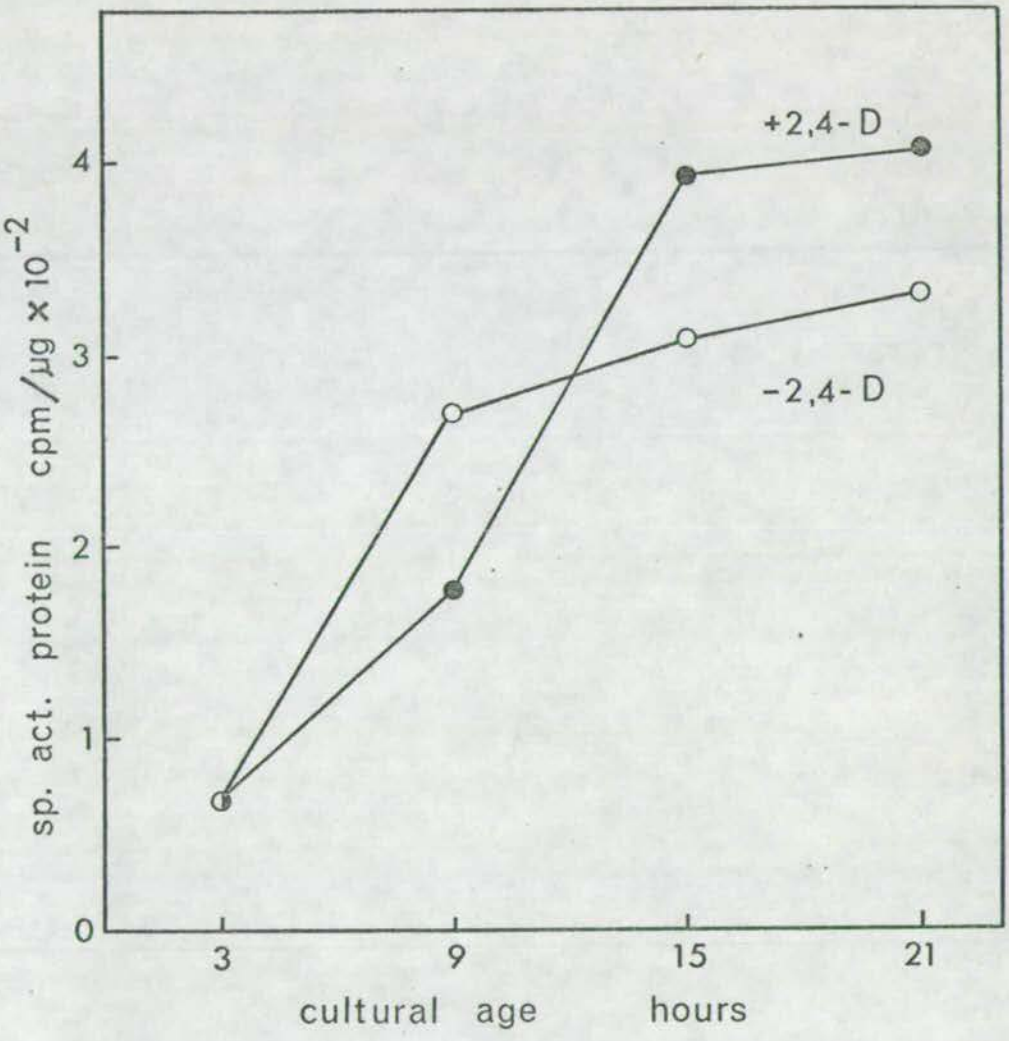

3
2
1
0
µg protein / cell x 10^3
-2,4-D
+2,4-D
9
18
27
36
45
54
63
72
81
culture time hours

FIGURE 15.

Specific activity of ^{35}S-methionine-labelled proteins during growth of auxin treated and non-treated tissue

Tissue was given a 3 hour pulse with radioactive methionine at various times up to the onset of S-phase (Methods, C-4-b). A TCA extraction and measurement of total protein was carried out (Methods, C-4-c and B-2). Radioactivity estimations were made as described in the Methods, (B-4-b-i).

FIG. 15


+2,4-D
-2,4-D
sp. act. protein cpm/µg x 10⁻²
0
1
2
3
4
3
9
15
21
cultural age hours

whether, in the non-treated tissue, the steady state level was a reflection of reduced synthesis or increased turnover or both. For the initial 24 hours the increase in protein is, apparently, independent of auxin.

CHAPTER 2. TWO METHODS OF CULTURE, DISC VERSUS EXPLANT

Two methods of tissue culture have been used in the investigation. For the experiments on ribosomal RNA synthesis 300 explants each of auxin treated and non-treated tissue were cultured in bottles (150 explants/bottle) in the routine manner (Section C-3-a). For the experiments on protein changes, a minimum of 80 discs (20 discs/plate) of artichoke tissue for each cultural state were cultured in 2.0 ml of media in petri dishes (Section C-4-a).

I found that it was necessary to use two methods of preparation because of the very different tissue requirements in the 2 sets of experiments. Since sufficient nucleic acid for a gel analysis could be extracted from only 5 to 10 explants, a single bottle containing some 100 to 150 explants provided enough material for a time course containing 10 points. In contrast, the isolation of nuclei and analysis of nuclear protein would have required from 50 to 100 explants/sample and a suitable time course would involve up to 10 bottles and 1000 explants. The time required to prepare such large samples would be prohibitively long and the chances of bacterial contamination greatly increased. Furthermore, since it is advisable to randomize the tissue before experimentation this long preparation time would increase variability between those discs cut early and those cut late. Consequently, a simple method was devised to cut discs of tissue under sterile conditions. Each disc was approximately 5 x the size of an explant and it took as long to prepare a time course consisting of 400 to 800 discs as it did to prepare the normal set of 4 to 6 bottles of explants. Thus, experimental times were similar in both methods. In addition, because of their larger size the discs were easier to

handle.

Since the original work on cell division was conducted on explants a number of experiments were performed to show that the proportion of cells and the degree of synchrony were comparable between the disc and the explant. The surface area to volume ratio of the disc was only slightly less than that of the explant (2.58/1 compared to 2.8/1). Since the results of Yeoman et al, (1968) suggested that the formation of the autolysate at the surface is the factor which determines the formation of the meristematic layer, the proportion of dividing cells in each should be similar. Indeed, this was found to be the case with a 5 fold increase in cell number after 96 hours of culture, as shown in Figures, (7 and 8).

The induction of a high proportion of dividing cells in the disc was complicated by one factor, a feature of the method of culture. The discs were placed, but not immersed, in a small volume of nutrient media in a petri dish. The media was drawn over the disc by capillary action which allowed for adequate aeration, but the lower side of the disc composing 38% of the total surface area was against the glass surface. Since this side did not have free access to the nutrients the cells remained dormant. This was exemplified by a hollowing out of the underside of the disc as the outer, exposed cells underwent division and the tissue expanded.

An effort was made to counteract this possible loss in division potential by increasing the concentration of 2,4-D from 10^{-6} M to 10^{-5} M. Yasuda et al, (1974) while investigating the induction of DNA synthesis in artichoke found the optimum concentration of 2,4-D for callus induction to be 10^{-5} M as did Fraser, (1968). The results presented in Table (5) agree with their findings. Although treatment

TABLE 5.

Effect of various concentrations of 2,4-D on cell division and synthesis of nucleic acids in cultured explants

The tissue was prepared as described in the Methods, (C-3-a) but with varying concentrations of the auxin, 2,4-D. After 48 hours of culture the explants were extracted with Kirbys medium (Methods, C-3-c) and the amount of nucleic acid was estimated from the absorbance at 260 nm. Cell counts for each sample were made from 5 explants and the amount of nucleic acid, expressed either on a cell basis or an explant basis was compared to the control with no added auxin.

TABLE 5.

Concentration of 2,4-D in media (Molarity)	Cells/explant	Estimation of total nucleic acids	
		µg/explant	µg/cell
0 (control)	1.4×10^{4}	1.9	1.3×10^{-4}
10^{-6}	2.7×10^{4}	3.9	1.4×10^{-4}
5×10^{-5}	2.9×10^{4}	4.0	1.4×10^{-4}
10^{-5}	3.2×10^{4}	4.8	1.5×10^{-4}
5×10^{-4}	1.1×10^{4}	2.7	2.4×10^{-4}
10^{-4}	1.2×10^{4}	2.2	1.9×10^{-4}

with 10^{-5} M 2,4-D effects the greatest increase in cell number, 10^{-6} M and 5×10^{-6} M are also adequate. The lower concentration of 10^{-6} M was used in the experiments with the explant in keeping with previous studies using this method of culture (Yeoman and Mitchell, 1970 ; Gore and Ingle, 1974) while 10^{-5} M 2,4-D was used for the discs. The disc system might be greatly improved by devising a method of bathing the entire piece of tissue in the nutrient medium.

CHAPTER 3. TWO PROBLEMS FOR INVESTIGATION

The majority of research on RNA stimulation by auxin has concentrated on polymerase activities, (O'Brien *et al*, 1968 ; Gore and Ingle, 1974 ; Guilfoyle *et al*, 1975) that is, regulation at the transcriptional level. Gore and Ingle, (1974) demonstrated that when artichoke tissue was treated with the auxin 2,4-D, RNA polymerase activity was enhanced 2.7 fold. Although from $^{32}P_i$ incorporation data, transcription of the rRNA precursor showed a similar increase, (Ingle *et al*, 1976) the level attained by the final rRNA product was enhanced several fold (Gore, 1972). Therefore, the increase in polymerase activity does not account for the much greater increase in the synthesis of mature RNA. It also, is apparently not due to an increase in the template size since Ingle and Sinclair, (1972) have shown that the number of rRNA genes is unaffected by the conditions of culture. Therefore, it was suggested that the difference in rRNA accumulation in control and auxin treated tissue was the result of regulation of gene activity at two levels; (1) that of transcription and (2) that of the processing of the precursors. An investigation of both the kinetics of rRNA maturation and the stability of the mature ribosomes seemed critical in discovering why rRNA failed to accumulate in the non-treated tissue despite its apparent synthesis. It appears, from Figures, (10 and 11) that auxin is not necessary for the initial increase in rRNA synthesis but is required for further accumulation of rRNA. It was hoped to gain some insight into whether the action of 2,4-D is dependent on rRNA synthesis as implied by Setterfield, (1963) and Masuda, (1966) or whether it is merely a coincidental effect as suggested by Fraser, (1975).

There has been considerable confusion in the literature regarding the timing and initial mode of action of 2,4-D on the induction of cell division in the artichoke. The data in Figures (10) and (13) show that the accumulation of RNA and protein in auxin treated and non-treated tissue is identical for the first 24 hours after excision. Results such as these have lead a number of workers (Adamson, 1962 ; Setterfield, 1963 ; Masuda, 1966) to suggest that artichoke tissue is unable to respond to auxin for the first few hours of culture. At this time the tissue is responding to a change in environment by increased cellular metabolism, and RNA and protein synthesis are probably required as the tissue forms a protective sheath of cells around its cut surface. In addition, it appears reasonable that some preparation for DNA replication must occur in the auxin treated tissue in this period of time prior to the onset of S phase at 18 to 21 hours. Evidence in support of this was initially presented by Mitchell, (1967) who found that the first G1 phase was abnormally long when compared to the second. Similarly, Yeoman and Mitchell, (1970) found a decrease in the mean division time when auxin was added 5 days after the start of culture even though the percentage of cells going into division was similar as when auxin was present from the start. Recently, Yasuda et al, (1974) have obtained results which correspond to the observations above. They found that if protein synthesis was delayed by cycloheximide then DNA synthesis was similarly delayed. Chloramphenicol, which selectively inhibits protein synthesis in chloroplasts and mitrochrondria (Newton, 1965) did not inhibit DNA synthesis at concentrations of 10^{-5} M and 10^{-4}M. Actinomycin D and 8-aza-guanine, inhibitors of RNA synthesis, both inhibited DNA synthesis by 50%. Although the use of inhibitors is

questionable in the interpretation of results, it, nevertheless, appears that some specific nuclear RNA and protein synthesis was necessary for the induction of DNA synthesis. Therefore, since the replication of DNA is an event which occurs in the nucleus, it suggested to me that the necessary changes occur in this organelle and probably involve proteins. In particular, I have examined changes in the acidic proteins, which, in the light of recent evidence, not only appear to be specific regulators of gene transcription (Stein et al, 1974) but are also implied in the control of DNA synthesis itself (Jeter and Cameron, 1974 ; Patel and Thomas, 1975).

PART IV

RIBOSOMAL RNA

THE EFFECTS OF AUXIN ON THE TRANSCRIPTION AND POST-TRANSCRIPTIONAL PROCESSING OF RIBOSOMAL RNA IN THE CULTURE OF ARTICHOKE CELLS

Introduction

When artichoke tuber cells are aged in nutrient medium, auxin can cause a 2.5 fold accumulation of rRNA during the first 50 hours of culture (Gore and Ingle, 1974 ; Hepburn, 1974) and Figure (10). Gore and Ingle, (1974) reported that during the same culture time the activity of RNA polymerase was increased 2.7 fold. They concluded that this higher rate of DNA transcription could account for the observed accumulation of rRNA and to support this, Ingle et al, (1976) noted that the incorporation of ^{32}P-orthophosphate into the initial 2.5×10^6 mol. wt. rRNA precursor was increased approximately 2 fold. Other experiments reported by Gore, (1972), showed that the level of $^{32}P_i$ in the mature rRNAs was, however, increased some 10 fold. If it is accepted initially that $^{32}P_i$ incorporation reflects a rate of synthesis there is an obvious discrepancy between the 2 sets of data presented above. A possible solution to this is that auxin not only increases the rate of transcription of precursor rRNA but also modifies the subsequent processing of RNA. The research in this chapter is an attempt to resolve the above difficulty.

I have attempted to measure the post-transcriptional regulation of rRNA accumulation by studying the kinetics of the maturation of the rRNA itself. This was accomplished in both auxin treated and non-treated tissue by labelling the precursor rRNA to steady state with ^{32}P-orthophosphate and following the rate of processing to the mature rRNA forms. Also, an effort was made to label the mature rRNAs of

auxin treated and non-treated tissue to constant specific activity in order to monitor the turnover rates of this RNA by the loss of radioactive phosphate. Because of the difficulties in equating incorporation of label with a rate of synthesis, I have attempted to measure, unambiguously, the rate of RNA synthesis by determining the specific radioactivity of a direct precursor to RNA and relating this to the amount of label incorporated. Labelling was conducted with (G-^{3}H) adenosine and the variation of the specific activity of adenine in ATP and RNA determined at various times.

Experimental design

(a) Artichoke tuber tissue, prepared as explants, was cultured in the presence or absence of 2,4-D for 48 hours when a pulse of ^{32}P-orthophosphate was given as described in the Methods, (C-3-a and C-3-b, respectively). At this time rRNA was still accumulating in the auxin treated tissue while accumulation had ceased in the non-treated tissue. Samples were removed from the labelling media after 15, 30, 60, 120, and 180 minutes and the nucleic acids were extracted by the Kirby method (Methods, C-3-c). The nucleic acids were fractionated on 2.3% acrylamide gels as described in detail in the Methods, (C-5-a). In order to resolve the mature 1.3×10^{6} rRNA and the 1.39×10^{6} precursor rRNA, which runs as a single peak in the acrylamide gels, the rRNA was denatured in formamide gels as previously described in the Methods, (C-5-b). The release of the 7S rRNA was monitored on 7.5% acrylamide gels (Methods, C-5-a). Following separation, the radioactivity in the nucleic acids was monitored by Geiger tube analysis as described in the Methods, (B-4-b-ii).

(b) In the cases where a pulse of label was to be followed by a long chase, the explants were only incubated for 24 hours when a 20 minute

pulse with ^{32}P-orthophosphate was given. The explants were transferred to the appropriate media containing 1 mM phosphate (Methods, C-3-b) and incubated a further 12 hours before removing samples at the total culture ages of 36, 41, 46, 50½, 60, 65, and 68½ hours. The nucleic acids were extracted and fractionated on 2.3% polyacrylamide gels as above.

(c) For the experiments where the explants were to be labelled with tritiated adenosine, the tissue was incubated for 25, 43, 48, and 70 hours when a pulse with 20 μCi/ml of carrier free (G-^{3}H) adenosine was given (Methods, C-3-b). Samples were removed after 30, 60, 120 and 180 minutes and frozen in liquid nitrogen pending a PCA extraction of the acid-insoluble nucleic acids and the acid soluble nucleotides, as described in detail in the Methods, (C-3-d).

Results

(a) Accumulation of rRNA in auxin treated and non-treated explants

(i) Fractionations of total nucleic acid from auxin treated and non-treated tissue are shown in Figure 16, (A,B,C,D,E,F,) and (G,H,J,K,L,M) respectively. The 265 nm OD profiles of both cultural states show the major peaks of the mature rRNAs with molecular weights of 1.3 x 10^6 and 0.7 x 10^6. The residual DNA peak which remained after salt precipitation, (Methods, C-3-c) is also evident, although the amount varied from preparation to preparation, showing the inconsistency of this technique for removing DNA. The radioactivity profiles show major peaks of the 2.5 x 10^6 mol. wt. precursor rRNA, the single unresolved mixture of the 1.39 x 10^6 precursor and the 1.3 x 10^6 mature rRNA, and 0.98 x 10^6 precursor rRNA and the 0.7 x 10^6 mature rRNA, which overlay a background of polydisperse RNA. The absence of radioactivity in the DNA peak in the control tissue is consistent with the lack of

FIGURE 16.

Fractionations of total nucleic acid from auxin treated and non-treated tissue and the incorporation of ^{32}P-orthophosphate into ribosomal RNA and into DNA.

After 48 hours of culture the explants were given a pulse (15 minutes to 180 minutes) with $^{32}P_i$ and the nucleic acids were fractionated on 2.3% polyacrylamide gels (Methods, C-5-a). The continuous scan shows the absorbance at 265 nm and the histogram, the radioactivity/1.0 mm slice (Methods, B-4-b-ii).

The gel scans are shown on the following 2 pages, with auxin treated and non-treated tissue being compared after a 15, 30 and 60 minute pulse (first page) and 90, 120 and 180 minute pulse (second page).

FIG. 16

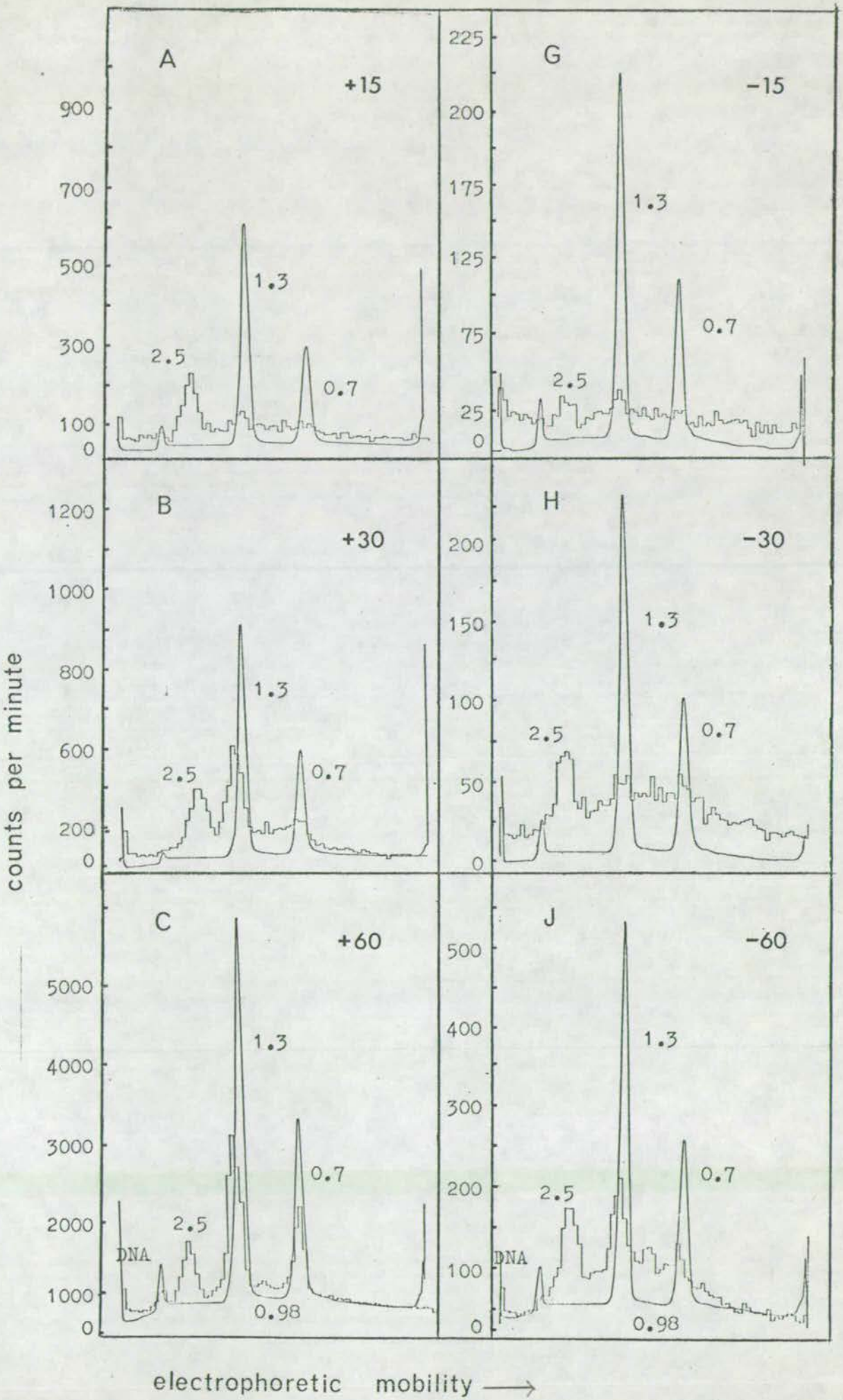
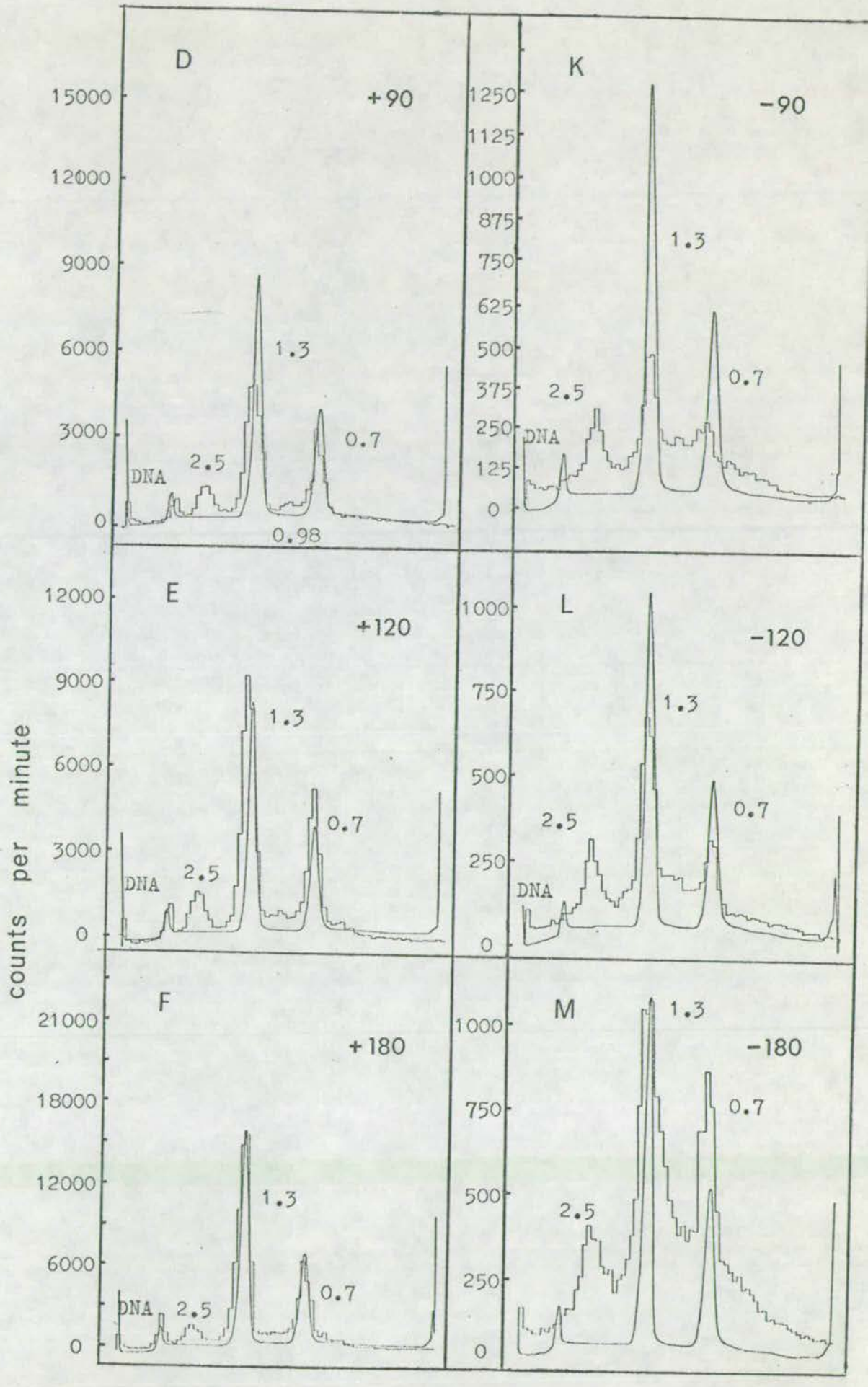

A
+15
900
700
500
300
100
0
2.5
1.3
0.7
G
-15
225
200
175
125
75
25
0
2.5
1.3
0.7
B
+30
1200
1000
800
600
200
0
2.5
1.3
0.7
H
-30
200
150
100
50
0
2.5
1.3
0.7
C
+60
5000
4000
3000
2000
1000
0
DNA
2.5
1.3
0.7
0.98
J
-60
500
400
300
200
100
0
DNA
2.5
1.3
0.7
0.98
counts per minute
electrophoretic mobility ⟶

FIG. 16a


D
+90
15000
12000
9000
6000
3000
0
1.3
2.5
0.7
DNA
0.98
K
-90
1250
1125
1000
875
750
625
500
375
250
125
0
1.3
2.5
0.7
DNA
E
+120
12000
9000
6000
3000
0
1.3
2.5
0.7
DNA
L
-120
1000
750
500
250
0
1.3
2.5
0.7
DNA
F
+180
21000
18000
12000
6000
0
1.3
2.5
0.7
DNA
M
-180
1000
750
500
250
0
1.3
2.5
0.7
counts per minute
electrophoretic mobility ⟶

division under these cultural conditions.

By comparing the proportion of label in each rRNA species after increasing periods of exposure to ^{32}P-orthophosphate, information about the pathway of processing of the precursor rRNAs to the mature forms may be obtained for both the dividing and non-dividing states. Regardless of the cultural state, after short labelling times a substantial proportion of the RNA detected as peaks was present in the 2.5 x 10^6 molecular weight precursor rRNA. The amount of radioactivity in this region of the gel increased as the tissue took up more isotope but the rate of increase declined as radioactivity accumulated in the mature rRNA. These results are consistent with the view that the rapidly labelled high molecular weight rRNAs are precursors to rRNA (Leaver and Key, 1970 ; Rogers et al, 1970 ; Grierson and Loening, 1974 ; Chapman and Ingle, 1976).

Figure (17) shows that the ratio of the 0.7 mature rRNA to the 2.5 x 10^6 precursor rRNA in auxin treated tissue increased rapidly as the tissue was exposed to longer periods of isotope. However, this rate was much reduced in the non-dividing tissue. This could either mean a slowed processing of the 2.5 precursor to the 0.7 mature rRNA, or that the mature rRNA was unstable in non-dividing tissue, or both. In addition, the fractionations demonstrated a 10 fold increase in the specific activity of the 0.7 mature rRNA of auxin treated tissue compared with the control, and this value remained constant throughout the 3 hour labelling period (Table 6).

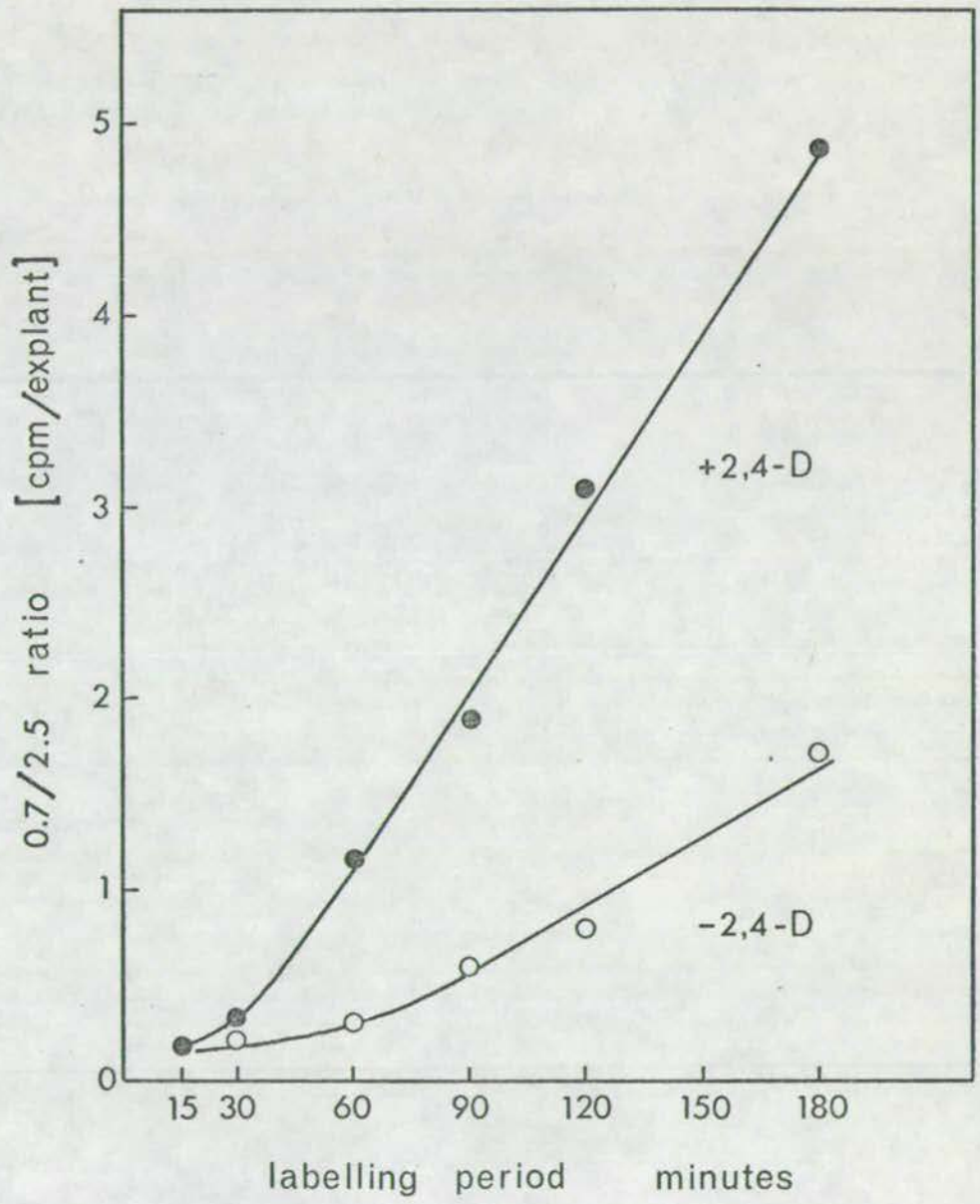
The specific activity of the mixed 1.39 x 10^6 molecular weight precursor rRNA plus the 1.3 x 10^6 mature rRNA peak was also increased by auxin but the ratio relative to the control varied throughout the labelling period (Table 6). At the early labelling periods the

FIGURE 17.

Comparison of the radioactivity in the mature rRNA to the precursor rRNA in auxin treated and non-treated explants after increasing exposure to ^{32}P-orthophosphate

From Figure (16) the amount of radioactivity in the 0.7 x 10^6 mature rRNA and 2.5 x 10^6 precursor rRNA was calculated on an explant basis and these values were compared after each labelling period.

FIG. 17


0.7/2.5 ratio [cpm/explant]
0
1
2
3
4
5
15
30
60
90
120
150
180
labelling period minutes
+2,4-D
-2,4-D

radioactive component migrated more slowly than the optical density peak suggesting a predominance of the 1.39 x 10^6 precursor. Denaturation of the nucleic acid sample by heat treatment at 60°C for 5 minutes releases the 7S (5 x 10^4 mol. wt.) rRNA from the 1.3 x 10^6 mature rRNA but not from the 1.39 x 10^6 precursor rrRNA (Hepburn and Ingle, 1975). An rRNA species of molecular weight 1.28 x 10^6 is produced and the 1.39 x 10^6 and 1.28 x 10^6 molecular weight rRNAs are resolved by polyacrylamide gel electrophoresis. This enables an assessment to be made of the quantity of precursor 1.39 x 10^6 rRNA processed to the mature rRNA. This method was attempted but the level of degradation of the mature rRNAs was too variable and the technique was complicated by the formation of a 2.0 x 10^6 molecular weight aggregate, (Figure 18). Separation was instead achieved by denaturation of the rRNA in formamide gels (Methods, C-5-b) as shown in Figure 19, (A,B,C,D,E,F) and (G,H,J,K,L) for dividing and non-dividing tissue respectively. This method was preferred since formamide maintains nucleic acids in an extended chain formation (Ts'o et al, 1962) and therefore did not demonstrate the problem of aggregation associated with heating. Also, the release of 7S rRNA was monitored by polyacrylamide gel electrophoresis on 7.5% gels as shown in Figure 20, (A and B). From the radioactivity profiles of the rRNAs denatured by formamide, the processing of the 1.39 x 10^6 precursor to the 1.3 x 10^6 mature rRNA was followed. After 60 minutes of incubation in ^{32}P-orthophosphate only the 1.39 x 10^6 precursor had incorporated label in the non-treated tissue. In the auxin treated tissue both the precursor and the mature rRNAs were labelled to almost equal amounts. After 90 minutes a small proportion of the label had been incorporated into the mature 1.3 x 10^6 rRNA of the control and this level increased with

FIGURE 18.

Effect of denaturation of the nucleic acid sample by heat treatment

Total nucleic acid, at a concentration of 500 µg/ml, was heated at 60°C for 5 minutes in electrophoresis buffer containing 10% sucrose prior to electrophoresis on 2.3% polyacrylamide gels (Methods, C-5-a). A 2.0×10^6 mol. wt. aggregate is evident on the OD scan (265 nm) and overlaps the radioactivity incorporated into the 2.5×10^6 rRNA precursor.

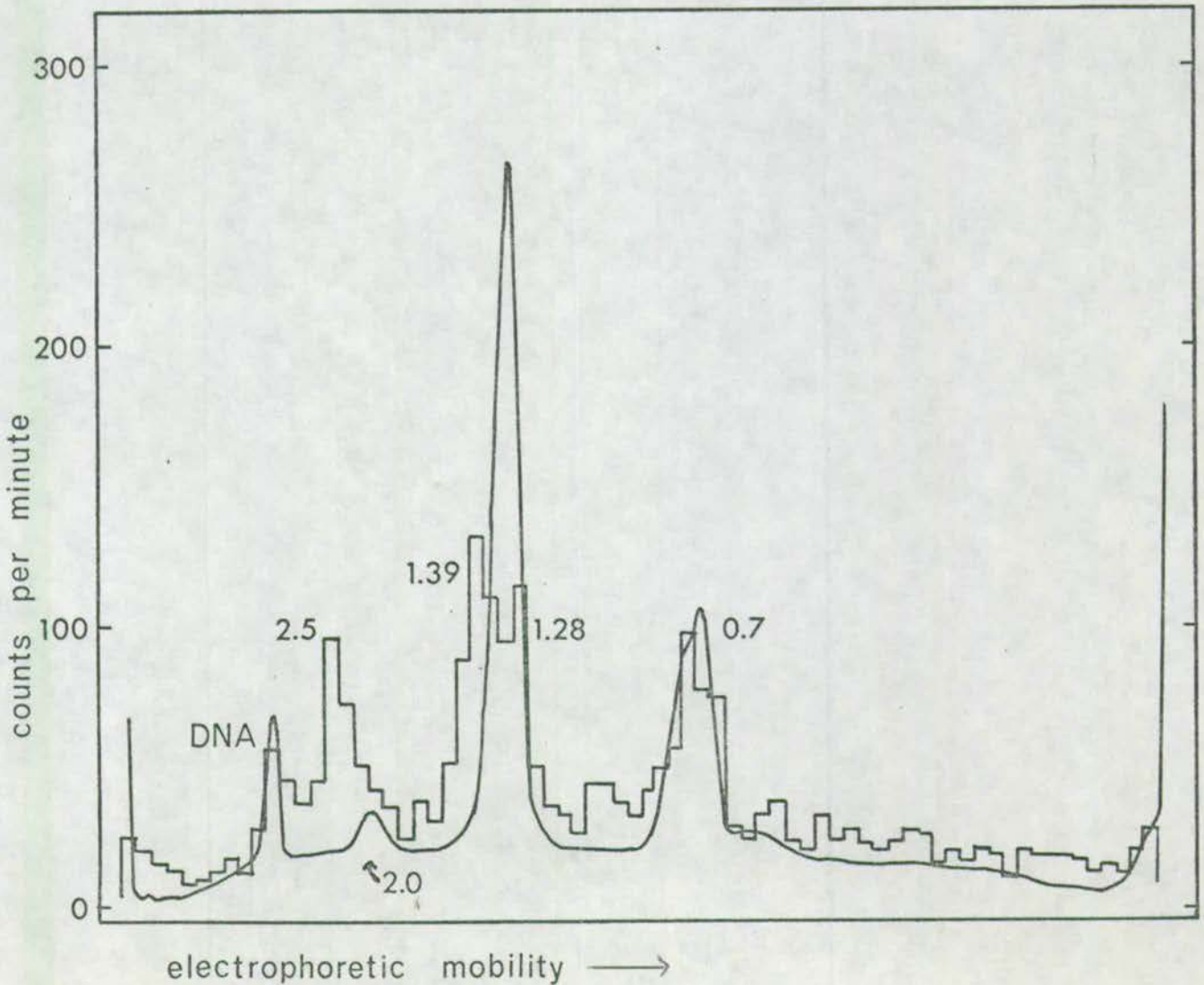

300
200
100
0
counts per minute
DNA
2.5
1.39
1.28
0.7
2.0
electrophoretic mobility ⟶

FIG. 18

FIGURE 19.

Denaturation and fractionation of $^{32}P_i$-labelled nucleic acids from auxin treated and non-treated tissue on polyacrylamide-formamide gels

After 48 hours of culture the explants were given a pulse (15 minutes to 180 minutes) with ^{32}P-orthophosphate and the nucleic acids were fractionated on 3.2% polyacrylamide-formamide gels (Methods, C-5-b). The continuous scan shows the absorbance at 265 nm and the histogram, the radioactivity/0.5 mm slice (Methods, B-4-b-ii).

The gel scans are shown on the following 2 pages, with auxin treated and non-treated tissue being compared after a 15, 30 and 60 minute pulse (first page) and 90, 120 and 180 minute pulse (second page).

FIG. 19

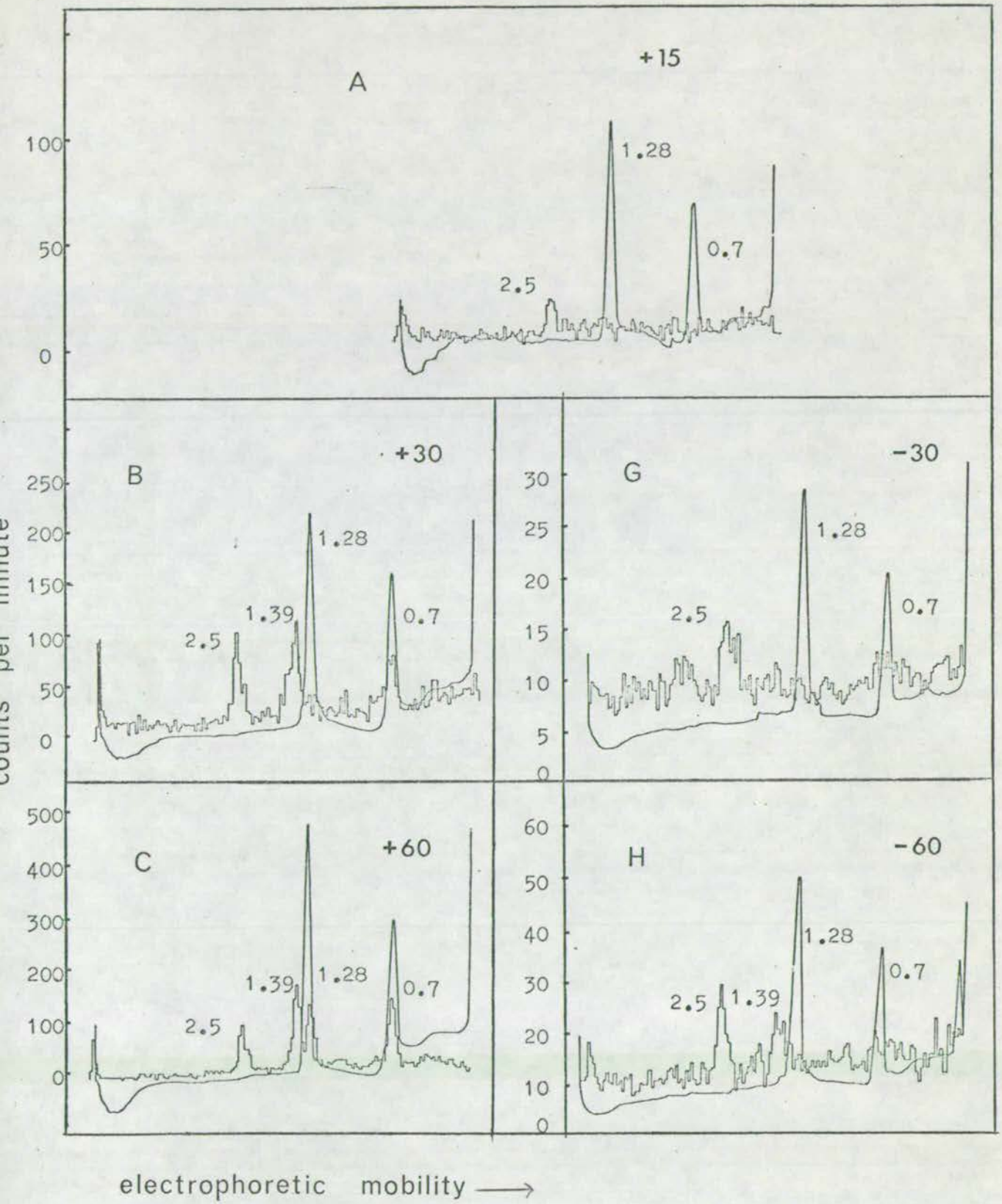

A
+15
1.28
0.7
2.5
B
+30
1.28
1.39
0.7
2.5
G
-30
1.28
0.7
2.5
C
+60
1.39
1.28
0.7
2.5
H
-60
1.28
0.7
2.5
1.39
counts per minute
electrophoretic mobility ⟶

FIG. 19 a

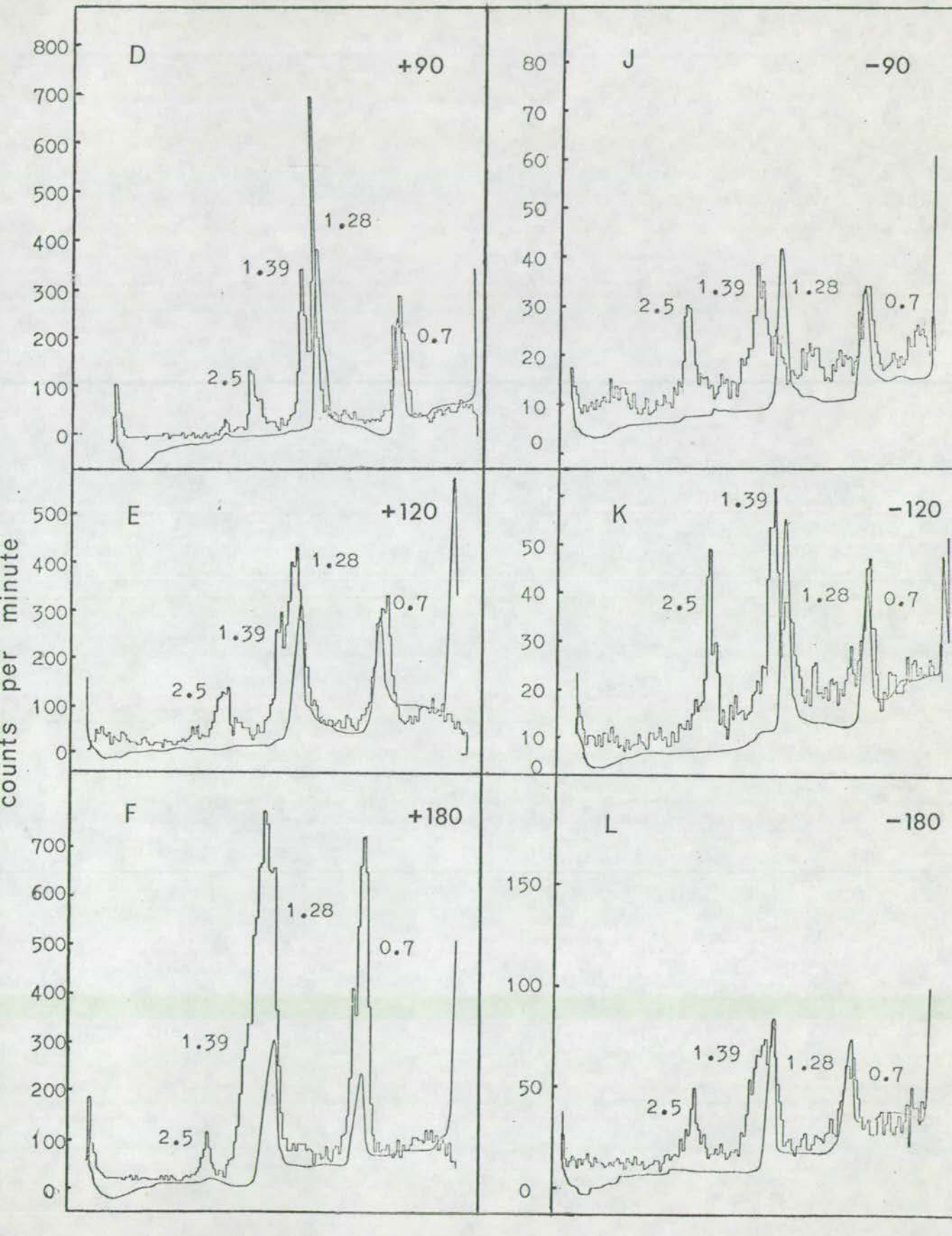
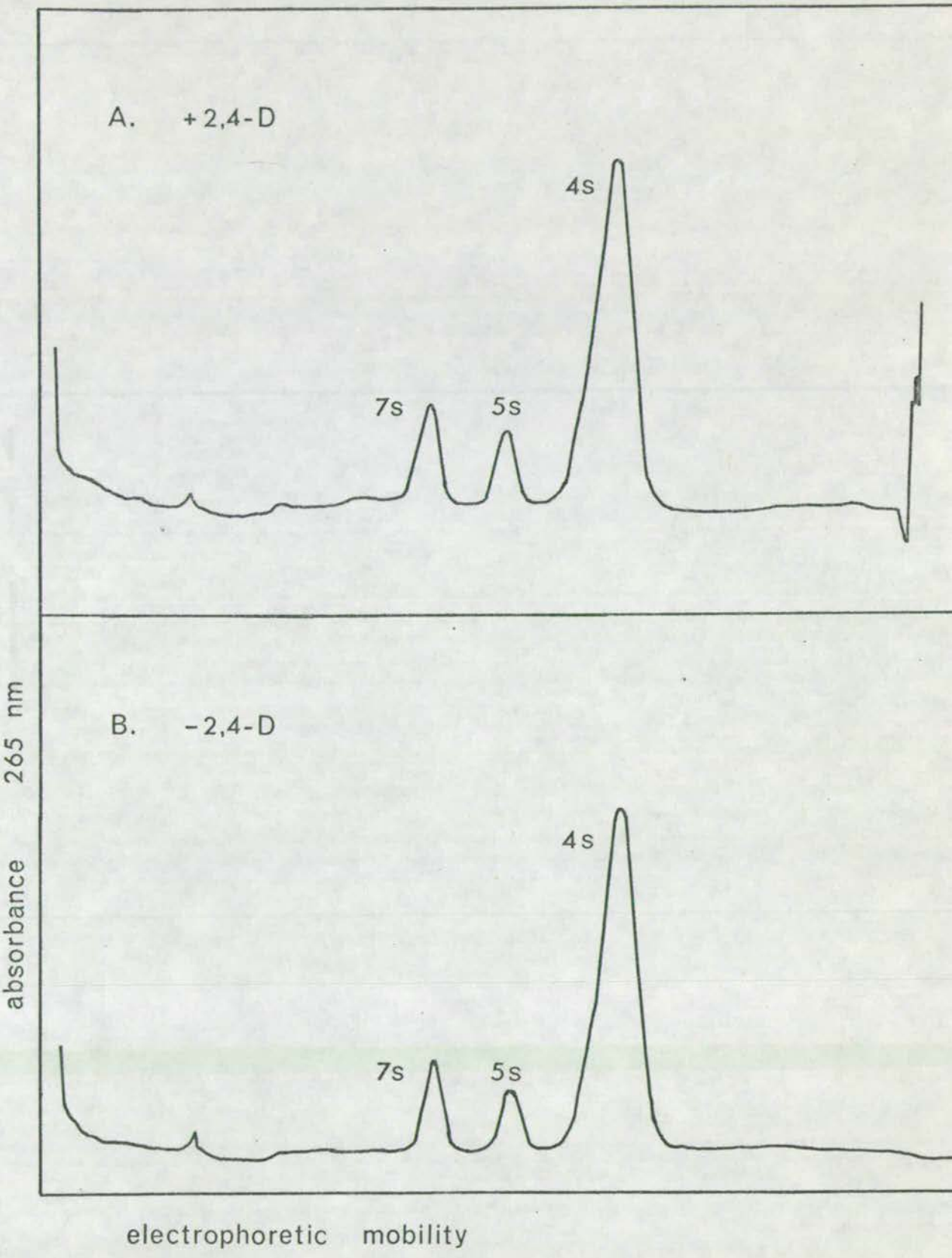

D
+90
J
-90
E
+120
K
-120
F
+180
L
-180
2.5
1.39
1.28
0.7
counts per minute
electrophoretic mobility

FIGURE 20.

Release of the 7S ribosomal RNA after denaturation of the total nucleic acid sample in formamide buffer

A nucleic acid sample from auxin treated and non-treated tissue was dissolved for 5 minutes in formamide buffer (Methods, C-5-b) prior to electrophoresis on 7.5% polyacrylamide gels (Methods, C-5-a). The continuous scan shows the absorbance at 265 nm.

FIG. 20


A. +2,4-D
4s
7s
5s
B. -2,4-D
4s
7s
5s
absorbance 265 nm
electrophoretic mobility

longer pulse times. In the dividing tissue, however, the majority of label was processed through to the mature rRNA after the same length of time.

Table 6 shows a comparison of the specific activities of the mature rRNAs in auxin treated and non-treated tissue as the labelling time was increased. The 40 fold greater specific activity of the mature 1.3×10^6 molecular weight rRNA of auxin treated tissue after a 60 minute pulse fell to a value of 10 fold after 3 hours. It had, at this point, reached the same differential exhibited by the 0.7×10^6 mol. wt. rRNA throughout the course of the 3 hour labelling. This is strong evidence for a slower processing of the 1.39×10^6 mol. wt. rRNA in the non-dividing tissue.

The total incorporation of label into the various rRNAs shown in Figures (16) and (19) may be expressed as counts/minute/cell (Figure 21, A and B). After a 90 minute pulse the incorporation of radioactivity into the 2.5×10^6 precursor had reached steady state in the auxin treated tissue and was closely approaching steady state in the non-treated tissue. At this time auxin treatment had increased incorporation into the 2.5×10^6 mol. wt. precursor rRNA 3.6 fold over the control. The mature 0.7 rRNA which is a good measure of the direct processing of the precursor was, however, increased approximately 11 fold. This suggests a 3 fold increase in the rate of processing of the rRNA in the auxin treated tissue.

(ii) Because rRNA was accumulating in the auxin treated tissue and the steady state level of accumulation of label into the 2.5 precursor rRNA was only increased approximately 3 fold, the enhancement of label in the 0.7 mature rRNA was probably not due to an increased turnover of rRNA in auxin treated tissue. It could instead, indicate

TABLE 6.

Comparison of the amount of ^{32}P-orthophosphate incorporation into mature rRNA in auxin treated and non-treated tissue during a time course labelling

Specific activity of the mature 1.3 x 10^6 rRNA and the 0.7 x 10^6 rRNA was calculated from the polyacrylamide-formamide gels presented in Figure (19). The combined peak of the 1.39 x 10^6 precursor rRNA and 1.3 x 10^6 mature rRNA from Figure (16) was also calculated. In each case the specific activity of the particular rRNA of the auxin treated tissue was compared to that of the control or non-treated tissue.

It was assumed that the specific activity of the 1.28 rRNA species (Figure 19) resulting from the release of the 7S particle upon treatment with formamide was equivalent to the intact 1.3 x 10^6 mature rRNA and hence, further reference is made to the intact form.

TABLE 6.

Label pulse (minutes)	Specific activity of rRNA in cpm/μg from formamide gels				Relative increase of incorporation of $^{32}P_i$ in rRNAs of auxin treated tissue over the control.		
	Control		Auxin				
	1.3	0.7	1.3	0.7	1.39 + 1.3	1.3	0.7
15	0	0	0	0	3.7	-	-
30	0	0	4.5	16.9	15.9	-	-
60	0.6	4.5	26.1	47.1	14.1	43.5	10.5
90	3.1	12.0	93.5	126.9	12.1	30.2	10.6
120	8.7	18.7	198.5	188.5	10.5	22.8	10.0
180	39.6	44.6	377.0	412.5	7.9	9.5	9.2

FIGURE 21.

Incorporation of radioactivity into the various rRNAs after increasing exposure to ^{32}P-orthophosphate

From Figure (16) and (19) the incorporation of label into the various ribosomal RNA species was re-expressed as counts/minute/cell, and these values were plotted against the length of the pulse in minutes in both auxin treated and non-treated tissue. The following notations are used:

2.5×10^6 - ●

1.39×10^6 - ○

0.98×10^6 - △

1.3×10^6 - ■

0.7×10^6 - □

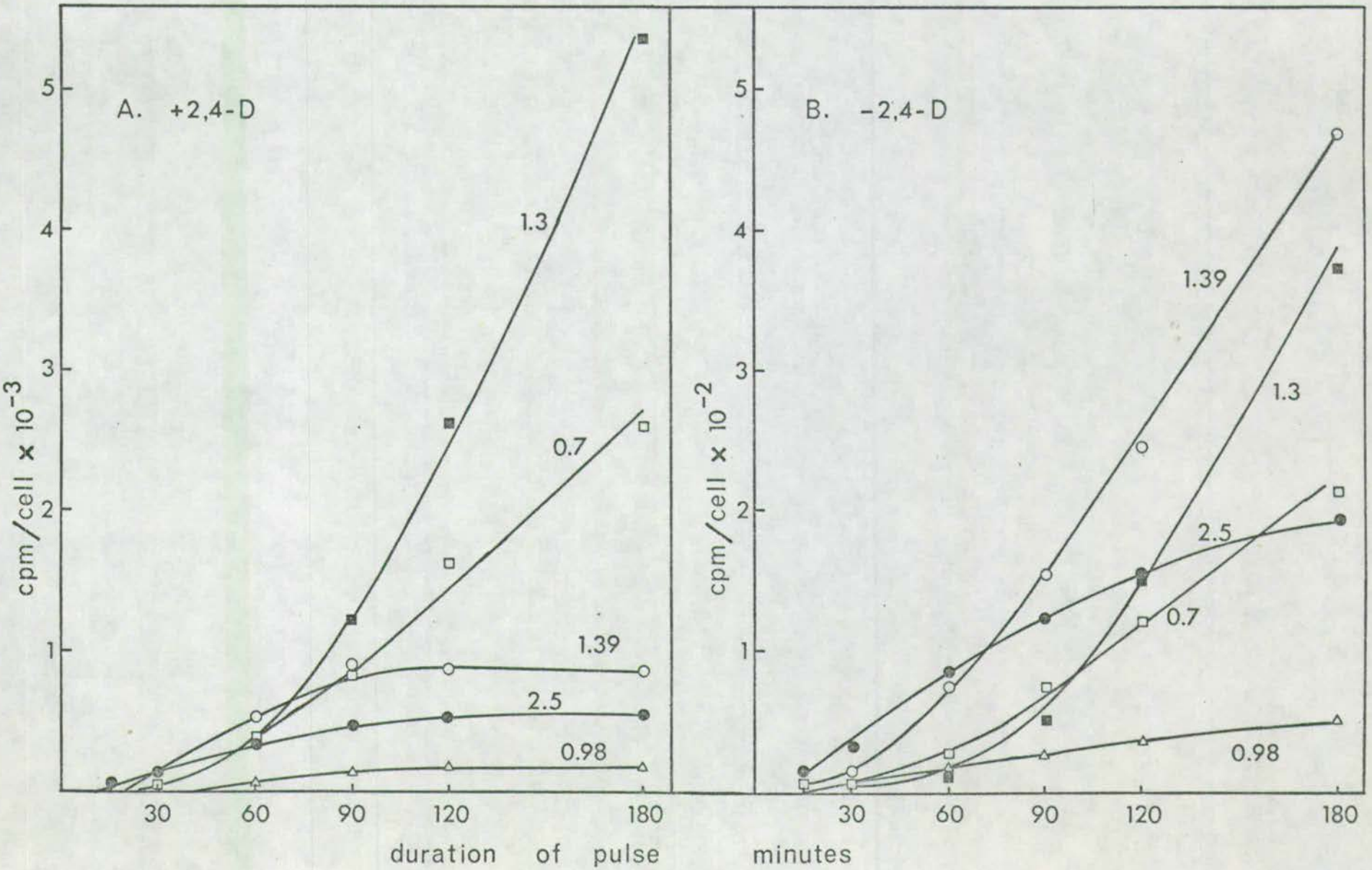

A. +2,4-D
B. -2,4-D
cpm/cell × 10^{-3}
cpm/cell × 10^{-2}
1.3
0.7
1.39
2.5
0.98
30
60
90
120
180
duration of pulse
minutes

FIG. 21

a decrease in the stability of this final rRNA product in the non-treated tissue. An effort was made to label the 0.7 x 10^6 rRNA to constant specific activity by giving the tissue a short pulse with $^{32}P_i$ followed by a long chase in order to stop further incorporation of label. Then it was hoped to compare the turnover rates of the mature 0.7 rRNA in auxin treated and non-treated tissue during further incubation. If there was no further incorporation of label upon establishment of the chase the only event that would alter the specific activity would be the synthesis of unlabelled RNA. Figure 22, (A,B,C.) shows the results of the experiment. Since the mature 0.7 rRNA of both cultural states continued to incorporate $^{32}P_i$ (Figure 22, B) the chase was incomplete with only a gradual dilution of label. Therefore, it was not possible to obtain a measure of the stability of rRNA in either cultural state. There was, however, one interesting observation. Although there was no increase in cell number in the non-treated explants and the accumulation of rRNA had reached steady state (Figure 22, A), the tissue was still incorporating radioactivity into stable rRNA (Figure 22, B). Also, in Figure 22, (c), the specific activity of the 0.7 x 10^6 ribosomal RNA was decreasing with exposure to chase conditions. These results suggest that rRNA was being synthesized in the non-treated tissue but was relatively unstable.

(b) Kinetics of labelling of rRNA precursors

The stability and labelling of the rRNA precursors in auxin treated and non-treated artichoke tissue was measured as described in detail by Chapman and Ingle, (1976). For these measurements I used the relative proportions of the various rRNAs depicted in Figure 21, (A and B). I did, however, correct these values to constant specific activity on the basis of linear incorporation of label into

FIGURE 22.

Results of the attempt to measure stability of the 0.7×10^6 mature rRNA in auxin treated and non-treated tissue

The explants were incubated for 30 hours in media containing or lacking 2,4-D when a 20 minute pulse was given with ^{32}P-orthophosphate. They were subsequently washed with sterile distilled water and transferred to fresh media containing 1 mM phosphate (Methods, C-3-b). The explants were incubated a further 12 hours before samples were removed. The nucleic acids were fractionated on 2.3% polyacrylamide gels (Methods, C-5-a) and the radioactivity in the 0.7×10^6 mol. wt. rRNA peak was determined (Methods, B-4-b-ii).

A. Level of mature rRNA accumulated at various cultural ages and expressed as µg/explant.

2,4-D 1.3×10^6 rRNA, (■) ; 0.7×10^6 rRNA, (●).

control 1.3×10^6 rRNA, (□) ; 0.7×10^6 rRNA, (○).

B. Amount of radioactivity incorporated into the mature 0.7×10^6 rRNA in cpm/explant at various times after the removal of the isotope.

C. Specific activity of the 0.7×10^6 rRNA in cpm/µg with increasing exposure to chase conditions.

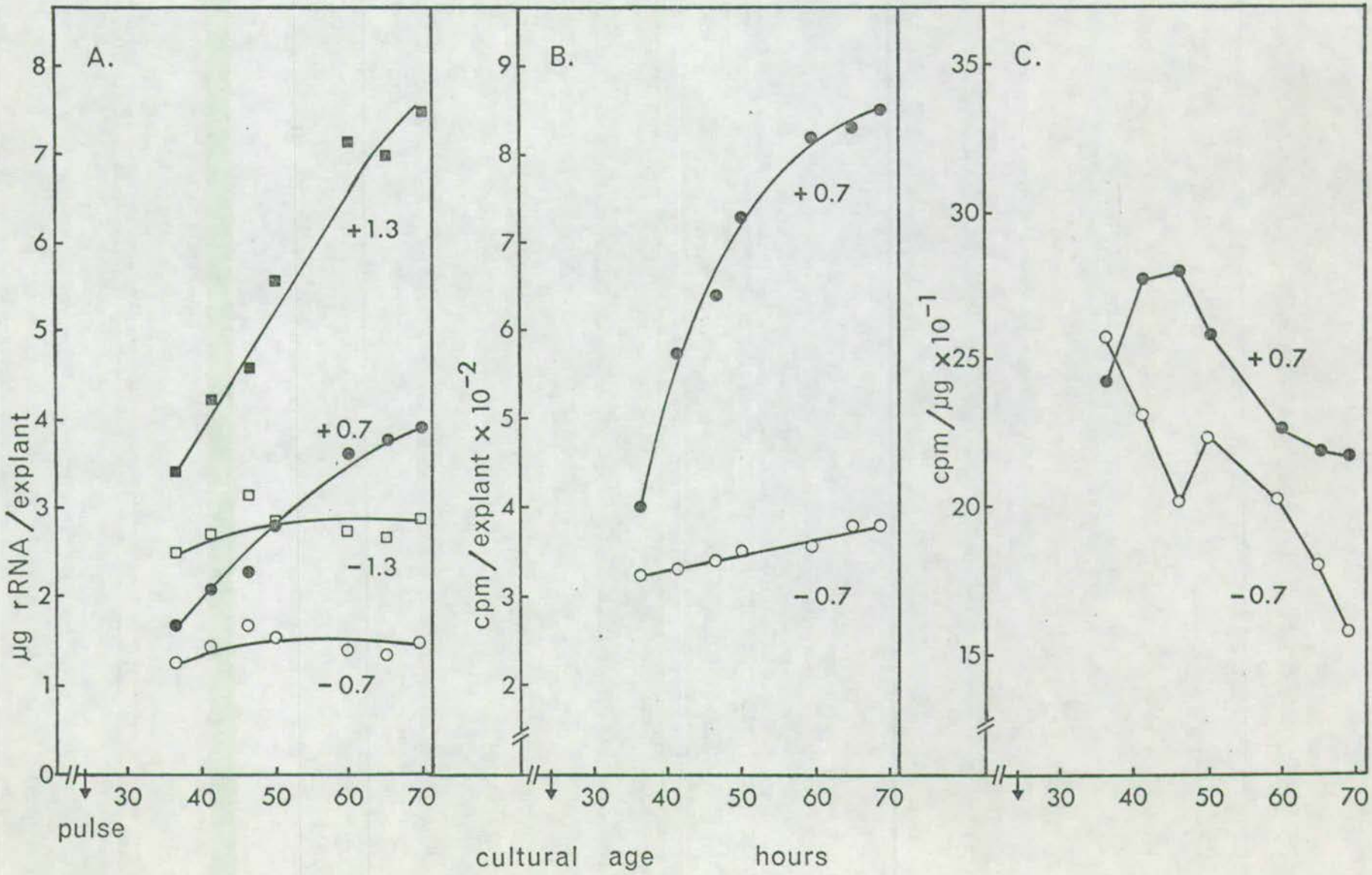

A.
B.
C.
µg rRNA/explant
cpm/explant x 10^{-2}
cpm/µg x 10^{-1}
+1.3
+0.7
-1.3
-0.7
+0.7
-0.7
+0.7
-0.7
pulse
cultural age hours

FIG. 22

DNA (Table 7). Although Chapman and Ingle, (1976) found that the accumulation of label into DNA was linear after 60 minutes, here a slightly longer time of at least 90 minutes was required (Figure 23, A). Correction for the lower specific activity of the nucleotide pool at the shorter labelling periods was made from this curve by comparing the slope of the tangent to the curve at various times with the slope between 120 and 180 minutes. After correcting the values in Figure 21, (A and B) for specific activity of the nucleotide pool, the new values for the incorporation of ^{32}P-orthophosphate into the rRNAs per cell is presented in Figure 23, (C and B). Since DNA remained unlabelled in the non-dividing tissue, it had to be assumed that the rate of equilibration of the precursor pool was the same as in the auxin treated tissue. As the uptake levels were similar for $^{32}P_i$ in each cultural state and the precursor rRNAs had reached steady state, this assumption appeared reasonable. In addition, the levels of ATP in the tissue were also similar (Figure 26).

From Figure 23, (B and C) the 2.5 x 10^6 molecular weight precursor was the most rapidly labelled rRNA in both cultural states, although it reached a steady state after approximately 40 minutes in the 2,4-D treated explants compared to about 90 minutes in the non-treated tissue. The most obvious difference in the processing rates between the two cultural states is shown by the 1.39 x 10^6 molecular weight precursor rRNA. After 60 minutes the 1.39 precursor in the auxin treated explants reached a steady state level, and accumulation of label into the 1.3 x 10^6 rRNA was linear. In the control tissue, however, the steady state value of the 1.39 x 10^6 precursor had not been reached by 180 minutes and linear accumulation of label into the 1.3 x 10^6 rRNA was just beginning. The approach to steady state labelling of the

TABLE 7.

Data for the incorporation of label (cpm/cell) into the precursor and mature rRNAs of auxin treated and non-treated tissue. Correction of these values on the basis of incorporation of label into DNA

Using the data presented in Figure (23,A) the incorporation of ^{32}P-orthophosphate into DNA was taken to be approximately linear between 120 and 180 minutes of labelling. By comparing the tangent to the curve at the various labelling times with the slopes between 120 and 180 minutes, correction values were obtained for the lower specific activity of the nucleotide pool at the shorter labelling periods. These values are expressed as a percentage of the slope of linear incorporation. The radioactivity in the various rRNAs depicted in Figure 21, (A and B) and in the Table were corrected to constant specific activity using the correction values.

TABLE 7.

Treatment	Duration of pulse with $^{32}P_i$ (minutes)	Various species of ribosomal RNA ($y \times 10^6$ mol. wt.). radioactivity incorporated (cpm/cell)						Correction to constant specific activity in percent.
		2.5	1.39 + 1.3	1.39	1.3	0.98	0.7	
2,4-D	15	67	26	26	-	6	11	
	30	138	205	157	47	24	45	
	60	338	903	534	369	53	394	
	90	446	2117	909	1209	117	817	
	120	519	3454	847	2607	160	1613	
	180	534	6214	837	5377	163	2600	
2,4-D corrected values	15	420	161	161	-	40	71	16
	30	531	787	605	182	90	172	20
	60	734	1964	1162	802	116	857	46
	90	637	3025	1298	1726	168	1167	70
	120	530	3525	864	2661	164	1646	98
	180	534	6214	837	5377	163	2600	100
control	15	16	10	10	-	-	5	
	30	33	15	15	-	7	14	
	60	84	86	75	11	18	26	
	90	123	206	155	51	26	75	
	120	157	398	245	153	36	121	
	180	195	841	467	374	50	215	
control corrected values	15	101	59	59	-	-	32	16
	30	138	58	58	-	27	55	20
	60	183	186	163	23	38	56	46
	90	176	295	221	73	36	107	70
	120	160	406	250	156	37	124	98
	180	195	841	467	374	50	215	100

FIGURE 23.

The incorporation of radioactivity into the various ribosomal RNAs of auxin treated and non-treated tissue after correction to constant specific activity

The specific activity of the nucleotide pool was corrected on the basis of the incorporation of ^{32}P-orthophosphate into DNA (A). Using this correction, explained for the previous Figure, and the results presented in Figure 21, (A and B) and Table (7), the incorporation of label into the various ribosomal RNAs of control (B) and auxin treated (C) tissue were found. The following notations are used :

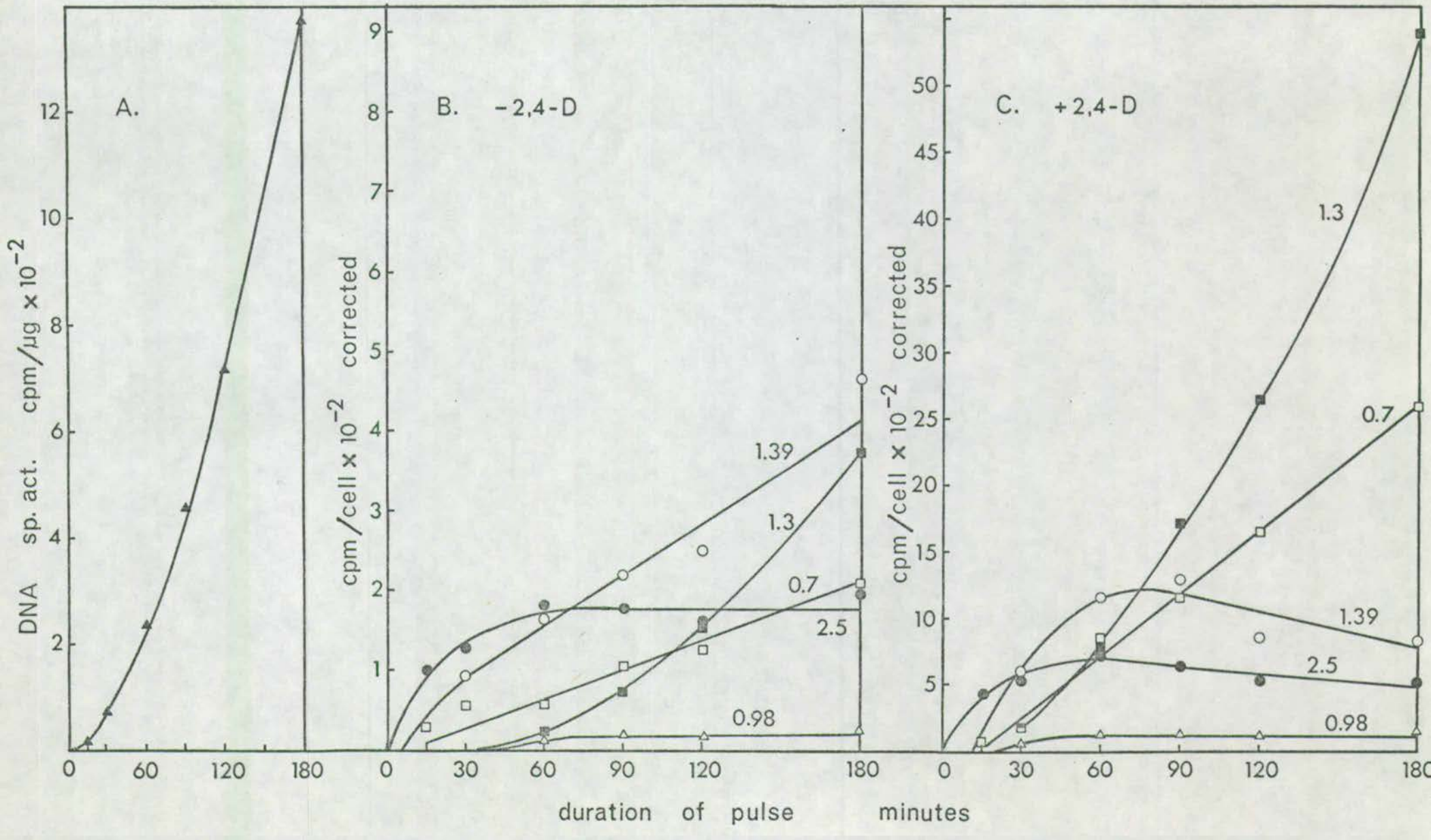
2.5×10^6 - ●

1.39×10^6 - ○

0.98×10^6 - △

1.3×10^6 - ■

0.7×10^6 - □


A.
B. -2,4-D
C. +2,4-D
DNA sp. act. cpm/µg x 10⁻²
cpm/cell x 10⁻² corrected
cpm/cell x 10⁻² corrected
1.39
1.3
0.7
2.5
0.98
1.3
0.7
1.39
2.5
0.98
duration of pulse minutes

0.98×10^6 precursor rRNA appeared similar in both cultural states with the plateau being reached after 60 minutes. Since the incorporation of ^{32}P-orthophosphate into the 0.98×10^6 rRNA was very low and somewhat variable in these experiments, further analysis on this species of rRNA was not carried out. A more comprehensive study of the labelling pattern of this rRNA in the nucleus has been done by Chapman and Ingle, (1976). Incorporation of radioactivity into the 0.7×10^6 mature rRNA was linear after 60 minutes in both control and auxin treated tissue, but the rate of incorporation of label was increased 12 fold over that of the control.

A first-order-decay analysis for the 2.5×10^6 precursor rRNA and the 1.39×10^6 precursor rRNA in auxin treated and non-treated tissue is shown in Figure 24, (A,B,C), as described in detail by Chapman and Ingle, (1976). From the slopes of the lines, the half-lives of the rRNAs were calculated and are presented in Table (8). It was not possible to measure the half-life of the 1.39×10^6 precursor rRNA in the non-dividing tissue since steady state had not been reached within the experimental period.

The rate of synthesis of the 2.5×10^6 precursor was related to that of the mature rRNAs also as described by Chapman and Ingle, (1976). From Table (8), it is evident that close to 100% of the 2.5×10^6 precursor was conserved in the 0.7×10^6 mature rRNA while approximately 90% of the label was conserved in the 1.3×10^6 rRNA of the auxin treated tissue. In the non-treated tissue only 50% of the label initially incorporated into the 2.5×10^6 precursor was conserved in the 0.7×10^6 mature rRNA. The results provide additional evidence for increased ribosome turnover in artichoke tissue which has not been stimulated to divide by auxin.

FIGURE 24.

First-order-decay analysis of the precursor ribosomal RNAs of auxin treated and non-treated tissue

The radioactivity-accumulation curves for the 2.5×10^6 and 1.39×10^6 precursor rRNAs were analysed according to Chapman and Ingle, (1976) using an equation developed by Greenberg, (1972) and Brandhorst and McConkey, (1974).

If the specific activity of the nucleotide precursor pool is constant over the period of labelling a class of RNA synthesized after a specific time, t=0, will accumulate according to equation 1.

$$S_t = S_\infty \left[1 - e^{-\ln 2 (1/T_D + 1/T_{\frac{1}{2}})t} \right] \qquad 1.$$

where S_t = the amount of RNA of that class/cell accumulated at time t.

S_∞ = steady state amount of that class of RNA/cell.

$T_{\frac{1}{2}}$ = half-life of that class of RNA.

T_D = Cell doubling time (15 hours for H. tuberosus in this culture system).

This equation describes a molar accumulation curve in which it is assumed that RNA decays with first-order kinetics and is synthesized with zero-order kinetics.

The data presented in Figure 23, (B and C) was plotted as $\ln(S_\infty - S_t)/S_\infty$ against time. From the slopes of the lines the half-lives for the 2.5×10^6 rRNA precursor in both cultural states and that of the 1.39×10^6 precursor in the auxin treated tissue were calculated and are presented in Table (8).

2.5×10^6 precursor rRNA of auxin treated (A) and control (B)

1.39×10^6 precursor rRNA of auxin treated explants (C)

FIG. 24

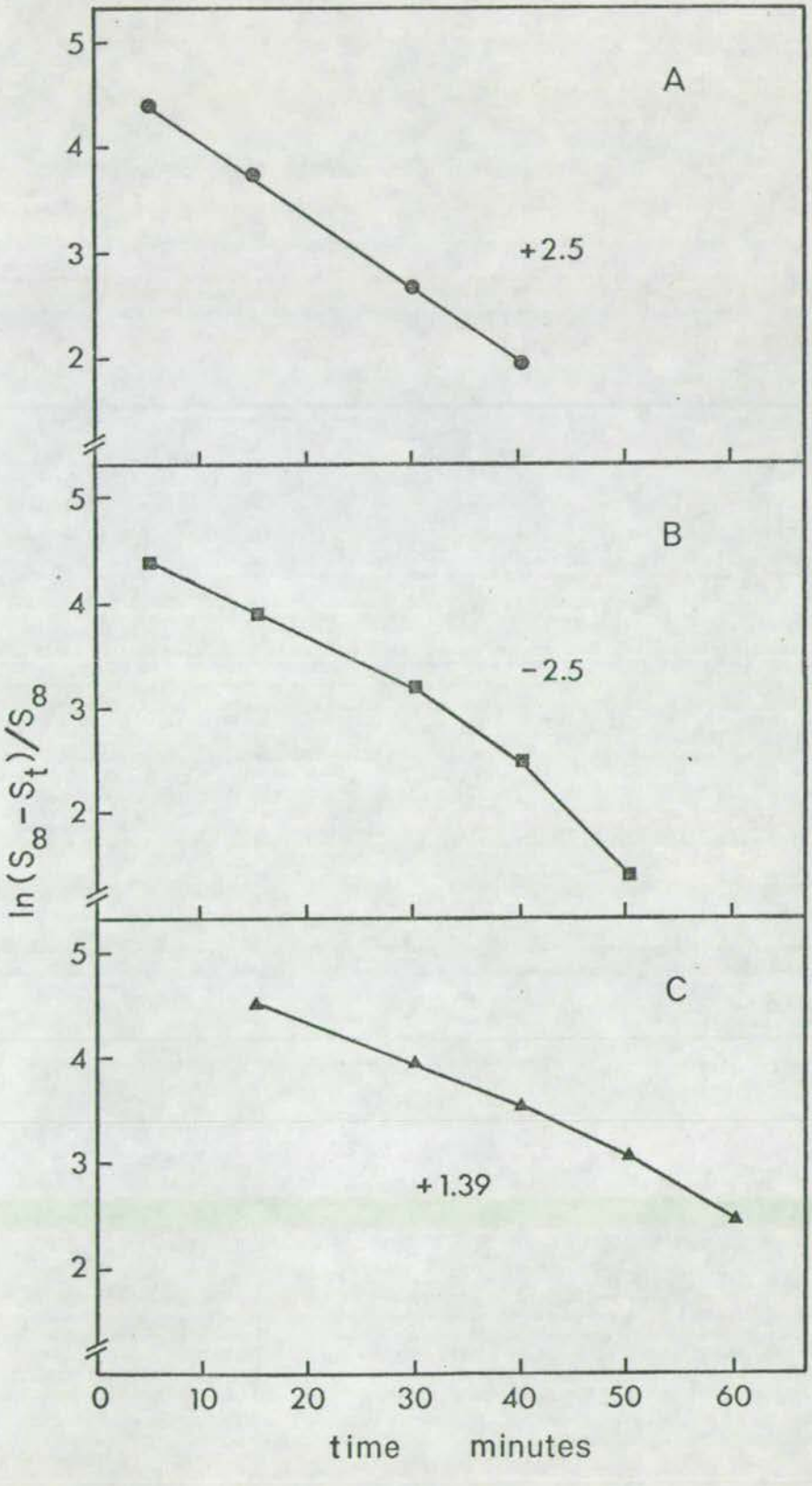

A
+2.5
B
−2.5
C
+1.39
$\ln(S_\infty - S_t)/S_\infty$
5
4
3
2
0
10
20
30
40
50
60
time minutes

TABLE 8.

Data on the kinetics of labelling of the precursor ribosomal RNA of auxin treated and non-treated tissue

Using the differentiated form of the equation (1.) :

$$\frac{dS}{dt} = S_{\infty} \ln 2(1/T_D + 1/T_{\frac{1}{2}})$$

the synthesis of the precursor rRNAs was calculated and related to that of the mature rRNAs. The rate of synthesis of the mature rRNA was taken from Figure 23, (B and C) and expressed as cpm/cell/hour. The amount of radioactivity which was being incorporated into the mature forms was used as an estimate of the percent of rRNA that was conserved from the precursor state and not degraded in some way.

The calculations for the proportion of mature rRNA in the precursor are shown below :

2,4-D : rate of synthesis of 0.7×10^6 = 920 cpm/cell/hour

$$920 \times \frac{2.5}{0.7} = 3285.7$$

: rate of synthesis of 1.3×10^6 = 2000 cpm/cell/hour

$$2000 \times \frac{2.5}{0.7} = 3846.2$$

control : rate of synthesis of 0.7×10^6 = 110 cpm/cell/hour

$$110 \times \frac{2.5}{0.7} = 392.9$$

TABLE 8.

TREATMENT	Precursor rRNA x 10^6	Plateau level = S_∞ (cpm/cell)	Slope (Δ /hour)	Half-life or $T_{\frac{1}{2}}$ (minutes)	Rate of synthesis cpm/cell/hour		Percent conserved (%)
					$\frac{dS}{dt}$ precursor rRNA	mature rRNA	
2,4-D	2.5	700	-4.2	10.0	2940	3285.7	100
	1.39	1300	-2.3	18.6	4248	3846.2	90
control	2.5	180	3.0	14.0	775.2	392.9	50

(c) Quantitative measurement of the synthesis of RNA

In the previous experiment, the kinetics of turnover of the rRNA precursors were calculated by correcting the precursor pool to constant specific activity on the basis of 3 observations (see previous) which Chapman and Ingle, (1976) regarded as adequate for the artichoke. I was then able to use the incorporation of ^{32}P-orthophosphate in RNA as a measure of synthesis. Since there are certain misgivings in relying on a single technique to measure a particular event, an alternative method of measuring RNA synthesis was attempted. Here, the specific radioactivity of ATP, a direct precursor to the macromolecule, was measured after labelling the tissue with tritiated adenosine and this was related to the amount of label incorporated. The technique has been described in detail by Humphreys, (1973) and is outlined in the Methods, (C-3-d).

The specific activity is, by definition, counts/minute/µg. To determine this I needed an estimate of total ATP and its radioactivity after each labelling period. This was achieved by using the luciferin-luciferase assay (Methods, C-3-d-ii) to measure ATP in a small, partially purified extract of nucleotides after separation by thin-layer chromatography. The radioactivity in the ATP sample was subsequently determined by liquid scintillation (Methods, B-4-b-i). In these experiments the total ATP pool was measured and related to the synthesis of RNA, disregarding possible compartmentalization of the ATP serving as RNA precursor. While this assumption may not be totally valid, the difficulty in testing for this occurrence and the unreliability of the results are points in favour of using the specific radioactivity of the total ATP pool. Also, measurements of RNA synthesis in mammalian systems, Brandhorst and Humphreys, 1971 ;

Greenberg, 1972) indicate that compartmentalization is highly transient or non-existent.

Figure 25, (A,B,C,D) shows that the specific radioactivity of the total ATP pool of non-treated tissue was consistently higher than that of auxin treated tissue. The slightly smaller pool size in the non-treated tissue would, apparently, account for this (Figure 26, A and B). It is important to note that the specific radioactivity of the ATP pool did not reach steady state until after 3 hours of labelling. Since the accuracy and vigour of the Humphreys technique depends on the rapid equilibration of the radioactive ATP pool this method was not suitable for an investigation of the half-lives of the short-lived rRNA precursors in the artichoke.

I did attempt to measure the synthesis of total cellular RNA, which includes precursor rRNA, mature rRNA, soluble RNA and the various polydisperse RNAs. I then compared the calculated rate of synthesis of RNA by the Humphreys method to the observed rate of accumulation of rRNA. The radioactivity in RNA was measured and corrected for the conversion of adenosine to guanosine (Table 9) to give the radioactivity of AMP in RNA/explant (Table 10) which was subsequently converted to counts/minute/µg RNA (Methods, C-3-d-iii and iv). The specific radioactivity of RNA is plotted in Figure 27, (A,B,C,D). After determining the rate of change in the specific radioactivity of RNA for each sample time and using the appropriate specific activity of ATP in counts/minute/µg (Figure 25), I calculated the rate of synthesis of RNA in µg/hour as described in the Methods, (C-3-d-i and iv), and as shown in Table (11). It is evident from these results that I have an error in the calculations since the rate of synthesis of RNA, which should have been the same at each

FIGURE 25.

Specific activity of total cellular ATP at various cultural ages of auxin treated and non-treated tissue

The luciferin-luciferase assay was used to measure ATP in a small, partially purified extract of nucleotides obtained by thin-layer chromatography (Methods, C-3-d-ii). An estimate of the radioactivity in each eluted sample was made by liquid scintillation (Methods, B-4-b-i). The specific activity of ATP in auxin treated and non-treated tissue at the cultural ages of 25, 43, 48 and 70 hours were compared.

2,4-D - ●

control - ○

FIG. 25

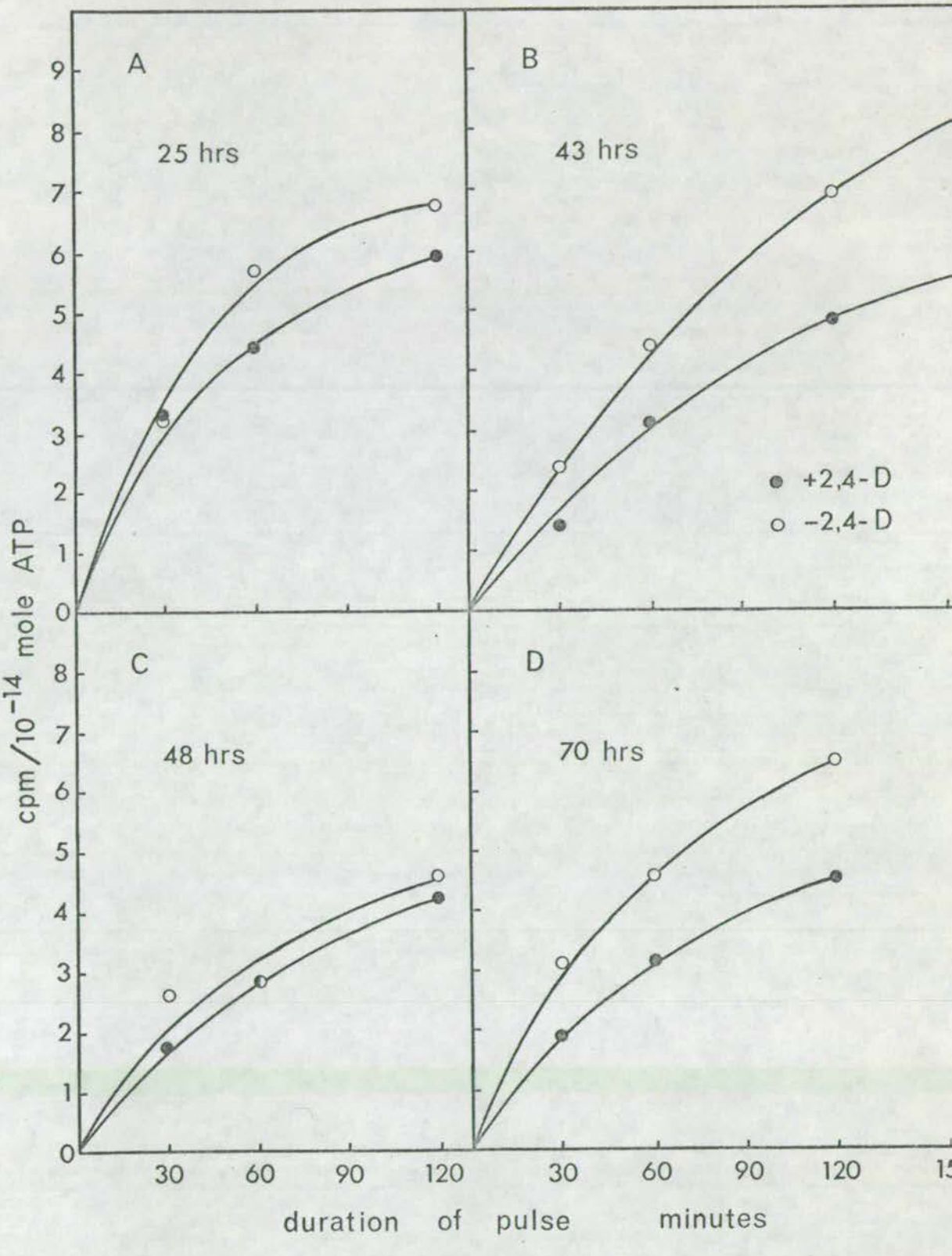

A
25 hrs
B
43 hrs
C
48 hrs
D
70 hrs
+2,4-D
-2,4-D
cpm/10⁻¹⁴ mole ATP
duration of pulse minutes
0
1
2
3
4
5
6
7
8
9
30
60
90
120

FIGURE 26.

Estimation of total cellular ATP in auxin treated and non-treated explants at various cultural ages

A perchloric acid (PCA) extract of nucleotides from total tissue was partially purified by thin-layer chromatography. The spot corresponding to standard ATP was eluted and the luciferin-luciferase assay was used to measure the amount of ATP in the sample by comparing it to a set of standards (Methods, C-3-d-ii). The values shown are an average of 3 to 4 replicates.

A. - 2,4-D

B. - control

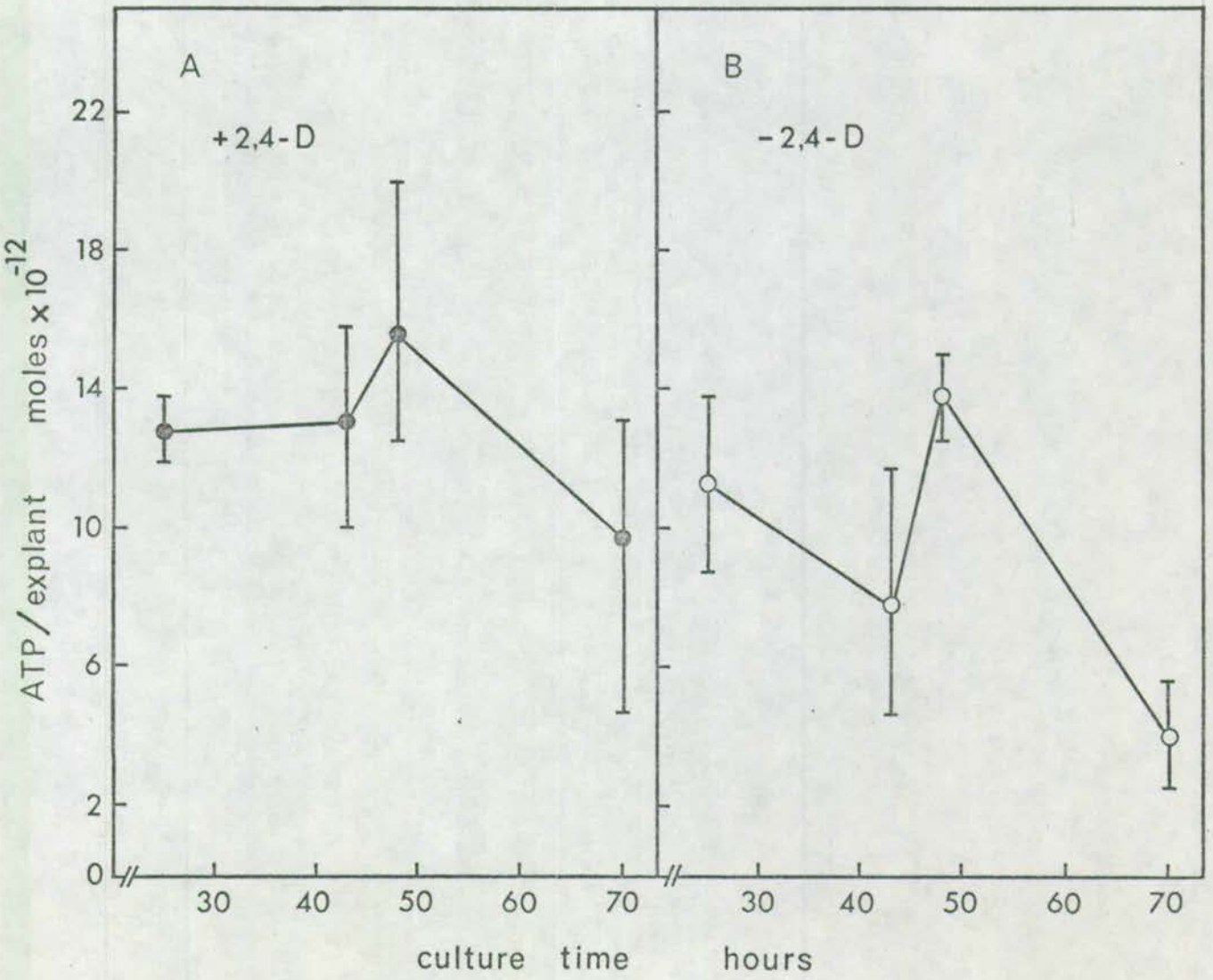

A
+2,4-D
B
-2,4-D
ATP / explant moles x 10^-12
22
18
14
10
6
2
0
30
40
50
60
70
culture time hours

FIG. 26

TABLE 9.

Estimation of the conversion of adenosine to guanosine in auxin treated and control tissue after increasing exposure to (G-^{3}H) adenosine

To determine the amount of radioactive AMP in RNA, the perchloric acid (PCA) pellet from a total tissue extraction was hydrolysed overnight in alkali and the nucleotides separated by thin-layer chromatography (Methods, C-3-d-iii). The radioactivity in the AMP and GMP spots was measured and compared (Methods, B-4-b-i).

TABLE 9.

Treatment	Cultural age (hours)	Duration of pulse (minutes)	Radioactivity in AMP spot (cpm)	Radioactivity in GMP spot (cpm)	Fraction of cpm in RNA in AMP
2,4-D	25	30	2492	768	0.76
		60	6868	2452	0.74
		120	19668	7816	0.72
	43	30	16177	2132	0.88
		60	35839	5202	0.87
		120	185455	51500	0.78
		180	290734	91633	0.76
	48	30	3220	1236	0.72
		60	10516	4240	0.71
		120	22844	9696	0.70
	70	30	44229	5071	0.89
		60	89752	21659	0.80
		120	274654	70347	0.77
control	25	30	1420	452	0.76
		60	5144	1556	0.77
		120	9256	3268	0.74
	43	30	9042	5330	0.63
		60	20164	6941	0.74
		120	110237	17412	0.86
		180	130178	29288	0.81
	48	30	1384	404	0.77
		60	2916	884	0.77
		120	7940	1824	0.81
	70	30	106755	53287	0.66
		60	115388	75604	0.60
		120	128363	142322	0.46

TABLE 10.

Calculations for the incorporation of radioactive adenosine into the nucleic acids of auxin treated and non-treated tissue

Using the formulas presented in the Methods, (C-3-d-iv) the counts per minute in DNA and the counts per minute in AMP in RNA were calculated by dividing the radioactivity in DNA and RNA by the number of explants and multiplying the latter by the fraction which was in AMP. The data for the specific activity of ATP with increasing exposure to (G-^{3}H) adenosine, shown in Figure (25), is also presented.

TABLE 10.

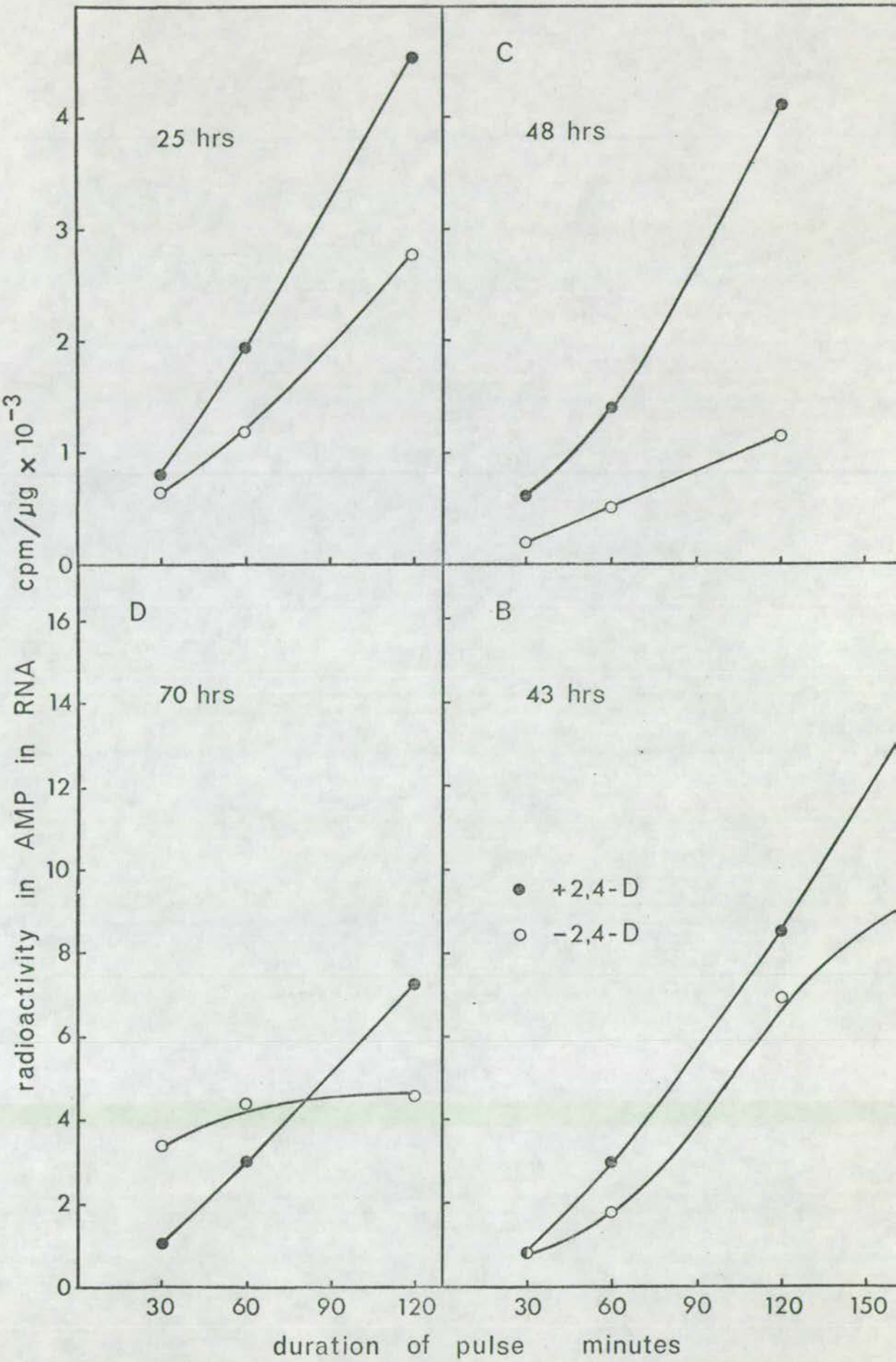
Treatment	Cultural age (hours)	Duration of pulse (minutes)	Radioactivity in DNA (cpm/explant)	Radioactivity in RNA (cpm/explant)	Radioactivity in AMP in RNA (cpm/explant)	Specific activity of ATP (cpm/10^{-12} mole)
2,4-D	25	30	120	3830	2926	330
		60	226	12247	9026	441
		120	814	37549	26885	593
	43	30	4.67×10^5	9.61×10^3	8.46×10^3	142
		60	2.87×10^6	2.16×10^4	1.88×10^4	311
		120	6.13×10^7	6.70×10^4	5.23×10^4	484
		180	1.25×10^8	1.08×10^5	8.21×10^4	575
	48	30	202	5064	3661	176
		60	711	12917	9210	286
		120	1909	39217	27530	424
	70	30	6.51×10^5	1.29×10^4	1.15×10^4	294
		60	9.82×10^6	3.38×10^4	2.70×10^4	315
		120	1.30×10^8	9.03×10^4	6.95×10^4	450
control	25	30	135	2828	2147	328
		60	201	8696	6678	573
		120	411	22367	16529	675
	43	30	1.82×10^5	7.88×10^3	4.96×10^3	236
		60	2.54×10^5	1.40×10^4	1.04×10^4	438
		120	2.63×10^6	3.51×10^4	3.02×10^4	688
		180	7.89×10^6	5.15×10^4	4.17×10^4	852
	48	30	72	1079	835	260
		60	169	3571	2739	288
		120	375	7287	5924	456
	70	30	2.21×10^6	3.86×10^4	2.55×10^4	317
		60	2.04×10^6	4.81×10^4	2.89×10^4	458

FIGURE 27.

Change in the specific radioactivity of AMP in RNA in auxin treated and control explants with increasing exposure to tritiated adenosine

The counts per minute in AMP in RNA/explant was calculated (Methods, C-3-d-iv and Table 10) and this was divided by the amount of RNA in a single explant (Table 11). Data for both auxin treated and non-treated tissue after 25, 43, 48 and 70 hours of culture are plotted : 2,4-D (●) and control (○).

FIG. 27


A
25 hrs
C
48 hrs
D
70 hrs
B
43 hrs
+2,4-D
−2,4-D
radioactivity in AMP in RNA cpm/µg x 10⁻³
0
1
2
3
4
0
2
4
6
8
10
12
14
16
30
60
90
120
150
duration of pulse minutes

pulse time, was in fact decreasing as the length of the pulse was increased.

Both Brandhorst and Humphreys, (1971) and Walbot et al, (1972) have reported that the ATP pool is normally equilibrated with externally-added adenosine within 10 minutes and after this time period, incorporation of label into RNA was linear. As can be seen from Figure (27), incorporation of label into RNA was linear after 30 minutes but the specific activity of ATP continued to increase for a further $2\frac{1}{2}$ hours (Figure 25). This behaviour is kinetically incompatible and suggests to me that the ATP spot was probably contaminated by some other slowly-equilibrating nucleotide. As a consequence the specific activity of ATP was over-estimated and became progressively worse with time resulting in a declining value for the calculated rate of RNA synthesis. I suspect that this contaminant may be labelled GMP which apparently does not separate from ATP in this chromatographic system. The conversion of labelled adenosine to GMP appears fairly active in the artichoke (Table 9) and since GMP fails to be detected in the luciferin-luciferase assay the net effect is an over-estimation of the specific activity of ATP. This in turn would result in a gross under-estimation of the actual rate of RNA synthesis. In order to reduce the effect due to the radioactive contaminant to a minimum I have used the 30 minute pulse sample from Table (11), to compare with the observed rate of accumulation of rRNA at each cultural age as shown in Figure (10). From Table (12), it would appear that rRNA was, on average, accumulating at 6 fold the rate of total RNA synthesis, whereas in reality synthesis should far exceed accumulation.

In conclusion, the Humphreys technique was not suitable for the estimation of either the half-lives of the rRNA precursors or the

TABLE 11.

Data : Calculated rates of synthesis of RNA at various times in the culture of auxin treated and non-treated tissue

After 25, 43, 48 and 70 hours of culture both auxin treated and non-treated tissue were pulse labelled with tritiated adenosine for 30, 60, 120 and 180 (for the 43 hour sample) minutes. An estimate of RNA in a PCA-insoluble extract was made and the amount of radioactive AMP in RNA was calculated (Methods, C-3-d-iii and Iv). The specific activity of ATP (PCA-soluble extract) depicted in Figure (25) as cpm/pmole was re-expressed as cpm/µg (S_A); and after determining the rate of change in the specific radioactivity of RNA at each sample time, the rate of synthesis of RNA in µg/hour was calculated (Methods, C-3-d-i) by the following equation :

$$\frac{dS_R}{dt} = S_A \times K_S, \quad \text{where } K_S = \text{rate constant of synthesis}$$

TABLE 11.

Treatment	Cultural age (hours)	Duration of pulse (minutes)	RNA µg/explant	Specific activity of AMP in RNA cpm/µg	Rate of synthesis of RNA cpm/µg/hour	Specific activity ATP cpm/µg	K_S/hour	Rate of synthesis of RNA µg/hour
2,4-D	25	30	3.6	806	1900	1.0×16^6	0.0019	0.014
		60	4.6	1954	2500	1.3×10^6	0.0019	0.009
		120	5.9	4534	3800	1.8×10^6	0.0021	0.006
	43	30	7.9	1068	3600	4.3×10^5	0.0084	0.132
		60	6.3	2965	4500	9.4×10^5	0.0048	0.030
		120	6.1	8574	6000	1.5×10^6	0.0040	0.012
		180	5.3	15375	9600	1.7×10^6	0.0056	0.010
	48	30	5.8	631	1500	5.3×10^5	0.0028	0.032
		60	6.5	1410	2100	8.7×10^5	0.0024	0.016
		120	6.7	4103	3000	1.3×10^6	0.0023	0.0075
	70	30	10.3	1117	2700	8.9×10^5	0.0030	0.062
		60	8.9	3044	3900	9.5×10^5	0.0041	0.036
		120	9.7	7165	5400	1.4×10^6	0.0039	0.019
control	25	30	3.3	656	1260	1.0×10^6	0.0013	0.008
		60	5.6	1193	1440	1.7×10^6	0.0008	0.004
		120	5.9	2787	1840	2.0×10^6	0.0009	0.003
	43	30	5.8	854	2200	7.2×10^5	0.0031	0.036
		60	5.8	1790	3300	1.3×10^6	0.0025	0.015
		120	4.4	6895	4500	2.1×10^6	0.0021	0.005
		180	4.4	9521	5000	2.6×10^6	0.0019	0.003
	48	30	4.0	209	500	7.9×10^5	0.0006	0.004
		60	5.4	504	660	8.7×10^5	0.0007	0.004
		120	5.1	1166	850	1.4×10^6	0.0006	0.002
	70	30	7.4	3427	2700	9.6×10^5	0.0028	0.021

TABLE 12.

Comparison of the observed rate of accumulation of rRNA to the calculated rate of synthesis using the Humphreys method, (1973).

The rate of accumulation of ribosomal RNA in auxin treated and non-treated explants was measured by drawing a tangent to the rRNA curves (Figure 10) at 25, 43, 48 and 70 hours of culture time. This rate was compared to the calculated rate of synthesis of RNA (Methods, C-3-d) when a 30 minute pulse with (G-^{3}H) adenosine was given.

TABLE 12.

Treatment	Cultural age (hours)	Rate of accumulation of rRNA µg/hour	Calculated rate of RNA synthesis µg/hour	Increase in observed rate over calculated rate
2,4-D	25	0.19	0.014	13 x
	43	0.25	0.132	2 x
	48	0.15	0.032	5 x
	70	0.38	0.062	6 x
control	25	0.05	0.008	6 x
	43	0	0.004	-
	48	0	0.036	-
	70	0	0.021	-

synthesis of total RNA in the artichoke tissue. This was particularly due to the long time taken for the specific radioactivity of the ATP pool to reach steady state. The situation would probably not have been improved by the addition of a higher concentration of radioactive adenosine; firstly because adenine is a known precursor of various plant growth substances and secondly, because of the build up of labelled contaminants with increasing exposure to the isotope. In retrospect, I should have hydrolysed the radioactive ATP spot and rechromatographed it to separate AMP from GMP thus obtaining a better measure of the proportion of label present as ATP. Due to low yield, however, it was not possible to do this since an entire ATP sample from 20 to 30 explants had to be used for the assay. From these experiments I was only able to conclude that the nucleotide precursor pool was slightly smaller in the non-treated tissue and therefore resulted in an enhanced specific radioactivity of the ATP pool.

Discussion

Because it was not possible to measure the rate of synthesis of RNA by the Humphreys method, (1973) it was necessary to rely on the ^{32}P-orthophosphate data and the method of Chapman and Ingle, (1976) for the calculation of the stability and processing of the rRNA precursors. I found that the control of rRNA accumulation operates at both the level of transcription and post-transcription. From the plateau levels of the radioactivity-accumulation curves I observed a 3.8 fold increase in the incorporation of $^{32}P_i$ into the initial 2.5×10^6 rRNA transcript, a value in close agreement with the polymerase data of Gore, (1972) and Gore and Ingle, (1974). Regulation at the post-transcriptional level appears to operate in several ways. Firstly, in the auxin treated tissue the rate of synthesis of the

0.7×10^6 mature rRNA was equal to the rate of synthesis of the 2.5×10^6 precursor rRNA. Therefore, the 10 fold increase in the incorporation of label into the mature rRNA of auxin treated discs was not due to an increase in the processing of the 2.5 precursor, but was, instead, due to a processing fault in the non-treated tissue. As the rate of synthesis of the 0.7×10^6 rRNA was only half that of the initial 2.5×10^6 transcript in the control, this appears to be the case. Furthermore, since the 10 fold difference in the incorporation of label was maintained throughout a 180 minute pulse, this fault does not, apparently, reside in a slower processing to the mature 0.7×10^6 rRNA. If this were so, a decrease in this differential would be seen with longer labelling times. Therefore, the rate of processing of the 2.5×10^6 precursor rRNA to the 0.7×10^6 mature rRNA was approximately the same in the two cultural states. As a result the observed 10 fold reduction in the incorporation of label into the mature 0.7 rRNA of control tissue was mainly due to 2 factors: (1) the normal processing of the 2.5×10^6 rRNA but the final 0.7×10^6 product was unstable. (2) the abnormal processing of the precursor whereby the initial cleavage product to the mature rRNA was unstable and degraded. This could possibly be due to a deficit in the proteins required for the maturation of the rRNA. Since a similar conclusion was reached by Leaver and Lovett, (1974) some knowledge of the synthesis of the ribosomal proteins would have been desirable. Post-transcriptional regulation also appears to operate by a slower processing of the precursor rRNA but this was mainly evident in the maturation of the 1.3×10^6 rRNA from the 1.39×10^6 precursor. Firstly, the half-life of the 1.39×10^6 rRNA of the control tissue was far too long to be measured under these experimental conditions, whereas an estimate of

19 minutes was found for the auxin treated tissue. Secondly, after a 60 minute pulse the incorporation of label into the 1.3 x 10^6 mature rRNA of the control was 40 fold less than that of the treated discs. After 180 minutes of labelling, however, this difference had dropped to 10 fold, a level now at par with the 0.7 x 10^6 rRNA. Apparently, this was not due to a decrease in the processing of the 1.39 precursor to the mature 1.3 x 10^6 rRNA in the auxin treated tissue since the rate of synthesis of the mature rRNA was nearly equal to the synthesis of the initial 2.5 x 10^6 transcript. As was the case above, the limitation in processing of the precursor rRNA may be due to a deficiency of proteins in the nucleoli. Therefore, in addition to the 2 reasons stated previously, the reduction in incorporation of label into the mature rRNA was also the result of a slower processing of the precursor rRNA. In conclusion, the switching on of rRNA accumulation in the artichoke in response to auxin is in part due to an increase in the transcription of the precursor rRNA and in part due to post-transcriptional control involving an increase in the rates and the degree of processing of rRNA.

There are several criticisms which I must make in regard to the technique used here and as described by Chapman and Ingle, (1976) for the study of the kinetics of rRNA maturation in the artichoke. Firstly, the assumption that the specific activity of the precursor pool had reached steady state on the basis of linear incorporation of label into a stable molecule, DNA, may not be totally correct. For instance, from the half-lives (Table 8) and the molecular weights of the rRNA species, I can predict that the amounts of the precursor 2.5 x 10^6 : 1.39 x 10^6 rRNAs of auxin treated tissue should be in the proportion of 1.00 : 1.03, but the values calculated from the radioactivity-accumulation

plateaus are 1.00 : 1.85. This indicates that the half-life of the 2.5×10^6 precursor rRNA has been over-estimated and this is probably due to the time required for the nucleotide-precursor pool to reach constant specific activity. It would have been preferable to have had a measure of the specific activity of the ^{32}P-orthophosphate in the α-position of the nucleotide precursors since it probably takes a long time for the radioactive phosphate to equilibrate, first in the γ-position, then in the β-position and finally in the α-position, which is the phosphate that is actually incorporated. As a result the rise to steady state of radioactivity in the 2.5×10^6 precursor rRNA would be composed of two factors, (1) the approach to constant specific activity of the ^{32}P-orthophosphate in the α-position of the nucleoside triphosphates and (2) the rate of turnover of the RNA molecules themselves. The second criticism is the use of simple kinetics in terms of an equation to describe events in a cell which are probably much more complicated. For instance, Greenberg, (1972) simplified the kinetics of the stability of RNA by deriving this equation (Figure 24 and Table 8) for an exponentially growing, unsynchronized population of cells, which has, in effect, averaged out any differences. Auxin treated artichoke cells do not become asynchronous until after 50 hours of culture. In addition, a certain proportion of the cells never divide and in the non-treated tissue there are few cell divisions at all. Although artichoke cultures do not comply strictly to the requirements of the equation it seemed insignificant in the light of the large error imposed by the equation itself. In addition, the equation was derived to calculate the half-lives of known amounts of RNA. As it was not possible to obtain an estimate of the specific activity of the precursor rRNAs, since the amount was too small

to be measured, I had to assume, as did Chapman and Ingle, (1976), that the amount of precursor remained constant over the period of the experiment (3 hours). Since the incorporation of label into DNA was almost linear and the precursor rRNA radioactivity accumulation curves reached steady state plateau values this assumption appears reasonable. Therefore, the plateau levels were regarded as synonomous to molar amounts of the precursors and were used as such in the equation.

For reasons quoted above, I do not feel that the actual values obtained for the half-lives and processing of the rRNA precursors in the artichoke are entirely reliable. The technique can only give a relative estimate of the stability of RNA and must be used with some reservation.

PART V

INDUCTION OF DNA SYNTHESIS :

CHANGES IN PROTEINS

CHAPTER 1. TIMING OF AUXIN INDUCED DNA SYNTHESIS

The induction and maintenance of cell division in the artichoke is entirely dependent on the presence of auxin (Yeoman and Mitchell, 1970). For DNA synthesis to be induced at all, 2,4-D must be present within a concentration range of 5×10^{-7} to 10^{-4}M (Yasuda et al, 1974). The first division, which is the most synchronous is unusual in that it has an extended G-1 phase (Mitchell, 1967). There is a lag period of at least 16 hours before DNA replication occurs if auxin is added at zero time. Is this 16 hour period obligatory or does it consist of an 8 hour lag period plus 8 hours for the wound events to occur? Some investigators have argued that wounding events are mediated first and that auxin only effects after the wound response has developed (Setterfield, 1963 ; Masuda, 1966). This would suggest that auxin need not be added at zero time. In trying to locate suitable times at which to look for auxin-induced changes we need some idea of the length of time the tissue must be treated with auxin before DNA synthesis occurs. Therefore, an experiment was constructed whereby auxin was added to the tissue at various times after excision and the time of the onset of DNA replication was determined.

Experimental design.

The discs were cultured at a concentration of 20 discs/2 ml media either with or without auxin for 9 hours. At this time the transitory effect of wounding on DNA, RNA, and protein synthesis was over (Mitchell, 1969) and see also Table (3) and Figure (8). The discs were subsequently transferred to fresh media containing 10 µCi/ml (CH_3-^{3}H) thymidine in 1 µM thymidine carrier. The transfer was made so as to effect 4

different experimental situations. Half of the auxin treated discs were placed in media containing 2,4-D and the other half placed in media lacking the auxin. The same procedure was followed for the non-treated tissue. The discs were further incubated and samples removed after 18, 21, 24, and 27 hours of total culture time. A total tissue extract of nucleic acids and protein was accomplished by the Schmidt-Thannhauser procedure as described in the Methods, (C-4-c), and protein and DNA content were measured as previously outlined also in the Methods, (B-2 and B-3) respectively. The amount of radioactive thymidine incorporated into a TCA-insoluble precipitate was measured (Methods, B-4-b-i), and the specific activity of DNA, corrected for uptake, was calculated.

Results

Figure (28) shows the specific activity of DNA in counts/minute/µg at each sample time. In tissue which had not been stimulated to divide by auxin DNA synthesis was absent for the duration of the experiment (curve 1). When auxin was added after 9 hours of culture, (curve 2) DNA synthesis was initiated but was delayed by 6 hours when compared to continuous auxin treatment (curve 4). From the slopes of the curves, it appears that roughly the same proportion of cells were stimulated to divide, despite the delay. When 2,4-D was removed from the medium (curve 3) the specific radioactivity fell as 2,4-D leaked out of the tissue and fewer cells went into division.

An estimate of total protein in each case, presented in Table (13), does not offer any conclusive evidence on the role of protein synthesis in the induction of DNA replication. It appears, however, that protein synthesis declined when 2,4-D was removed from the media. Similarly, in tissue where auxin was not added until later there was

FIGURE 28.

Effect of the auxin, 2,4-D on the timing of DNA synthesis

Both auxin treated (● , ■) and non-treated (○ , □) tissue were cultured for 9 hours when they were transferred to fresh media containing tritiated thymidine, The transfers, either to media promoting cell division (+ 2,4-D) or to that which did not (- 2,4-D), were made according to the following schemes:

curve 1. - 2,4-D ⟶ - 2,4-D

curve 2. - 2,4-D ⟶ + 2,4-D

curve 3. + 2,4-D ⟶ - 2,4-D

curve 4. + 2,4-D ⟶ + 2,4-D

Discs were further incubated and samples removed after 18, 21, 24, and 27 hours of total culture time. DNA was extracted and the specific radioactivity estimated and plotted against cultural age.

FIG. 28

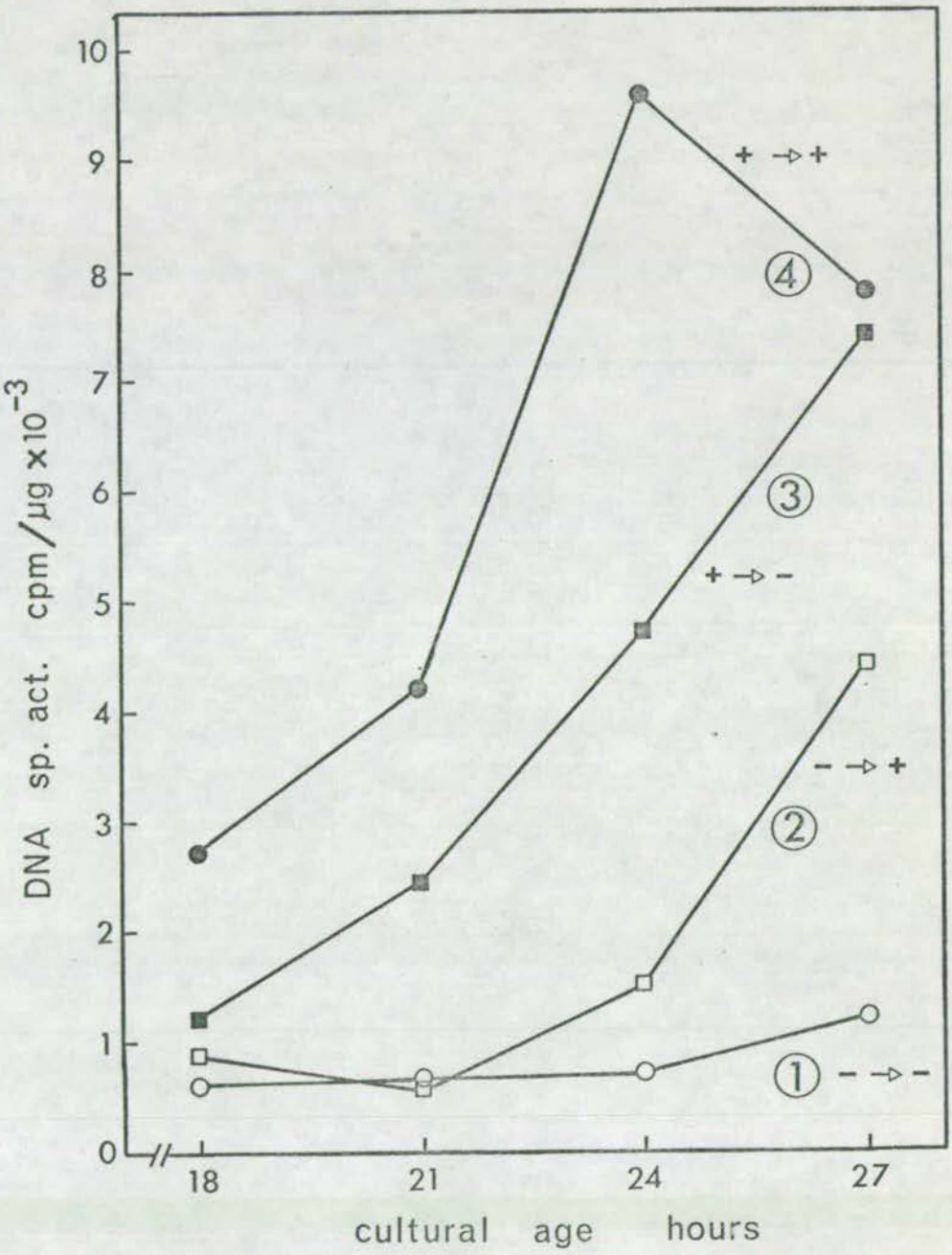

DNA sp. act. cpm/μg ×10⁻³
0
1
2
3
4
5
6
7
8
9
10
18
21
24
27
cultural age hours
+ → +
④
③
+ → -
- → +
②
① - → -

TABLE 13.

Changes in total protein levels when auxin was added or removed from the media at a later time in culture

The tissue was treated as previously described for Figure (28). Protein from each sample was extracted (Methods, C-4-c) and estimated by the Lowry procedure (Methods, B-2). The amount of protein in each sample was compared.

TABLE 13.

Cultural Age (hours)	Amount of protein (µg/disc)			
	Transfer from +2,4-D to :		Transfer from -2,4-D to :	
	+2,4-D	-2,4-D	+2,4-D	-2,4-D
18	264	287	273	287
21	282	251	291	302
24	288	249	272	266
27	288	252	300	285

apparently, increased protein synthesis concomitant with the induction of DNA replication.

Discussion

From these results 2 hypothesis may be drawn:

1. The induction of DNA synthesis involves an obligatory sequence of metabolic events apart from the response to wounding.
2. 2,4-D must accumulate to some critical value before DNA replication is induced.

Evidence in favour of the first hypothesis comes mainly from the cell number data presented in Figures (7) and (8). It is apparent from these graphs that the timing of DNA synthesis and cell division was the same regardless of whether the tissue was treated with 10^{-5}M 2,4-D or the lower concentration of 10^{-6}M. Not only was the length of the lag phase the same but, as shown in Table (5), nearly the same percentage of cells divided. This suggests that the time taken to reach the required concentration level was probably quite short. In addition, I have used the higher 10^{-5}M level of 2,4-D in this experiment whereas concentrations as low as 5×10^{-7}M are known to induce DNA replication (Yasuda et al, 1974). Also, the results in Figure (28) show that although auxin was absent from the media for 9 hours and DNA synthesis began approximately 6 hours later than if auxin was present from the start of culture, a similar percentage of cells have been stimulated to divide. This result combined with the knowledge that only the peripheral 6 or 7 layers of cells divide, (Mitchell, 1967) suggests that concentration effects were minimal. In conclusion it appears essential that 2,4-D be present in the tissue for a certain obligatory period of time prior to the onset of DNA synthesis. From the results presented here, it was not possible to determine whether

2,4-D was able to act immediately upon addition to freshly excised tissue or not until the wounding response (between 6 to 9 hours of culture) was over. Nevertheless, the time delay of auxin-induced DNA synthesis appears to occur as a separate event from wounding. For instance, regardless of whether 2,4-D was added at the start of culture or after 9 hours of culture, it still required from 18 to 21 hours before the synthesis of DNA was initiated. The induction of DNA replication, then, apparently relies on a certain sequence of metabolic events, the timing of which cannot be altered.

CHAPTER 2. NUCLEAR PROTEIN/DNA RATIOS

Introduction

Since DNA replication occurs in the nucleus it appeared essential to investigate changes in this organelle during the period of time between the addition of auxin and the onset of S. There are increasing numbers of reports which provide evidence that various activities of the nucleus are controlled by a specific protein fraction, the acidic proteins (Stein et al, 1974). It appeared reasonable, therefore, that preparation for DNA synthesis might involve specific accumulation of these proteins either during replication itself, or both. For instance, the mechanism of DNA synthesis would require the appropriate enzymes and cofactors. This is followed by the association of the newly synthesized DNA with both non-histone proteins and histones and RNA to form chromatin (Bonner et al, 1968). It seemed necessary, then, to have a quantitative measure of the ratio of nuclear protein to DNA as the cells progressed through the pre-replication phase to the onset of DNA replication.

Experimental design

A total of 40 discs for each sample were incubated in batches of 20, in 2 ml media containing or lacking the auxin 2,4-D for 0, 6, 12, and 18 hours. At each of these times a 3 hour pulse with 10 µCi/ml (CH_3-^3H) thymidine in 1 µM thymidine carrier was given. 5 discs from each sample were removed for a total tissue extraction of nucleic acids and protein (Methods, C-4-c), while the remaining 35 were chopped in Honda medium to isolate the nuclei as described in the Methods, (C-4-d-ii). The nuclei were lysed by thoroughly suspending the

preparation in 0.6 ml 0.1 N NaOH at room temperature and the macromolecules were precipitated overnight with 5% TCA in the cold. The estimations of protein and DNA were made as previously described, (Methods, B-2 and B-3).

Results

The results of the experiments are presented in Table (14). From the total tissue extraction of nucleic acids the average amount of DNA in a single disc was estimated to be 2.7 µg. This agrees closely with a value of 24 pg DNA/telophase obtained by Ingle et al, (1976) which approximates 2.4 µg for a disc with 100,000 cells. The average DNA content of the isolated nuclei was 0.65 µg/disc, which would give a nuclear yield of 27%. Since this is a reasonable value when compared to previous results shown in Table (1), the precipitation of the macromolecules with TCA must have been nearly quantitative. The amount of nuclear protein was corrected for yield by letting 2.7 µg DNA/disc be equal to 100% yield. When this was compared to the total level of protein in a disc it is evident that from 7 to 10% of the protein in the tissue was associated with the nucleus. When the amounts of nuclear protein and DNA were compared over the time course it was found that the ratio of total nuclear protein/DNA remained at a near constant value of 6/1 in the non-treated tissue for the duration of the 21 hour culture period. In the treated tissue, however, this ratio was increased to approximately 9/1 at the onset of DNA replication. Figure (29) shows the accumulation of nuclear protein in both cultural states during the incubation. Initially there was a small increase in both auxin treated and non-treated tissue, which probably represents a response to excision (Part III, Chapter 1., Section B). This value of 16.5 µg/disc was more or less maintained in the non-treated tissue,

TABLE 14.

Nuclear protein/DNA ratios during the early culture of auxin treated and non-treated tissue

The discs were given a 3 hour pulse with tritiated thymidine after 0, 6, 12, and 18 hours of culture time. Total tissue protein and DNA were extracted as described in the experimental design. Similarly, nuclei were isolated and the protein and DNA estimated.

From the TCA extracts, the average DNA level = 2.7 µg/disc, compared to a value of 2.4 µg/disc (24 pg/telophase cell) from Ingle et al, (1976).

The average DNA content of isolated nuclei = 0.65 µg/disc which approximates a yield of 27%.

TABLE 14.

Treatment	Cultural age at end of 3 hour	Nuclear protein extracted	Nuclear DNA extracted	Nuclear protein to DNA	Yield of DNA	Corrected nuclear protein	Total tissue protein	TCA extract DNA	Specific activity DNA corrected for uptake.
	hours	µg/disc	µg/disc	ratio	percent	µg/disc	µg/disc	µg/disc	cpm/µg
2,4-D	3	3.3	0.6	5.8	21.0	15.8	186	2.1	456.5
	9	2.9	0.5	6.3	17.0	16.8	208	3.6	383.0
	15	4.9	0.8	6.1	29.6	16.6	220	2.7	639.9
	21	5.6	0.7	8.7	21.0	26.6	240	2.6	2393.0
control	3	3.3	0.6	5.9	21.0	15.8	136	2.3	231.8
	9	3.3	0.6	6.0	20.0	16.6	206	2.6	397.1
	15	4.2	0.7	6.2	25.6	16.5	230	2.9	304.6
	21	5.4	0.9	6.1	29.6	18.1	268	3.4	367.4

FIGURE 29.

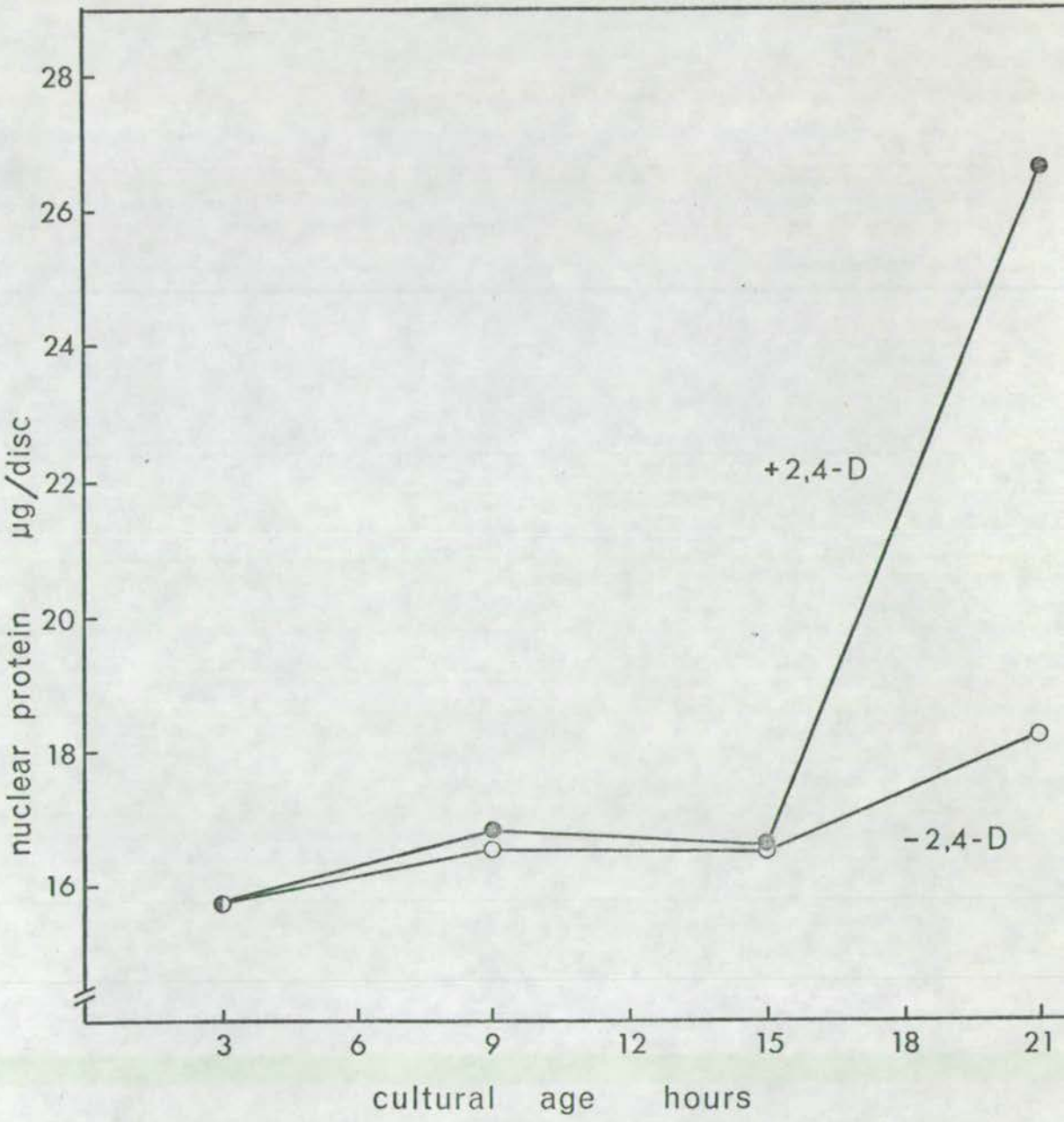
The amount of nuclear protein in an auxin treated and non-treated disc at various culture times up to the onset of S-phase in the treated tissue

The nuclei were isolated and the amount of protein estimated (experimental design). The values obtained were corrected for 27% yield (Table 14) and plotted against the cultural age.

2,4-D - (●)

control - (○)

FIG. 29


28
26
24
22
20
18
16
nuclear protein µg/disc
+2,4-D
-2,4-D
3
6
9
12
15
18
21
cultural age hours

increasing to 18.1 µg after 21 hours of culture. In the treated tissue, however, there was a 1.7 fold enhancement of nuclear protein at the onset of DNA replication, to reach a level of 26.6 µg/disc at 21 hours. This represents a 1.5 fold difference in the amount of nuclear protein between the two cultural states during S-phase in the auxin treated tissue. This increase is probably a reflection of the acidic protein fraction, including the enzymes and cofactors required for DNA synthesis and histone synthesis.

CHAPTER 3. - TWO-DIMENSIONAL GEL SYSTEM

SECTION A. Experiments to determine the iso-electric points of the majority of cytoplasmic proteins

1. Iso-electric focusing gels of pH range 3.5 to 10.0

In the initial experiments wide range iso-electric focusing gels using ampholines pH 3.5 - 10 were used to establish the pI (iso-electric point) range of the majority of the urea-soluble proteins in the cytoplasm.

Experimental design

The acetone washed soluble (cytoplasmic) proteins (Methods, C-4-e) were dissolved in lysis buffer (Methods, C-4-f) to a final concentration of 3 mg/ml, and 50 µl containing approximately 150 µg of protein was layered on top of each first dimension iso-electric focusing gel. See the Methods section, (C-5-c), for the detailed procedure. Following electrophoresis overnight at 200 V, the gels were prepared for separation in the second dimension (Methods, C-5-c). The cylindrical gel was then horizontally embedded in a 11.25% acrylamide slab gel and separated according to molecular weight by SLS discontinuous gel electrophoresis in the vertical direction. The pH gradient of a first dimension gel was determined in a control gel as described in the Methods, (C-5-c-ii) and shown in Figure (30), and a representative picture of a stained and dried gel is shown in Figure (31). The molecular weight scale for the second dimension was constructed by the use of marker proteins of known molecular weight combined with the stained double band, clearly visible at 65,000 and 60,000 mol. wt. (Methods, C-5-c-iii).

FIGURE 30.

Sample pH gradient of a pH 3.5 to 10 iso-electric focusing gel

Following electrophoresis in the first dimension, a blank gel was used to determine the pH gradient. The cylindrical iso-electric focusing gel was sliced as 0.5 cm segments into distilled water, left several hours, and the pH measured, (Methods, C-5-c-ii). The pH was plotted against gel length, the more acidic values at the top, graduating to the more basic values at the bottom of the gel.

FIG. 30

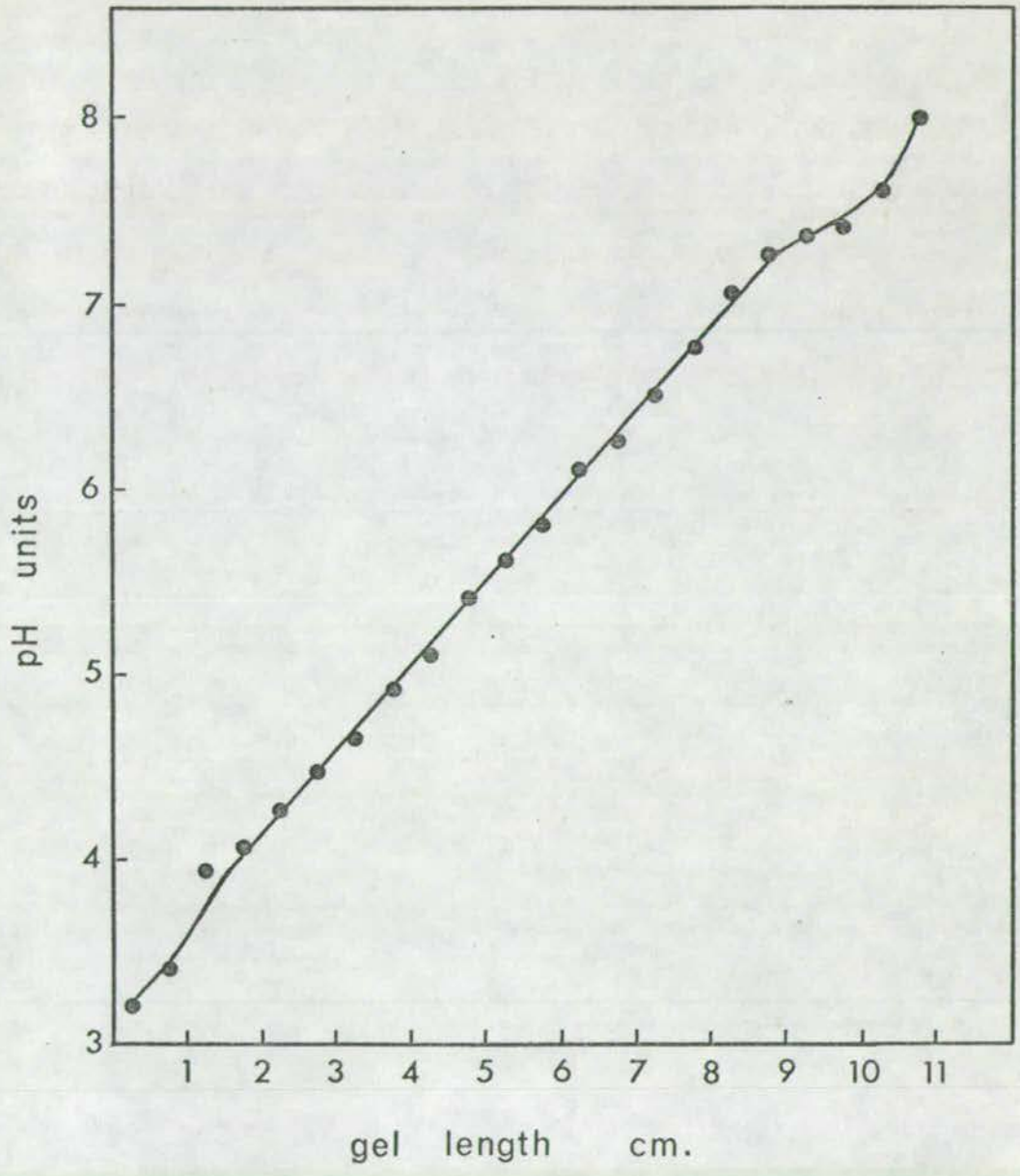
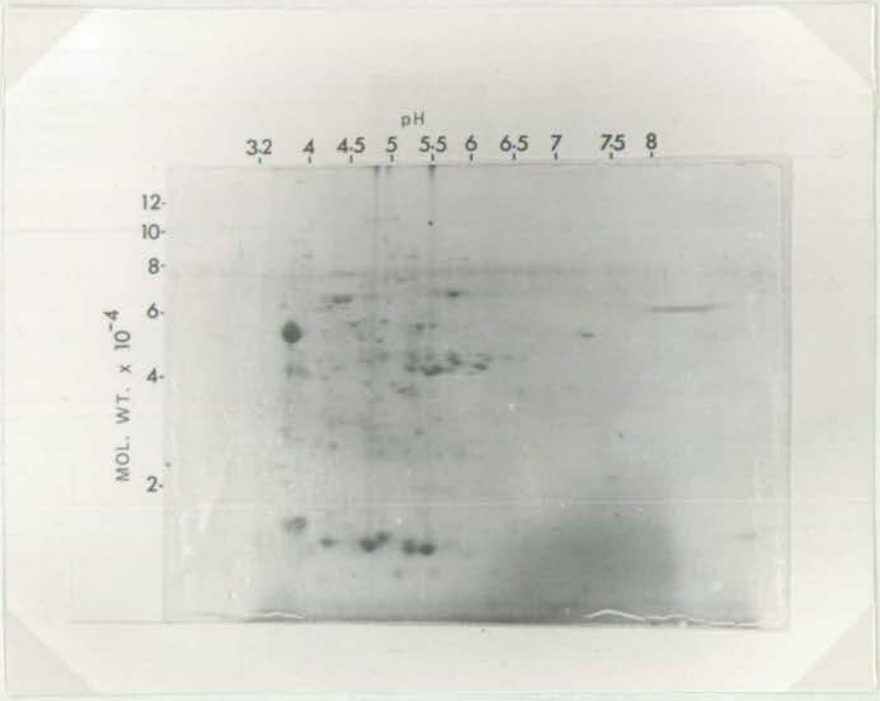


FIGURE 31.

Photograph of a stained and dried slab gel. Separation of proteins in the first dimension was on a pH 3.5 to 10 iso-electric focusing gel

The soluble proteins from artichoke tuber tissue were first separated in the horizontal direction on the basis of their iso-electric points; then in the vertical direction by molecular weight (see experimental design and Methods, (C-5-c). The proteins are evident as stained spots.

FIGURE 31.



Results

After staining and destaining approximately 150-200 protein spots could be detected with a loading of 150 µg of protein. Figure (31) shows that the majority of 10 M urea soluble cytoplasmic proteins have iso-electric points between pH's 4 and 6, and molecular weights of less than 60,000. The vertical streaks through the gel have probably been caused by the relative insolubility of those 15,000 molecular weight proteins they are associated with. At the basic end of the gel a streaked protein of 57,000 mol. wt. illustrates the loss of the pH gradient in this region. This was caused by the leakage of urea and ampholines from the bottom of the gel during the run which resulted in the shortening of the upper pH range from pH 10 to pH 8 (Figure 30). This abnormality did not, however, affect the majority of proteins which were separated on a gradient that changed linearly at a rate of 0.45 pH units/cm.

2. Iso-electric focusing gels of pH range 5 to 7

To enhance resolution of the proteins two improvements were made :

(a) In the first dimension, proteins were separated on iso-electric focusing gels of pH 5 to 7, since the majority of proteins were focused in this range.

(b) In the second dimension an exponential gradient of 10.5% to 15% acrylamide was used to improve resolution of those proteins in the 20,000 to 50,000 mol. wt. range.

Experimental design

The preparation of soluble (cytoplasmic) proteins for electrophoretic separation by iso-electric focusing was carried out as described in the previous experiment. Approximately 150 to 200 µg of protein in 50 µl lysis buffer was loaded on each first dimension gel. Nuclear proteins

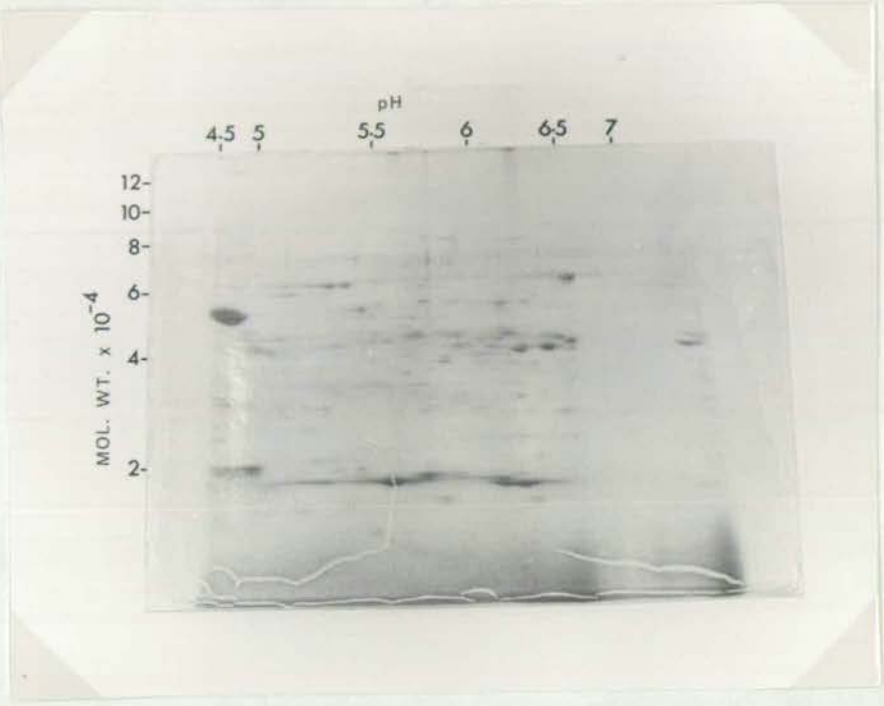
FIGURE 32.

Effect on the separation of the proteins by shortening the electrophoresis time for the first dimension iso-electric focusing gels, pH 5 to 7

Total tissue soluble proteins from artichoke tuber cells were prepared and separated 2-dimensionally as previously described for Figure (31). In the first dimension, however, the total volt-hours used was only 5100, compared to the normally used 8000 V-hours.

The photograph of the stained and dried gel shows, by the streaking, that a large number of proteins had not yet reached their respective iso-electric points.

FIGURE 32.



were prepared from the isolated nuclei (Methods, C-4-d-ii) of 40 discs. Following suspension in 110 µl lysis buffer and removal of the chromatin by centrifugation (Methods, C-4-f) the urea-soluble proteins from the nuclei of 20 discs, equivalent to about 50 to 60 µg of protein was loaded on each gel.

Electrophoresis was carried out as before (above and Methods, C-5-c) excepting the first dimension gels were run at 400 V for at least 8000 V-hours to allow all of the proteins to become focused. If shorter times were used such as 5100 V-hours (Figure 32) broadening of the protein spots in the first dimension was evident.

Results

A sample pH gradient of a pH 5 to 7 iso-electric focusing gel is shown in Figure (33). Here, the gradient increased by 0.2 pH units/cm which allowed better separation of those proteins with similar pI values. Figure (34) shows a photograph of the stained proteins which are now distributed uniformly over the gel and it was possible to detect over 200 spots with the same loading used before. Although perhaps 5 to 10 of the more basic proteins were lost from the bottom of the gel they were sacrificed in aid of better resolution of the major protein population.

Figure (35) shows a photograph of a stained gel of a preparation of the 10 M urea-soluble proteins of the nucleus. Here too, the proteins were spread almost over the entire gel. In contrast to the cytoplasmic or soluble proteins, the majority of the nuclear proteins have molecular weights greater than 40,000. It appears that the urea-soluble or acidic proteins of artichoke nuclei demonstrate similar iso-electric points (pH 4 to 6) and molecular weights ($>$ 40,000) as the barley embryo (Trewavas, 1976a) and animal systems (MacGillivray and Rickwood, 1974).

FIGURE 33.

Sample pH gradient of a pH 5 to 7 iso-electric focusing gel

Following electrophoresis in the first dimension, a blank gel was used to determine the pH gradient. The cylindrical iso-electric focusing gel was sliced as 0.5 cm segments into distilled water, left several hours, and the pH measured (Methods, C-5-c-ii). The pH was plotted against gel length, the more acidic values at the top, graduating to the more basic values at the bottom of the gel.

FIG. 33

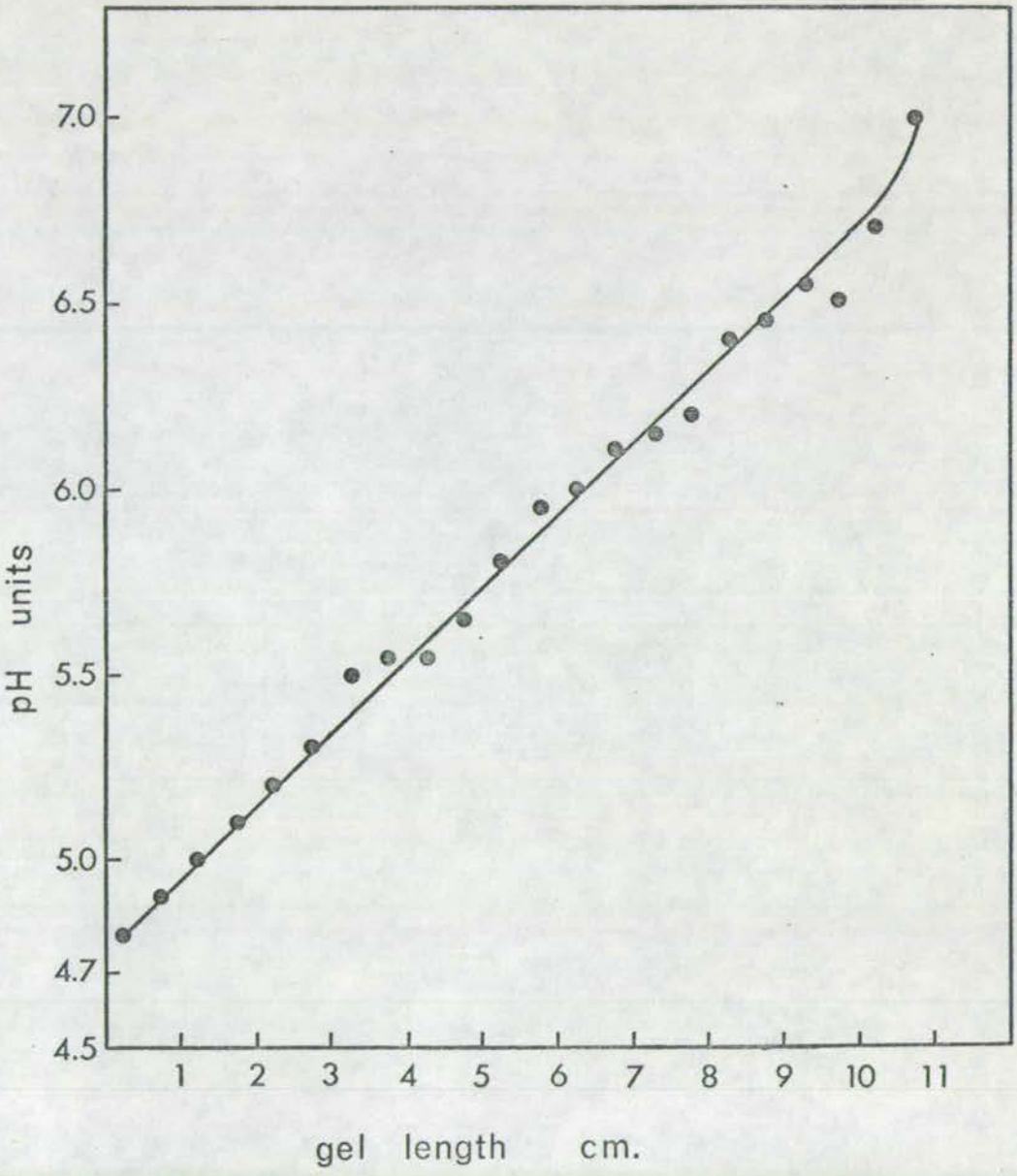


FIGURE 34.

Photograph of a stained and dried slab gel. Proteins were separated in the first dimension on a pH 5 to 7 iso-electric focusing gel

The soluble proteins from artichoke tuber tissue were separated in the horizontal direction on the basis of their iso-electric points, and in the vertical direction by molecular weight (see experimental design and Methods, C-5-c). An iso-electric focusing gel, pH gradient, 5 to 7 was used in the first dimension and a 10.5% to 15% gradient slab gel, which resolved proteins in the approximate range of 130,000 (top) to 15,000 (bottom) molecular weight, was used in the second. The proteins are evident as stained spots.

FIGURE 34.

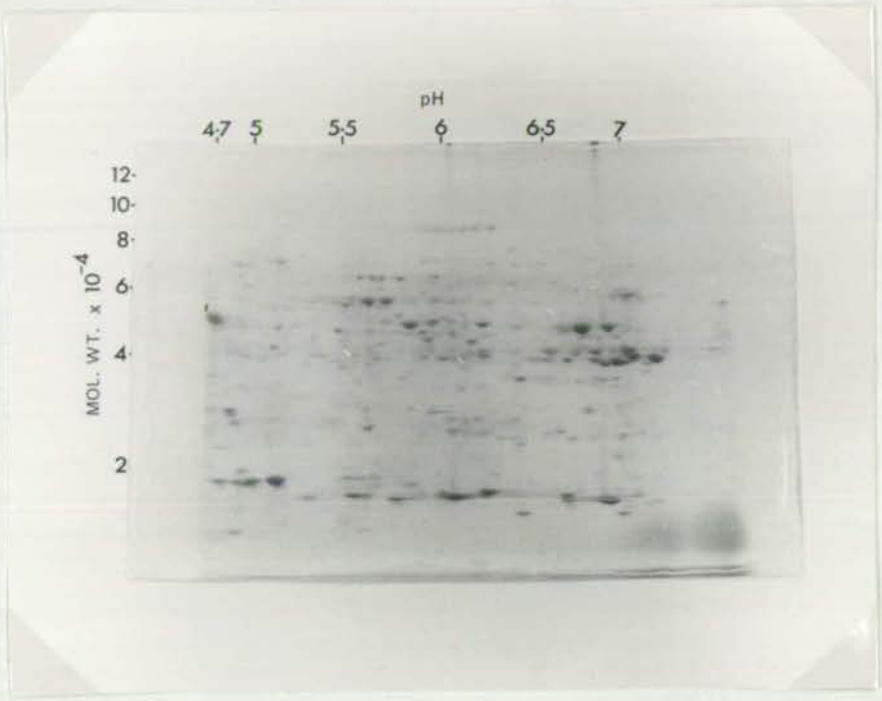
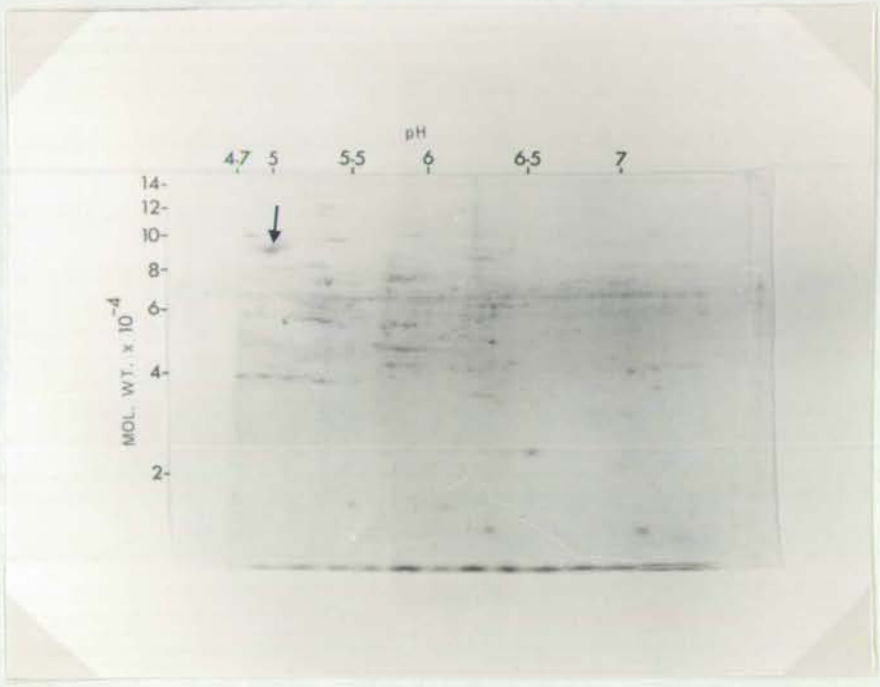


FIGURE 35.

Photograph of a stained and dried slab gel after separation of the urea-soluble proteins of the nucleus

Nuclei of artichoke tuber cells were isolated (Methods, C-4-d-ii) and the 10 M urea-soluble nuclear proteins (Methods, C-4-f) were separated 2-dimensionally (Methods, C-5-c). Separation in the first dimension was achieved on an isoelectric focusing gel of pH 5 to 7, and in the second dimension on a 10.5% to 15% gradient slab gel as in Figure (34). The molecular weight range is approximately 130,000 (top) to 15,000 (bottom). The proteins are evident as stained spots.

FIGURE 35.



Discussion

Separation on a pH 5 to 7 iso-electric focusing gel better resolved the proteins with more proteins evident as discrete spots. The majority of the cytoplasmic urea-soluble proteins focused between pH 4.5 and 6, and since the protein fraction from the nucleus displayed a similar distribution it was decided to use the first dimension gel with a 5 to 7 pH gradient for subsequent experiments. Although the modifications improved resolution, staining alone was not adequate for measuring small changes in the proteins, so detection by the use of radioisotopes was employed as described in the Methods section, (B-4-b-iii and iv).

SECTION B. Reproducibility and matching of gels

1. Terminology to locate spot positions

Figure (36) are pictures of autoradiograms of ^{35}S-methionine labelled cytoplasmic proteins after two-dimensional separation. Proteins have been separated in the horizontal direction on the basis of their iso-electric points and in the vertical direction by molecular weight. Throughout the concourse of this thesis the positions of the protein spots on the gels shall be referred to in the following way:

5.0/75 or 5.0-5.3/100

where : 5.0 = pH of the iso-electric point of the protein

5.0-5.3 = a range of pH values

75 and 100 = molecular weight x 10^{-3}

2. Effect of different loadings

The gels in Figure (36) serve to illustrate a number of points about the reproducibility and matching of gels of different loadings and different types of preparations. In general, the less protein loaded on the gel, the better the resolution. Since the cytoplasmic protein preparation was a complex mixture of proteins only a small number species were present as major components. Therefore, the gel could be overloaded with little loss in total resolution. As the amount of a component protein is increased it expands symetrically in the iso-electric focusing direction but enlarges mainly on the low molecular weight side in the SLS dimension. This is readily seen in Figure (36) of the more prominent proteins.

When a protein becomes focused at the pH of its iso-electric point it changes the pH gradient at this point and if the protein is a major component this change is quite pronounced either side of the spot.

FIGURE 36.

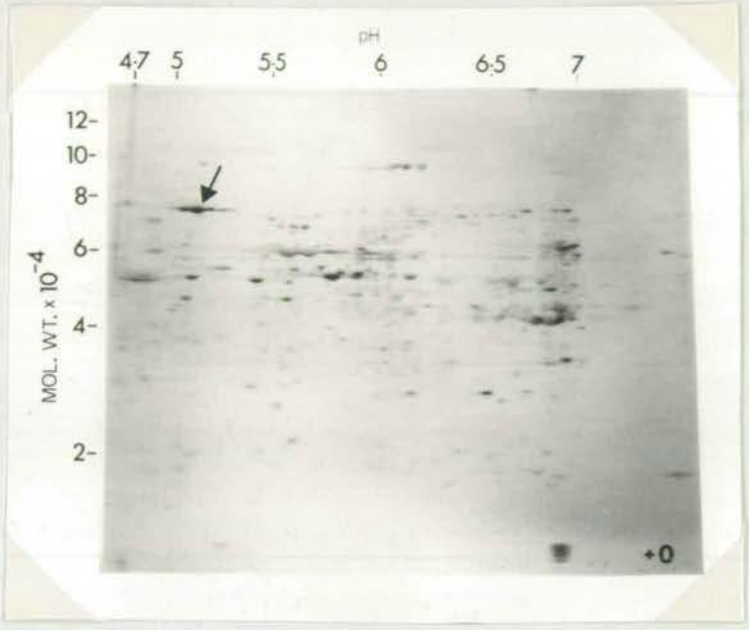
Photographs of autoradiograms of 2-dimensionally separated ^{35}S-methionine labelled cytoplasmic proteins of auxin treated and non-treated tissue

Discs of artichoke tuber tissue were cultured for 12 hours when a 3 hour pulse with ^{35}S-methionine was given (Methods, C-4-b). Cytoplasmic proteins were prepared (Methods, C-4-e), taken up in 10 M urea (Methods, C-4-f) and separated in the first dimension on a pH 5 to 7 iso-electric focusing gel (horizontal direction) and in the second dimension on a 10.5% to 15% polyacrylamide SLS slab gel (vertical direction) (Methods, C-5-c). The molecular weight range in approximately 130,000 (Top) to 15,000 (Bottom).

2,4-D - top gel

control - bottom gel

FIGURE 36.



As a result a minor protein next to it is not engulfed by it but shifted to its true position in the iso-electric focusing dimension. Therefore, the true position required for accurate matching of gels cannot be found for different preparations. The only way to overcome this would be to load less than 20 µg on each gel to minimize loading effects. Since it was not experimentally possible to enhance the specific radioactivity to such a level of detection, an alternative method of matching gels patterns of the spots was used.

3. An example of positioning by pattern matching

One type of pattern seen often was that of proteins possessing charge heterogeneity which was evident as a series of spots with the same molecular weight. The spacing of these spots is consistent with single charge differences (O'Farrell, 1975). For instance, one cytoplasmic protein in common between auxin treated and non-treated tissue was 5.0-5.3/75 (Figure 36) which had one major spot with 4 satellite spots. Since the pattern was very reproducible in both cultural states, with the progressive loss with culture of the more basic satellites then the charge differences were most likely due to *in vivo* modifications and were not artifactual. The *in vivo* modifications may include phosphorylation, acetylation, and the addition of charged carbohydrate groups. Since the smaller proteins have fewer targets for modification they tend to be less heterogenous than larger proteins. Indeed, few multiple spots were found below 55,000 molecular weight.

4. An example of an artifact

One artifact observed in all the two-dimensional gels was an aberration in the pH gradient from pH 5.2 to 5.5, where streaking of the proteins was quite pronounced. One explanation provided by Bobb and Hofster (1971) is that urea, which has a strong influence on the

carboxyl groups of amino acids, may have caused a large change in charge in the pH 5.2 to 5.5 range which is the pK_a of the major dissociating carboxyl groups of glutamic and aspartic acid. The pK_a values of carboxyl groups in 10 M urea are about 1 pH unit higher than in water.

5. Discussion

In conclusion, the actual pH scale in iso-electric focusing probably fluctuates under the influence of loading and other effects such as that above, and the ideal pH scale demonstrated by the control gels cannot be used to pin-point the true positions of the proteins. The two-dimensionally separated proteins of auxin treated and non-treated tissue were compared by relative spot positions and intensities, i.e., patterns, which was more easily accomplished and quite reliable.

CHAPTER 4. MODIFICATION OF PROTEINS BY PHOSPHORYLATION

Phosphorylation has been associated with various cellular components, including ribosomes, microtubules, membranes, and the nucleus. In particular, phosphorylation has been implicated as a controlling feature in a number of processes such as the activation and inactivation of enzymes, selective transport across membranes, regulation of DNA synthesis, specific regulation of gene transcription, and meiosis (Allfrey et al, 1973 ; Trewavas, 1976b).

In animal cells phosphorylation has been regarded as a rapid method of switching the cell from one state to another in response to certain external stimuli. For example, some animal hormones apparently operate in this way. The transformation by auxin of a resting cell to an actively dividing cell may also depend, in part, on the phosphorylation of certain proteins and enzymes. The increasing evidence that the phosphorylation of the acidic proteins of the nucleus are regulators of specific gene activity (Stein et al, 1974) is all the more cause for an investigation of the possible role of phosphorylation in the induction of DNA synthesis in the artichoke. Therefore, I wanted to discover whether, during any time after the addition of auxin, particular acidic proteins of the nucleus or cytoplasm were phosphorylated and whether this modification was associated with any single phase in the progress of the cell to DNA replication.

SECTION A. Phosphorylation of cytoplasmic proteins

Experimental design

Artichoke tuber discs were incubated with or without auxin for 0, 6, 12, and 18 hours, each time course sample consisting of 25 discs in 2.5 ml of media. Labelling was effected by transferring the discs, at the appropriate times, to 1 ml of pulse media containing 100 µCi of ^{32}P-orthophosphate and 10 µCi of (CH_3-^{3}H) thymidine plus 1 µM thymidine carrier for 3 hours. Thymidine was included to monitor the onset of DNA synthesis in the auxin treated tissue. After labelling, the tissue was washed free of excess isotope and 20 discs were used for the extraction of the cytoplasmic proteins as described in the Methods, (C-4-e). The washed protein sample was taken up in lysis buffer (Methods, C-4-f) and the urea soluble proteins were separated two-dimensionally (Methods, C-5-c) and the slab gels put on for autoradiography (Methods, B-4-b-iii). The remaining 5 discs were extracted with TCA to obtain a measure of total nucleic acids and protein (Methods, C-4-c). The radioactivity in the acid-insoluble precipitate was measured by employing two channels in the liquid scintillation counter. Since the isotopes, ^{3}H and $^{32}P_i$ have different energies of radiation they were resolved by pulse height analysis in the counter itself, and a measure of DNA synthesis was obtained by the incorporation of tritiated thymidine. Both DNA and protein become labelled with $^{32}P_i$, however, but the amount contributed by DNA should be minimal at all times except during DNA replication in the auxin treated tissue. The RNA was rendered acid soluble by alkali hydrolysis and so was removed from the calculations.

Results

After removal of the ribosomes by centrifugation (Methods, C-4-e) approximately 90% of the cytoplasmic proteins were recovered (Table 15) when compared to the total tissue extraction with TCA. The cytoplasmic proteins from 2 discs or approximately 175 µg to 200 µg was loaded on each first dimension gel. After 18 hours of culturing the tissue in 2,4-D, the specific activity of DNA, corrected for uptake, was enhanced nearly 2 fold over the non-treated tissue. DNA replication or S-phase was then regarded as having begun at this time. The incorporation of ^{32}P-orthophosphate into the acidic proteins of the cytoplasm, however, appears similar in both cultural states. As the cellular metabolism of the tissue increased with culture time so did the incorporation of the label into protein.

Pictures of the autoradiograms from the two-dimensional separation of the $^{32}P_i$ labelled cytoplasmic proteins are presented in Figure 37 (A,B,C,D,) and(E,F,G,H) for auxin treated and non-treated tissue respectively. Although there was an enhancement of phosphorylation from 15 labelled proteins labelled after 3 hours to about 70 after 9 hours (Figure 37, A,B,E,F,) no difference in the pattern of labelling was evident between the two cultural states. The enhancement of activity on the gels agrees with the measured increase in specific activity of the proteins. When the pulse was effected after 12 hours of culture (Figure 37, C,G) there was again no difference in the labelling pattern, with about 70 to 80 proteins labelled. After 18 hours, in the non-treated tissue, (Figure 37, H) only an additional one or two proteins were phosphorylated. There was, however, a marked change in the auxin treated tissue with a shift in the iso-electric point of many of the proteins to a more basic value. This indicates that a modification

TABLE 15.

Data for experiment on ^{32}P-orthophosphate-labelled cytoplasmic proteins of auxin treated and non-treated tissue

In order to monitor the possible changes in phosphorylation of the cytoplasmic proteins with culture in media containing or lacking auxin, the discs of artichoke tuber cells were pulse labelled with ^{32}P-orthophosphate at various times. Tritiated thymidine was included to monitor the onset of DNA synthesis (experimental design).

The estimated amount of protein loaded on each first dimension gel was calculated from a Lowry value (Methods, B-2) obtained for the sucrose buffer extraction of protein (Methods, C-4-e).

TABLE 15.

Treatment	Cultural age at end of 3 hour pulse (hours)	TCA extractions: protein	TCA extractions: DNA	Buffer extraction of protein	Amount of protein on gel (= 2 discs)	Specific activity corrected for uptake: $^{32}P_i$	Specific activity corrected for uptake: ^{3}H	Total protein to DNA	Percent uptake: $^{32}P_i$	Percent uptake: ^{3}H
		µg/disc	µg/disc	µg/disc	µg	protein cpm/µg	DNA cpm/µg	ratio		
2,4-D	3	97.0	2.7	92.0	184.0	67.0	141.4	36	46.4	62.3
	9	114.7	3.1	94.4	188.8	177.9	175.8	37	55.1	74.3
	15	111.7	2.7	100.8	201.6	394.4	323.4	41	63.9	72.1
	21	97.9	2.4	100.0	189.6	406.4	1009.1	41	77.0	71.4
control	3	97.0	2.1	87.2	174.4	66.5	186.6	46	46.2	59.7
	9	107.9	2.5	90.4	180.8	272.0	199.7	43	54.6	75.0
	15	95.4	1.7	92.0	184.0	309.2	274.3	56	79.0	78.7
	21	107.9	2.4	94.8	189.6	332.5	563.3	45	93.9	79.3

FIGURE 37.

Photographs of autoradiograms of 2-dimensionally separated $^{32}P_i$-labelled cytoplasmic proteins from auxin treated and non-treated tissue

^{32}P-orthophosphate-labelled cytoplasmic proteins were prepared and separated by 2-dimensional gel electrophoresis (experimental design). The proteins were resolved according to their iso-electric point in the first dimension (horizontal direction) and according to their molecular weight by SLS gel electrophoresis in the second dimension (vertical direction).

The gels are displayed on the next 2 pages, with auxin treated and non-treated tissue being compared when the pulse was effected between 0 - 3, 6 - 9 (first page), 12 - 15, and 18 - 21 hours (second page) of culture time.

2,4-D - A (0 - 3) ; B (6 - 9) ; C (12 - 15) ; D (18 - 21)

control - E ; F ; G ; H (the same)

FIGURE 37.

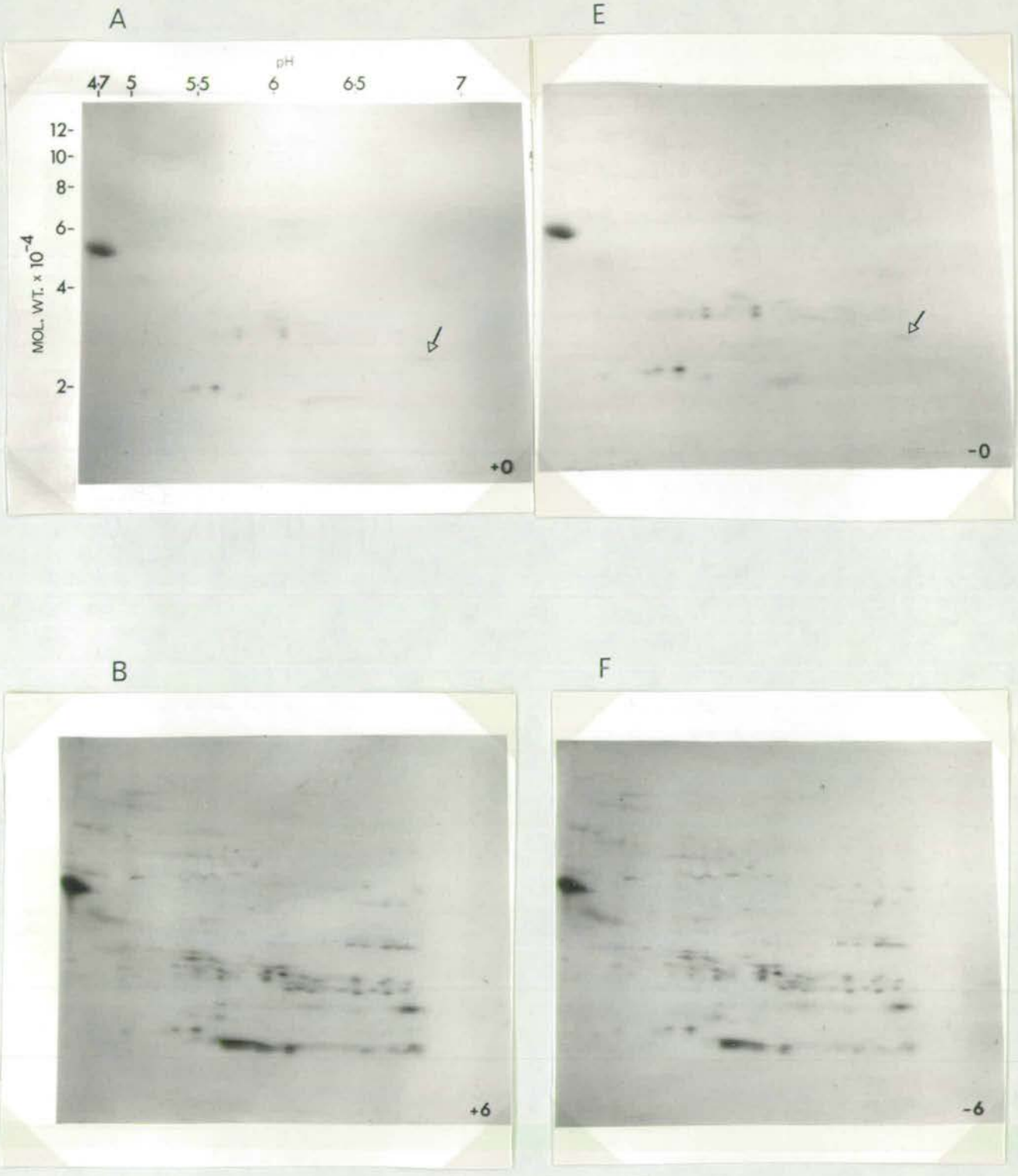
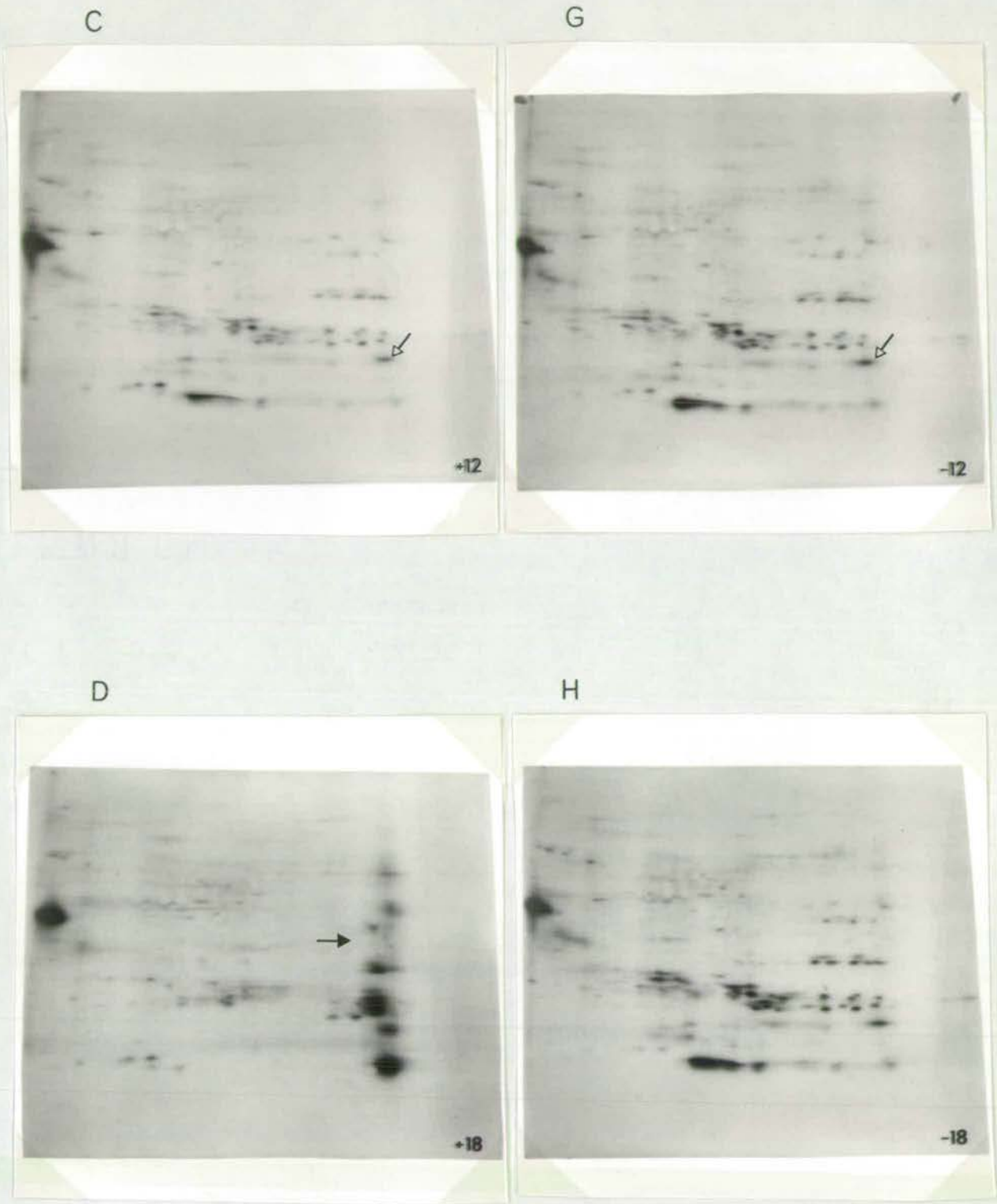


FIGURE 37 (continued)



perhaps in the degree of phosphorylation, has taken place and may bear some relevance to the onset of DNA replication.

The two-dimensional gels were exposed to autoradiography for 5 weeks. O'Farrell, (1975) recommended that a sample mixture containing 180,000 cpm of ^{14}C labelled proteins be loaded on each gel and put on for autoradiography for 4 weeks. The activity on the 0 to 3 hour pulse time gels was certainly very low, representing only 6% of this recommended value. Nevertheless, the activity on the remaining gels appeared adequate despite having only a maximum of 44% of the counts recommended by O'Farrell. In the $^{32}P_i$ experiment, however, the number of proteins which incorporated the label was very much reduced, with 80 to 100 labelled proteins compared to over 1000 of the ^{14}C labelled proteins (O'Farrell, 1975). Therefore, the specific activity of any single protein was enhanced several fold. In addition, the radiation energy of $^{32}P_i$ is over 10 fold higher than ^{14}C.

SECTION B. Phosphorylation of nuclear proteins

Experimental design

The modification of urea-soluble nuclear proteins by phosphorylation was investigated by the incorporation of ^{32}P-orthophosphate after 0, 6, 12, and 18 hours of culture. A total of 30 discs for each time course sample were incubated in 3.0 ml of media with or without auxin. The tissue was given a 3 hour pulse with 100 µCi/ml of $^{32}P_i$ and 10 µCi/ml of tritiated thymidine in 1 µM thymidine carrier as described in the previous experiment. The nuclei from 25 discs were isolated by chopping the tissue in Honda medium (Methods, C-4-d-ii) and the nuclear preparation was suspended in 110 µl of lysis buffer (Methods, C-4-f). Following centrifugation to remove the chromatin the urea-soluble proteins were separated by two-dimensional gel electrophoresis (Methods, C-5-c) and the slab gels put on for autoradiography (Methods, B-4-b-iii). As in the previous section the remaining 5 discs were removed for a TCA extraction of total protein and nucleic acids (Methods, C-4-c) and the radioactivity was measured for each (previous section and Methods, B-4-b-i).

Results

The experimental results are presented in Table (16). The incorporation of tritiated thymidine into DNA, after correction for uptake, was increased over 10 fold in the auxin treated as compared to the non-treated tissue after 18 hours of culture. The wound response is very evident between 6 and 9 hours of growth with both cultural states incorporating the thymidine label into the acid-insoluble precipitate. Owing to a lack of material, a quantitative estimate of the amount of protein loaded on each gel was not possible. However, an estimate of

TABLE 16.

Data for experiment on ^{32}P-orthophosphate-labelled nuclear acidic proteins of auxin treated and non-treated tissue

In order to monitor possible changes in the phosphorylation of the acidic nuclear proteins with culture in media containing or lacking auxin, the discs of artichoke tissue were pulse labelled with $^{32}P_i$ at various times. Tritiated thymidine was included to monitor the onset of DNA synthesis (experimental design). The nuclei were isolated (Methods, C-4-d-ii) and approximately 50 µg of urea-soluble proteins were loaded on each gel. An estimate of the amount of radioactivity on each gel was made from the radioactivity incorporated into an acid-insoluble precipitate.

TABLE 16.

Treatment	Cultural age at end of pulse	Estimate of total protein	Estimate of extracted nuclear protein	Ratio of total protein to nuclear protein	Incorporated of $^{32}P_i$ into total protein corrected uptake	Estimate of radio-activity on each gel	Incorporation of tritiated thymidine into DNA corrected uptake
	(hours)	(µg/disc)	(µg/disc)		(cpm/disc)	(cpm/15 discs)	cpm/disc
2,4-D	3	206	4.3	47.9	19383	6070	382
	9	215	4.5	47.8	47595	14936	1035
	15	240	4.5	53.3	58549	16477	969
	21	257	7.0	37.0	161299	65391	4235
control	3	206	4.3	47.9	18150	5684	279
	9	215	4.5	47.8	39448	12379	1083
	15	230	4.5	51.1	60298	17700	265
	21	250	4.9	51.1	61087	17932	330

the amount of nuclear proteins expected from 15 discs after a 27% yield of nuclei (PART V, CHAPTER 2.) is shown in Table (16) and represents only half the amount of cytoplasmic proteins which were loaded on the first dimension gels and similarly separated. Using the incorporation of $^{32}P_i$ into an acid-insoluble precipitate, expressed as cpm/disc, and the ratio of total protein to nuclear protein extracted, an estimate of the amount of radioactivity on each gel was made. This represented only 3 to 36% of the optimum level of activity recommended by O'Farrell (see previous section). These calculations are, however, based on the assumption that the specific radioactivity of the cytoplasmic and nuclear acidic proteins were equivalent. From the low specific radioactivity of the phosphorylated cytoplasmic proteins demonstrated in the previous experiment and the amount of activity present on the gels of the nuclear proteins it is obvious that this is not the case. It has been estimated that the nucleus contains greater than half the total protein phosphate of a cell (Langan, 1967). If the calculated radioactivity on each gel were increased 2 fold (Table 16) this would more approximate the observed activity on the autoradiograms. In addition, since the individual specific radioactivity was high, in that only a small proportion of the nuclear proteins were phosphorylated, a 5 week exposure time for these gels was more than adequate.

Photographs of the autoradiograms from the two-dimensional separation of the $^{32}P_i$ labelled nuclear acidic proteins are presented in Figure 38, (A,B,C,D) and (E,F,G,H) for auxin treated and non-treated tissue respectively. After a 3 hour pulse with $^{32}P_i$ at 0 and 6 hours of culture (Figure 38, A,B,E,F) no difference in the phosphorylation of the nuclear proteins was detected between the two cultural states. Approximately 15 proteins were labelled after 3 hours compared to about 25 to 30 after

FIGURE 38.

Photographs of autoradiograms of a 2-dimensional separation of $^{32}P_i$-labelled nuclear acidic proteins from auxin treated and non-treated tissue

^{32}P-orthophosphate-labelled nuclear urea-soluble proteins were prepared and separated by 2-dimensional gel electrophoresis (experimental design). The proteins were resolved according to their iso-electric point in the first dimension (horizontal direction) and according to their molecular weight by SLS gel electrophoresis in the second dimension (vertical direction).

The gels are displayed on the next 2 pages, with auxin treated and non-treated tissue being compared when the pulse was affected between 0 - 3, 6 - 9 (first page), 12 - 15, and 18 - 21 hours (second page) of culture time.

2,4-D - A (0 - 3) ; B (6 - 9) ; C (12 - 15) ; D (18 - 21)

control - E ; F ; G ; H (the same)

FIGURE 38.

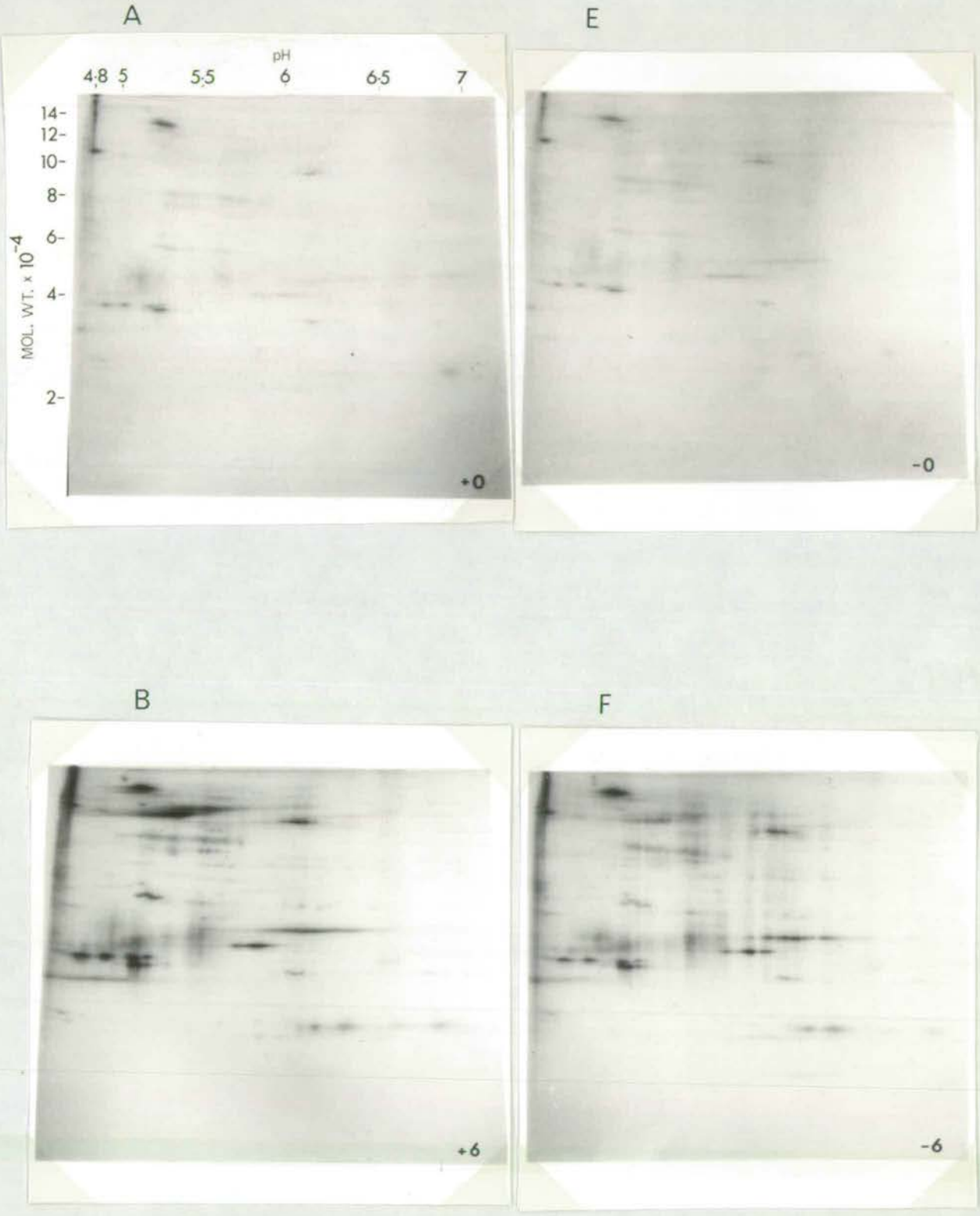


FIGURE 38 (continued)

C

G

D

H

9 hours, as the tissue adjusted to being cut and cultured. Similarly, after 15 hours (Figure 38, C,G) there was little change with about 30 proteins labelled in each. At 18 to 21 hours or during DNA replication in the auxin treated tissue there was a vast difference between the two cultural states with 6 additional proteins being phosphorylated in the dividing tissue. They are as follows :

1. 5.25/100
2. 5.35/110

These proteins are not only the most highly phosphorylated proteins but, as judged by staining (Figure 35) they are two of the most abundant proteins synthesized at this time.

3. 5.8/30
4. 6.0/30
5. 6.6/27
6. 4.8-5.2/36 This occurred as a streak at the acidic end of the gel and was probably one protein with different degrees of phosphorylation.

Discussion

I could not detect any phosphorylated proteins of the nucleus in common with those of the cytoplasm. There was, however, one possibility, a phosphorylated cytoplasmic protein of 27,000 mol. wt. which was evident from the start of culture in both cultural states. Although it had an iso-electric point of 7.0 it could still be the same protein as number 5, but with a different degree of phosphorylation. It is important to note that modification of either nuclear or cytoplasmic proteins by phosphorylation did not occur until S-phase when DNA synthesis had already begun. Perhaps phosphorylation is functional in the activation of those proteins or enzymes involved in the actual mechanics of DNA replication.

But, it is still the changes which may occur before S that would seem more important in the regulation of DNA synthesis.

SECTION C. In vitro phosphorylation of nuclear proteins

In the previous two sections it was demonstrated that the patterns of phosphorylated proteins in the nucleus and the cytoplasm were vastly different. This suggests that the phosphorylation of nuclear proteins takes place within the nucleus itself which is consistent with the results of other systems (Langan, 1967). Although the majority of phosphorylated cytoplasmic proteins had molecular weights of less than 50,000 while those of the nucleus were greater than 40,000, a selected few may be in common but demonstrate different degrees of modification in the two environments. There is also the possibility that some proteins in the cytoplasm may be phosphorylated while passing through the nuclear membrane. As a result they would remain undetected as phosphorylated proteins in the cytoplasm and in addition not be recognizable as proteins in common between the cytoplasm and the nucleus. Phosphorylation of the proteins would also tend to shift the iso-electric point to a more acidic value making absolute identification impossible.

It was decided to test for the phosphorylating capacity of the nucleus itself by incubating the isolated nuclei in media containing the terminal phosphate donor (γ -^{32}P)-ATP and separating the acidic proteins two-dimensionally.

Experimental design

Artichoke tuber tissue discs were incubated in media with or without 2,4-D for 0, 6, 12, 18, and 24 hours. Each time course sample consisted of 40 discs, 20 discs/2 ml media. Nuclei were extracted in the usual manner (Methods, C-4-d-ii) and incubation in 50 µCi/ml of (γ -^{32}P)-ATP as previously described, (Methods, C-4-g). The urea-soluble nuclear

proteins (Methods, C-4-f) from approximately 15 discs were separated by two-dimensional gel electrophoresis (Methods, C-5-c). A sample of the nuclear preparation prior to the addition of lysis buffer and equivalent to 10 discs was used for an estimation of protein and the radioactivity incorporated into protein (Methods, C-4-g).

Results

It is evident from the results presented in Table (17) that the amount of nuclear protein recovered was low and only approximated a 16% yield of nuclei. It was evident, however, that the auxin treated tissue still demonstrated a 1.5 fold increase in the amount of nuclear protein from the start of culture to S-phase. In this investigation of *in vitro* phosphorylation of nuclear acidic proteins the specific radioactivity was enhanced 5 to 7 fold when compared to the level of activity after an *in vivo* pulse with ^{32}P-orthophosphate for 3 hours (previous section). There was, however, a noticeable decrease in the specific activity of the nuclear proteins with an increase in the cultural age and may indicate the presence of increasing levels of protein phosphatase. Also, the adverse conditions imposed by the *in vitro* incubation of isolated nuclei may have prompted the activation of phosphorylase in the nuclei of the later cultural ages.

Pictures of the autoradiograms are presented in Figure 39, (A), (B,C,D,E) and (F,G,H,J) for the 0 time sample and the various culture ages of auxin treated and non-treated tissue respectively. The following observations were made and related to the *in vivo* phosphorylation of nuclear proteins (see previous section) by relative spot positions and patterns.

Firstly, two proteins were totally absent from the in vitro labelling experiment :

TABLE 17.

Data for in-vitro phosphorylation of urea-soluble nuclear proteins of auxin treated and non-treated tissue

This experiment was designed to test for the phosphorylating capacity of the nucleus itself, and to compare these results with the *in vivo* $^{32}P_i$-labelled proteins. Isolated nuclei from both cultural states at ages, 0, 6, 12, 18 and 24 hours were incubated with $(\gamma\text{-}^{32}P)$-ATP and the amount of protein and label incorporated were calculated (experimental design). The nuclei sample used for the protein estimation was precipitated with cold 10% TCA, then dissolved in 0.1 N NaOH. A sample was taken for a radioactivity estimate and the proteins and DNA were again precipitated with 10% TCA. A second radioactivity measurement was made when the macromolecules were re-dissolved for the Lowry (Methods, B-2) and the percent recovery of label (proteins) was estimated.

TABLE 17.

Treatment	Cultural age at time of labelling (hours)	Nuclear protein of recovered (µg/disc)	Specific activity of nuclear protein (cpm/µg)	Amount of protein on each gel µg	Radioactivity on each gel cpm	Percent counts recovered after TCA precipitation
-	0	2.2	2287	35.9	82107	78
2,4-D	6	1.7	3078	34.6	106485	62
	12	2.2	3072	31.1	95524	88
	18	2.3	2175	41.3	89811	71
	24	2.8	2031	51.1	103794	69
control	6	1.3	3852	19.6	75489	83
	12	2.1	2304	33.8	77885	77
	18	3.0	1839	49.3	90643	78
	24	2.3	1020	44.0	44876	65

FIGURE 39.

In vitro phosphorylation of nuclear acidic proteins of auxin treated and non-treated tissue. Autoradiograms of the 2-dimensionally separated proteins

The in vitro-labelled, (γ -^{32}P)-ATP, phosphorylated nuclear urea-soluble proteins were prepared and separated by 2-dimensional gel electrophoresis (experimental design). The proteins were resolved according to their iso-electric point in the first dimension (horizontal direction) and according to their molecular weight by SLS gel electrophoresis in the second dimension (vertical direction).

The gels are presented on the next 2 pages, with auxin treated and non-treated tissue being compared at the cultural ages of 6, 12, 18, and 24 hours. The proteins from freshly excised tissue are also presented.

fresh tissue (0 time) - A

2,4-D - B ; C ; D ; E for the time course

control - F ; G ; H ; J for the time course

FIGURE 39.

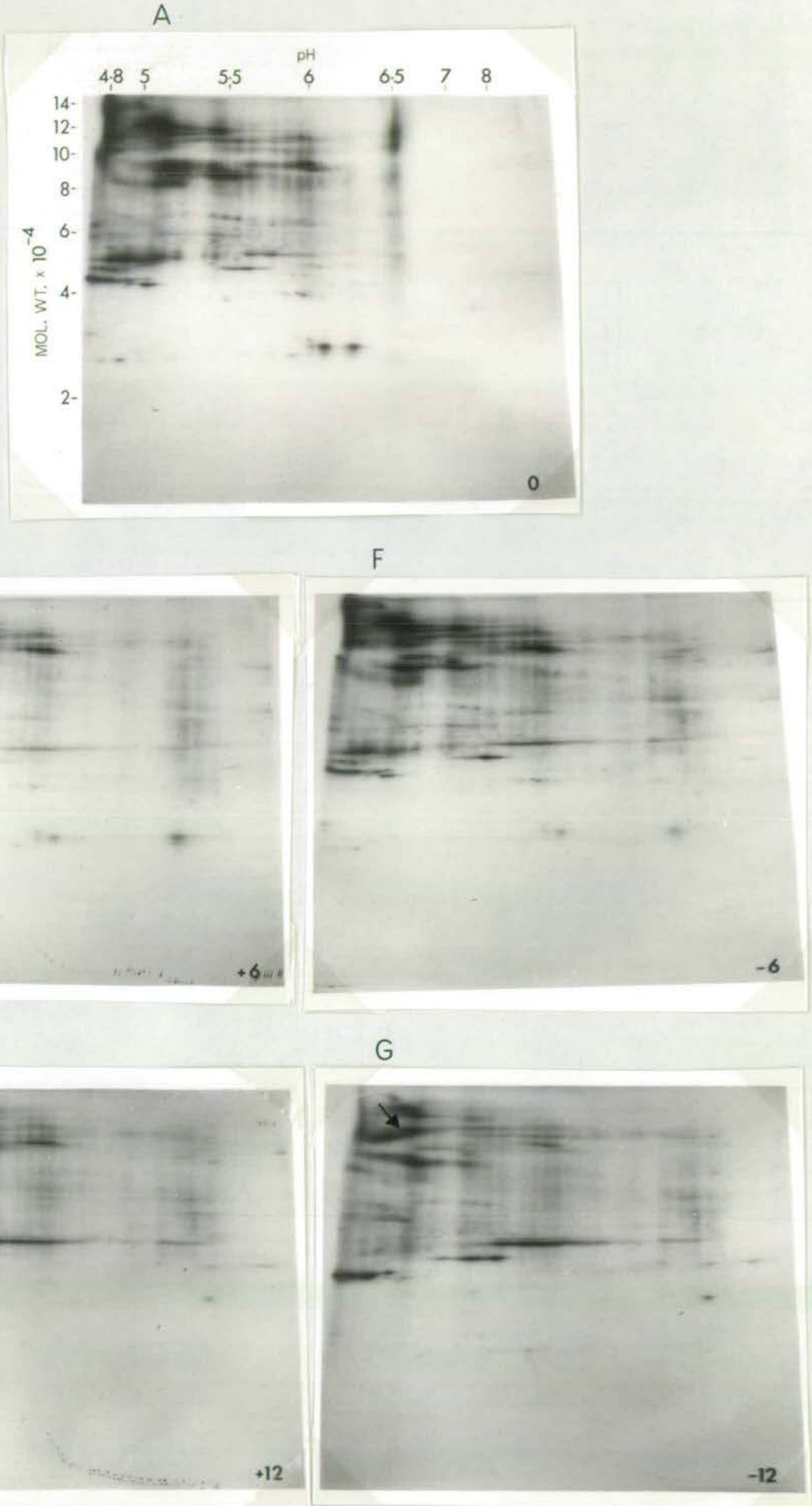
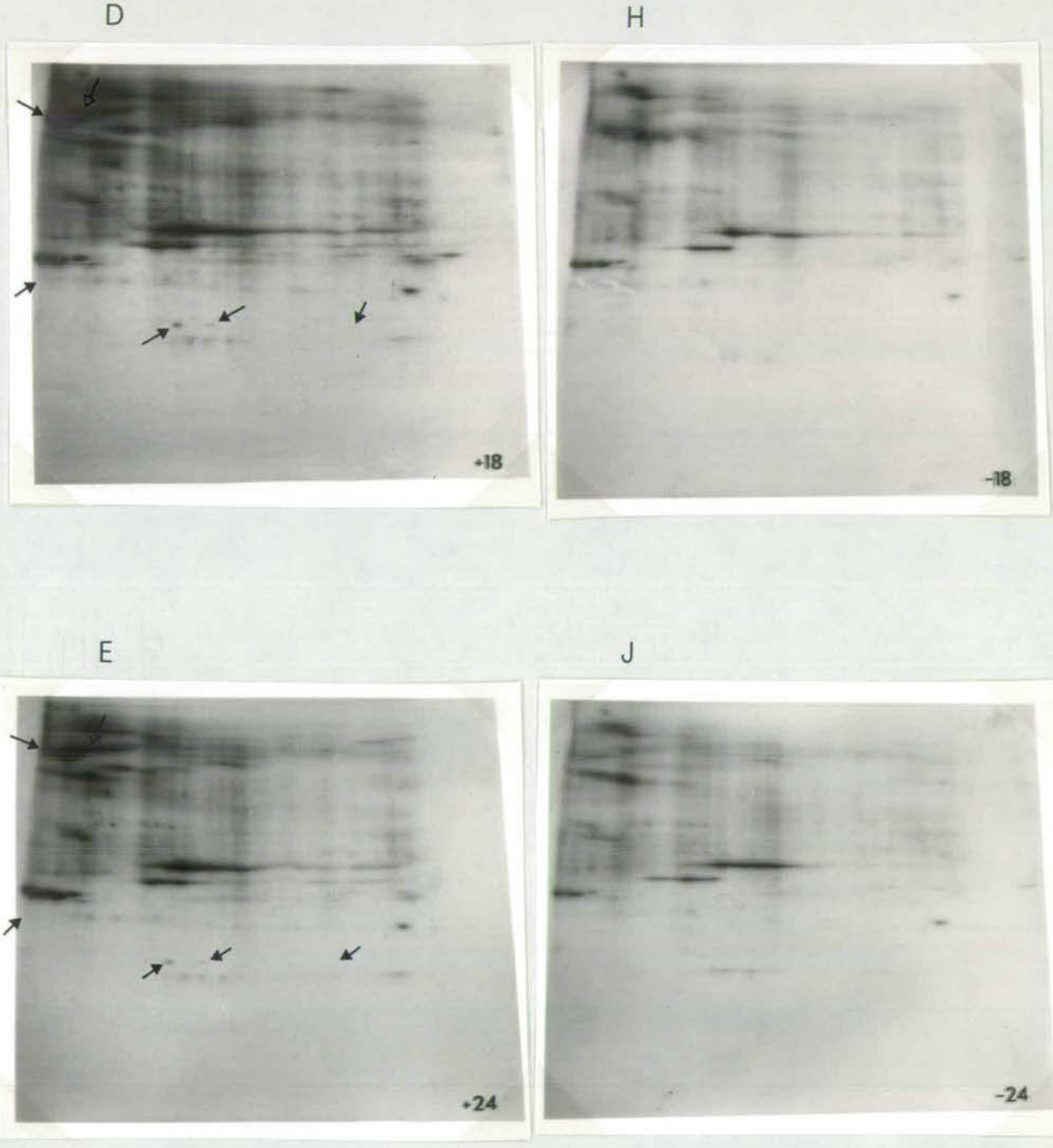


FIGURE 39 (continued)



5. 6.6/27

6. 4.8-5.2/36 (streak)

the absence of these proteins does not necessarily mean they were not present in the nucleus, for they may require a different phosphate donor than ATP or alternatively they may only be phosphorylated while entering the nucleus and so remained undetected in this experiment using isolated nuclei.

The following proteins were shown to be present in the auxin treated tissue at the onset of S-phase at 18 hours :

3.	5.8/30	present as :	5.5/30
4.	6.0/30		5.7/30

Although they have shifted to more acidic iso-electric points by 0.3 pH units, the spacing between the 2 proteins of 0.2 pH units was maintained. In addition, their association with another group of 6 phosphorylated proteins also present in the in vivo experiment makes it certain that they are indeed the same proteins. The shift in the iso-electric points to more acidic values probably indicates a greater extent of phosphorylation under these conditions.

Likewise, the appearance at S-phase of the two highly phosphorylated proteins:

1.	5.25/100	present as :	4.8/100
2.	5.35/110		5.0/110

also indicates a greater level of phosphorylation by the shift in the iso-electric points to more acidic values. The 5 to 7 fold increase in the specific activity of the proteins compared to the in vivo experiment is certainly evidence in the favour of a higher degree of phosphorylation which caused a shift in the iso-electric points. These two proteins (1. and 2.) are also labelled during the wound response in both cultural states but to a lesser degree, in keeping with the transient occurrence of DNA synthesis at this time.

SECTION D. Nuclei-protein kinase assay

Since the nuclear proteins demonstrated a high rate of phosphorylation at the onset of S-phase it suggested that the phosphorylation of certain proteins and enzymes may be critical to the initiation and continued replication of DNA. Various reports, summerized recently by Trewavas, (1976b) have suggested that as proteins are phosphorylated by protein kinases, the activity of these enzymes may ultimately be the controlling feature in the regulation of the genome. Following then, it was expected that the activity of the nuclear protein kinases may be enhanced at the onset of S and therefore represent a sort of switch in the timing of DNA synthesis.

Experimental design

Artichoke tuber discs were incubated in media, with or without auxin for, 0,3,6,9,12,15,18 and 21 hours. Each time sample consisted of 20 discs in 2.0 ml of media. The nuclei from each sample were isolated (Methods, C-4-d-ii) and assayed for protein kinase activity as described in detail in the Methods, (C-4-h). Casein was included in the assay to reduce to a minimum the effect of endogenous protein phosphorylation and thus give a reliable estimate of enzyme activity. From the 0.9 ml volume of nuclei in sucrose buffer, 0.15 ml remained for a Lowry estimation of protein (Methods, B-2).

Results

The incorporation of $^{32}P_i$ into an acid-insoluble precipitate after the various reaction times is presented in Table (18), while the amount of protein extracted and the initial reaction rates for each are shown in Table (19). Figure (40) is a graphic representation of protein kinase activity expressed as the initial reaction rate, counts/minute/µg

TABLE 18.

Protein kinase assay - Data for the incorporation of ^{32}P-orthophosphate

Nuclei were isolated from both auxin treated and control tissue at various cultural ages up until the onset of S-phase in the treated discs. The protein kinase activity in each sample of 20 discs was assayed by the incorporation of $^{32}P_i$ from (γ -^{32}P)-ATP into an acid-insoluble precipitate. The reaction was stopped after 5, 10, 20 and 30 minutes. In a duplicate sample excess casein was included as phosphate acceptor in order to reduce the effect of endogenous phosphorylation and thereby give a reliable estimate of enzyme activity.

TABLE 18.

	Treatment	Reaction time	Incorporation of $^{32}P_i$ label into nuclear proteins at the following cultural ages (hours) - expressed as cpm/20 discs							
		(minutes)	0	3	6	9	12	15	18	21
Added casein	2,4-D	5	775	1010	880	1850	1730	1350	2300	1700
		10	1400	1560	2130	3180	1980	2900	3850	3200
		20	2100	2520	2910	4850	2700	5050	7240	6840
		30	2860	3110	3910	7270	4500	6920	9780	9780
	control	5		1260	1200	1120	1480	1400	1400	1550
		10		1780	2160	1920	1380	2040	2450	2820
		20		2530	2750	3250	2630	4300	4800	5470
		30		3320	3810	4430	3990	6330	7150	8030
No casein	2,4-D	5	210	25	90	450	440	80	210	20
		10	130	10	40	520	490	400	650	700
		20	450	70	280	1030	550	440	990	400
		30	340	330	190	1150	780	750	850	280
	control	5		160	230	250	250	50	20	20
		10		270	330	520	280	100	60	450
		20		340	370	430	300	340	110	50
		30		400	560	520	280	410	470	20

TABLE 19.

Protein kinase assay; Initial reaction rates

The activity of nuclear protein kinase in both auxin treated and non-treated tissue at various cultural ages is expressed as the initial reaction rate. This rate was estimated from the curves of the incorporation of $^{32}P_i$ in the time course labelling and is shown either as cpm/minute/ 20 discs or cpm/minute/µg artichoke protein,

TABLE 19.

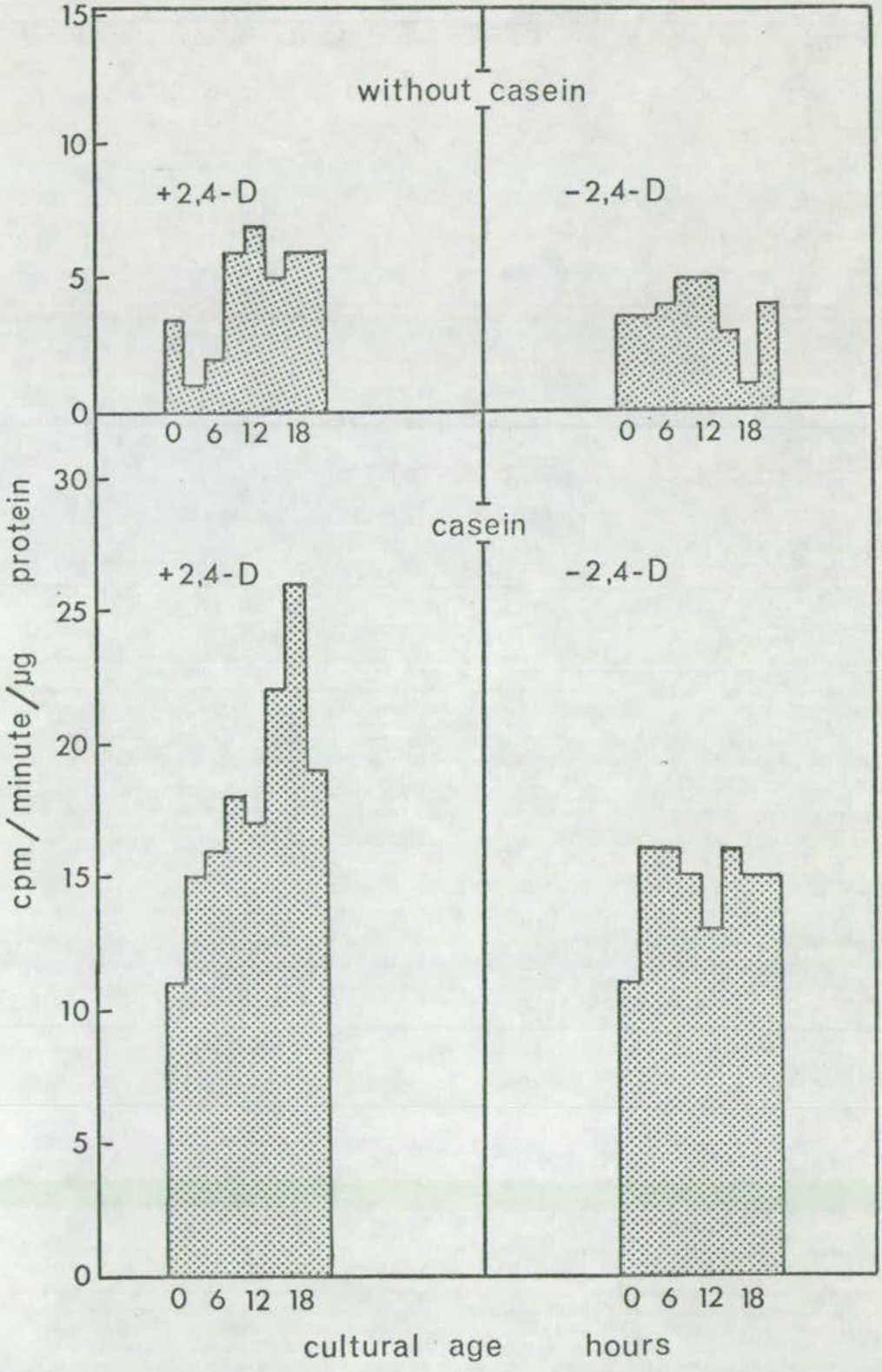
Treatment	Cultural age	Nuclear protein extracted	Initial Reaction Rate (cpm/minute/20 discs)		Initial Reaction Rate (cpm/minute/µg protein)	
	Hours	µg/disc	Casein	no casein	Casein	No casein
-	0	8.1	150	30	11	3.5
2,4-D	3	8.4	200	0	15	1.0
	6	8.1	240	20	16	2.0
	9	8.7	280	65	18	6.0
	12	9.8	260	55	17	7.0
	15	8.9	325	50	22	5.0
	18	9.8	425	75	26	6.0
	21	10.5	380	88	19	6.0
control	3	8.4	250	35	16	3.5
	6	8.6	240	40	16	4.0
	9	9.0	220	55	15	5.0
	12	9.6	205	33	13	5.0
	15	9.0	275	25	16	3.0
	18	9.9	275	0	15	1.0
	21	11.1	300	63	15	4.0

FIGURE 40.

Protein kinase assay: Histogram showing the change in protein kinase activity in nuclei with culture in media containing or lacking 2,4-D

The initial reaction rate in cpm/minute/ µg protein (Table 19) is plotted against the cultural ages of both auxin treated and non-treated tissue. The results from both reaction mixtures (with or without added casein) are shown.

FIG. 40


without casein
+2,4-D
-2,4-D
casein
+2,4-D
-2,4-D
cpm / minute / µg protein
cultural age hours
0 6 12 18
0 5 10 15 20 25 30

artichoke protein. In the auxin treated tissue protein kinase activity demonstrated a 3 fold increase from the start of culture to the onset of S-phase at 18 hours. The increase was linear and did not display a sudden enhancement in activity coincident with DNA replication. This suggested that the activity of protein kinase is not a controlling feature and perhaps the availability of substrate; i.e, the synthesis of nuclear proteins is the major factor. It does appear, however, that these results were influenced by the presence of protein phosphatase since the incorporation of counts dropped after 10 minutes incubation at the later cultural ages (Table 18). This was especially evident in the reaction mixtures without added casein. It is not certain whether the non-treated samples were also affected by phosphatase after 18 hours of culture since the values obtained were rather variable. Since both cultural states demonstrated a similar decrease in the specific activity of the in vitro phosphorylated nuclear proteins (previous section) at the later stages it appears that the increase in phosphatase activity is a general occurrence.

Discussion

The phosphorylation and dephosphorylation of proteins may compete and hence regulate the activity of certain enzymes and proteins. In fact, Szeszák, (1976) has demonstrated, also by in vitro studies, that the plateau value of phosphorus incorporation is the result of an equilibrium between phosphorylation and dephosphorylation. It is possible that, in the unfavourable conditions of an in vitro experiment, the phosphatase enzymes which may normally be compartmentalized became activated in the isolated nuclei especially at the later cultural ages. Conversely, the enzymes were not segregated, but in either case, as the ATP was used up in the kinase reaction the presence of the larger amount

of phosphatase reduced the level of phosphorylation (Figure 18). Since phosphatase activity was particularly associated with the onset of S-phase in the auxin treated tissue this could imply a type of regulation.

A comparison of the activity of protein kinase in the two cultural systems was made by using the initial rate of the reaction, (Table 19) and (Figure 40), which should be independent of the effects of phosphatase. The activity in the auxin treated tissue was enhanced 3 fold from cutting compared to a 2 fold increase in the non-treated tissue. This suggests that protein kinase activity was increased during DNA replication but it is not a major control mechanism for phosphorylation. Interpretation of these results may be hindered by the physical state of 2 different cell populations, dividing and inert, in the auxin treated tissue. Since the activity in non-treated tissue increased 2 fold and the centre core of auxin treated tissue is also in a non-dividing state, a sudden enhancement of protein kinase activity at the onset of S may have gone undetected in the observed gradual increase in kinase activity. In addition, the contribution of specific protein kinases remains unknown.

SECTION E. Nuclear ATP levels

To accompany the preceding experiment on protein kinase activity in the nucleus, the level of ATP in the nucleus was monitored throughout the pre-replication phase and on into S in the auxin treated as compared to the non-treated tissue.

Experimental design

Incubation of the tissue was as described in the previous section with samples of 20 discs used for a nuclear isolation (Methods, C-4-d-ii) after 0, 6, 12, 18 and 24 hours of culture. A PCA-soluble nucleotide extract from each sample of nuclei was prepared (Methods, C-4-k) and assayed for ATP by the luciferin-luciferase procedure (Methods, C-3-d-ii). The nucleic acid sample (PCA pellet) was estimated for DNA content (Methods, B-3).

Results

The results of Table (20) show that the amount of DNA (µg/disc) had almost doubled in the auxin treated tissue after 24 hours of culture. This value and the amount of ATP in the extracted nuclei were corrected for 27% nuclear yield (PART V, CHAPTER 2.) to give a value of pmoles of ATP in the nuclei per disc. Since cell division had not yet taken place the amount of ATP in each cell nucleus was calculated by dividing by 100,000, the average number of cells in each disc.

The levels of ATP in the nucleus depicted graphically in Figure (41) tend to follow the pattern of previous results. From the initial amount of 5×10^{-6} pmoles/cell nucleus of freshly excised tissue the level of ATP in the nuclei of auxin treated tissue had increased 5 fold at the onset of S-phase at 18 hours. At the same time, in non-treated tissue a 3 fold increase was noted. After 24 hours when DNA synthesis

TABLE 20.

Changes in the level of nuclear ATP with the culture of discs in media containing or lacking 2,4-D

The nuclei from 20 discs of auxin treated and non-treated tissue were isolated after 0, 6, 12, 18 and 24 hours of total culture time and extracted with perchloric acid (PCA). The PCA-soluble nucleotide fraction was assayed for ATP content by the luciferin-luciferase method (Methods, C-3-d-ii). The amount of DNA in the PCA pellet was also measured. The level of ATP was corrected for 27% nuclear yield and re-expressed on both a disc and cell basis.

TABLE 20.

TREATMENT	CULTURAL AGE	ATP in 20 discs	ATP/disc	AMOUNT OF DNA extracted	RATIO OF ATP to DNA	ATP/disc corrected to 27% yield	ATP/cell (100,000 cells/disc)
	hours	pmoles	pmoles	µg/disc	pmoles/µg	pmoles	pmoles
-	0	2.5	0.1	0.6	0.2	0.5	5×10^{-6}
2,4-D	6	7.0	0.4	0.9	0.4	1.3	13×10^{-6}
	12	7.3	0.4	0,8	0.5	1.3	13×10^{-6}
	18	13.3	0.7	0.9	0.8	2.5	25×10^{-6}
	24	9.0	0.5	1.0	0.5	1.7	17×10^{-6}
control	6	5.3	0.3	0.8	0.4	1.0	10×10^{-6}
	12	4.0	0.2	0.7	0.3	0.7	7×10^{-6}
	18	8.0	0.4	0.9	0.4	1.5	15×10^{-6}
	24	4.8	0.2	0.9	0.2	0.9	9×10^{-6}

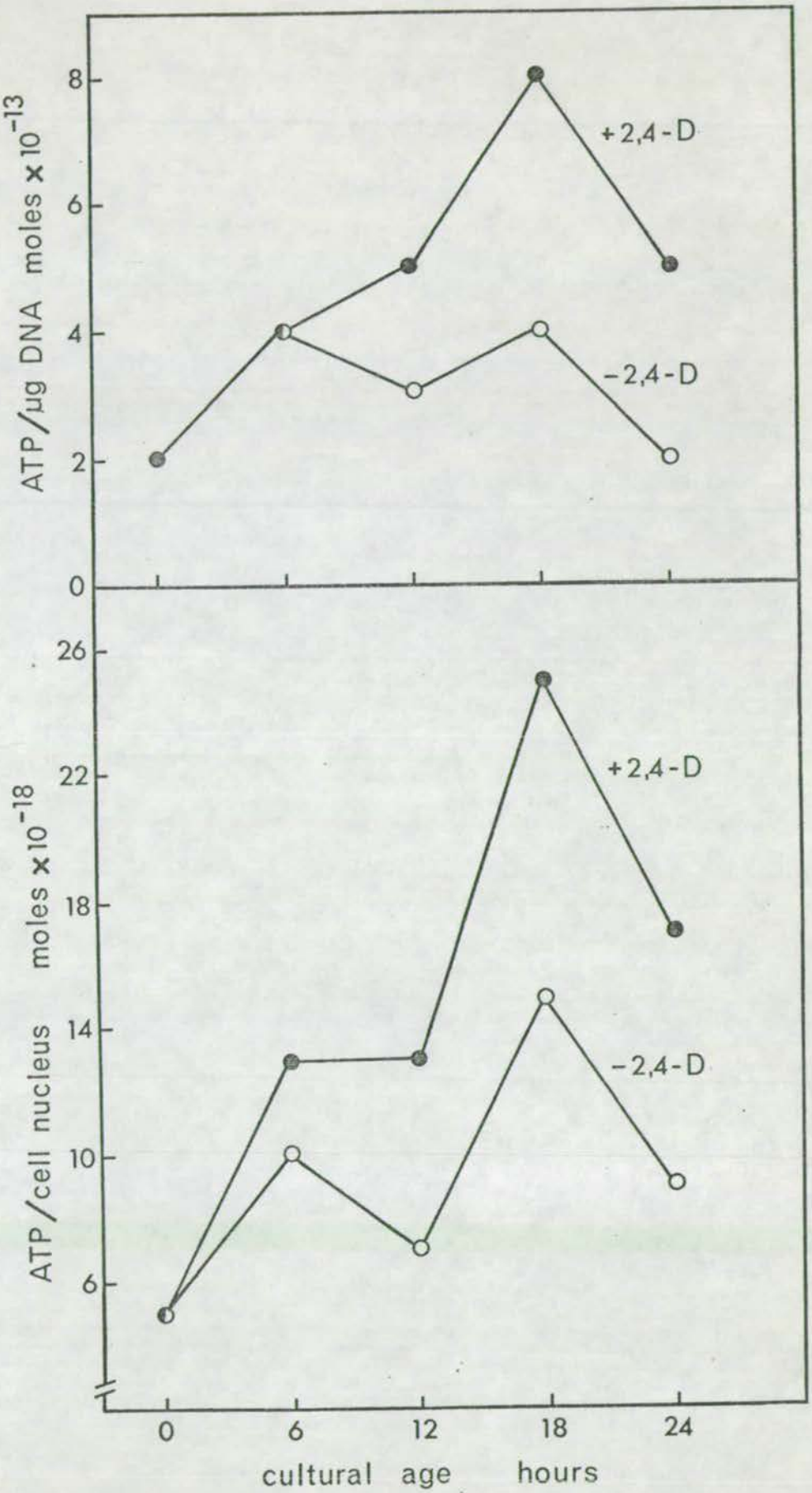
FIGURE 41.

Changes in nuclear levels of ATP with the culture of tissue in media containing or lacking auxin

The levels of ATP in the nuclei of auxin treated and non-treated tissue were compared up until the time when DNA synthesis was in progress in the treated tissue.

The values are expressed as the amount of nuclear ATP/cell and also as pmoles ATP/µg DNA.

FIG. 41


ATP/µg DNA moles x 10⁻¹³
8
6
4
2
0
+2,4-D
-2,4-D
ATP/cell nucleus moles x 10⁻¹⁸
26
22
18
14
10
6
+2,4-D
-2,4-D
0
6
12
18
24
cultural age hours

was in progress in the auxin treated tissue this level fell by 8 units, and similarly so with the non-treated tissue. Both cultural states demonstrated an enhancement of the amount of ATP consequent with the wound response at 6 hours of culture, but the effect in the auxin treated tissue was more pronounced. Since the non-treated tissue shows a similar pattern of response, the small proportion of cells which did divide as a result of wounding demonstrate a degree of synchrony parallel with that of the S-phase of auxin treated tissue. It was also noted that the amount of ATP in the nuclei of auxin treated tissue was only increased 1.6 fold over that of non-treated tissue, a result similar to that obtained for a total cellular estimate in Figure (26). Since there appears to be a substantial level of ATP in the nuclei of non-treated tissue (Figure 41), as well as in the whole cell (Figure 26), the unavailability of energy in the form of ATP does not appear to be the limiting factor in the determination of whether the cells divide.

No doubt the vast majority of the increase in ATP is consumed by the replication of DNA at 18 hours of culture and so not much may be gathered from these results on the availability of ATP for protein kinase.

CHAPTER 5. SYNTHESIS OF ACIDIC PROTEINS IN RESPONSE TO AUXIN

The addition of the auxin 2,4-D to artichoke tuber tissue caused a normal resting cell to be transformed to an actively dividing cell. It is highly probable that one of the changes in response to auxin is the synthesis of specific proteins and enzymes which are involved at some point in the initiation and maintenance of DNA replication. There are numerous reports that the specificity of gene expression may be regulated by certain acidic proteins and their modified forms and that the controlling influence is the movement of specific proteins from the cytoplasm to the nucleus (Goldstein, 1974). Accordingly, I have examined the synthesis of both nuclear and cytoplasmic proteins during the pre-replication period and the start of S to detect whether there are any specific changes which may be attributed only to that tissue which is preparing to divide. In addition I could detect in what part of the cell, the cytoplasm or the nucleus that these changes were associated.

SECTION A. Synthesis of cytoplasmic proteins

The synthesis of cytoplasmic proteins during the pre-replication phase and the onset of DNA synthesis in the auxin treated tissue was compared to that of the non-treated tissue during the same time period by monitoring the incorporation of ^{35}S-methionine into the acidic protein fraction.

Experimental design

A total of 20 discs in 2 ml were incubated in media containing or lacking the auxin, 2,4-D. After 0, 6, 12, and 18 hours of culture each sample was given a 3 hour pulse with 80 µCi/ml ^{35}S-methionine and 10 µCi/ml (CH_3-^{3}H) thymidine in 1 µM thymidine carrier by transferring the tissue to 1 ml of the labelling media. The cytoplasmic proteins from 15 discs were extracted (Methods, C-4-e), separated two-dimensionally (Methods, C-5-c) and the slab gels put on for autoradiography (Methods, B-4-b-iii). The remaining 5 discs were extracted in TCA (Methods, C-4-c) for a measure of total nucleic acids and protein (Methods, B-3 and B-2) and an estimate of the amount of label incorporated into both (Methods, B-4-b-i). The isolated cytoplasmic proteins were also measured for the amount of protein extracted.

Results

As shown in Table (21), cytoplasmic proteins from approximately 1.5 discs equalling amounts of 200 µg to 273 µg were loaded on each first dimension gel. These calculations were made directly from the acetone washed preparation of cytoplasmic proteins and represents nearly 70% of the total tissue protein as estimated from a TCA extraction. Although the amount of protein loaded on each gel was high, the proportion that would actually focus in the 5 to 7 pH range would be somewhat less.

TABLE 21.

Data for experiment on ^{35}S-methionine-labelled cytoplasmic proteins of auxin treated and non-treated tissue

In order to monitor possible changes in the synthesis of the soluble acidic proteins in auxin treated and control tissue up until the start of S-phase in the treated cells, the discs were pulse labelled with ^{35}S-methionine at various times. Tritiated thymidine was included to monitor the onset of DNA replication (experimental design).

The estimated amount of protein loaded on each first dimension gel was calculated from a Lowry estimation (Methods, B-2) of the proteins extracted with sucrose buffer (Methods, C-4-e).

TABLE 21.

Treatment	Cultural age at end of 3 hour pulse (hours)	TCA extractions protein µg/disc	TCA extractions DNA µg/disc	Buffer extraction of protein µg/disc	Amount of protein on gel (= 1.5 discs) µg	Specific activity corrected for uptake ^{35}S protein cpm/µg	Specific activity corrected for uptake ^{3}H DNA cpm/µg	Total protein to DNA ratio	Percent uptake ^{35}S	Percent uptake ^{3}H
2,4-D	3	191	5.0	137.2	205.8	227.1	149.4	38	59.5	65.8
	9	204	4.8	153.6	230.4	687.1	428.3	43	85.8	85.8
	15	241	5.4	149.3	224.0	639.8	402.3	45	97.2	92.4
	21	235	5.0	182.0	273.0	712.7	757.1	47	98.2	91.8
control	3	206	5.0	140.8	211.2	165.0	81.7	41	62.3	69.2
	9	204	4.8	140.8	211.2	725.9	465.2	43	85.8	87.1
	15	285	6.2	175.6	263.4	611.3	394.8	46	97.8	93.8
	21	260	5.6	150.8	226.2	610.4	406.9	46	98.7	93.7

O'Farrell, (1975) recommended that no more than 100 µg be loaded on each gel. The specific activity of DNA, corrected for uptake, was enhanced nearly 2 fold in the auxin treated tissue as compared to the non-treated tissue after 18 hours of culture. DNA replication or S-phase had apparently started at this time. The specific activity of the proteins also demonstrated a sudden increase with the onset of S. In both cultural states there appeared to be a surge of incorporation of label into both DNA and protein between 6 and 9 hours of culture, which coincided with the response to wounding. The yield of DNA was in excess of the usual 2.4-2.7 µg/disc and could be due to a contaminant which reacted with the diphenylamine.

Figure 42 shows pictures of the two-dimensional autoradiograms of cytoplasmic proteins from auxin treated (A,B,C,D) and non-treated (E,F,G,H) tuber tissue during the 21 hour time course. An increase from the initial 200 labelled proteins to about 500 was evident after 9 hours of culture, but no difference in the labelling pattern of the two cultural states was apparent (Figure 42, A,B,E,F). The enhancement of label was probably due to an increased metabolism of the tissue upon cutting and culturing as previously described in PART III, CHAPTER 1, SECTION B. Similarly, when the pulse was effected after 12 hours of culture and also after 18 hours, when DNA synthesis was in progress in the auxin treated tissue (Figure 42, C,D,G,H), still no difference in the pattern of labelling could be detected. From these gels I would estimate that from 800 to 1000 proteins were being synthesized in fairly large amounts.

These gels were subjected to autoradiography for 5 weeks. O'Farrell, (1975) used a sample mixture containing 180,000 cpm of ^{14}C labelled proteins for an exposure time of 4 weeks which he regarded as optimal.

FIGURE 42.

Photographs of autoradiograms of 2-dimensionally separated ^{35}S-methionine-labelled cytoplasmic proteins from auxin treated and non-treated tissue

^{35}S-methionine-labelled cytoplasmic proteins were prepared and separated by 2-dimensional gel electrophoresis (experimental design). The proteins were resolved according to their iso-electric point in the first dimension (horizontal direction) and according to their molecular weight by SLS gel electrophoresis in the second dimension (vertical direction).

The gels are displayed on the next 2 pages, with auxin treated and non-treated tissue being compared when the pulse was effected between 0 - 3, 6 - 9, (first page), 12 - 15, and 18 - 21 hours (second page), of culture time.

2,4-D - A (0 - 3) ; B (6 - 9) ; C (12 - 15) ; D (18 - 21)

control - E ; F ; G ; H (the same)

FIGURE 42.

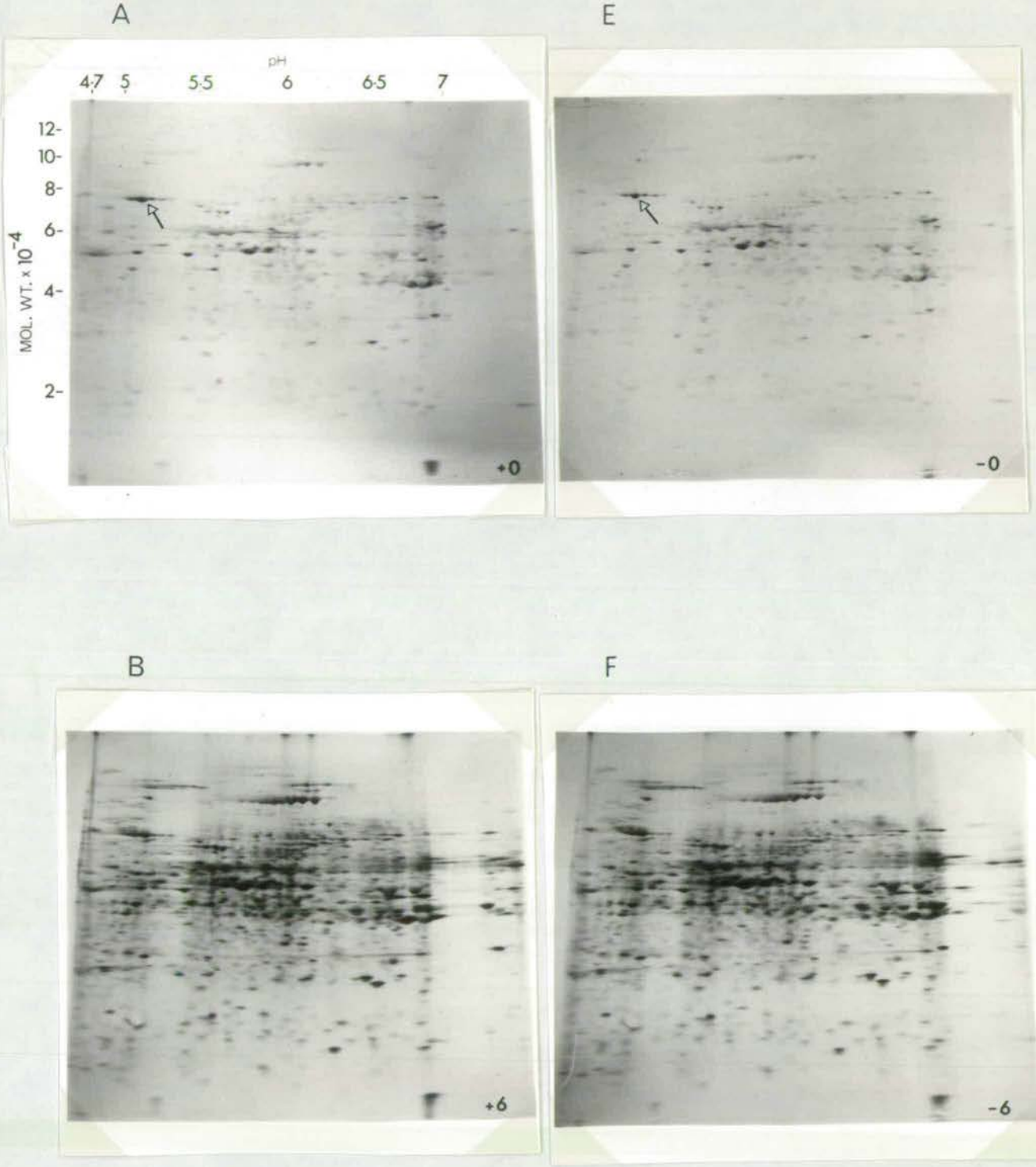
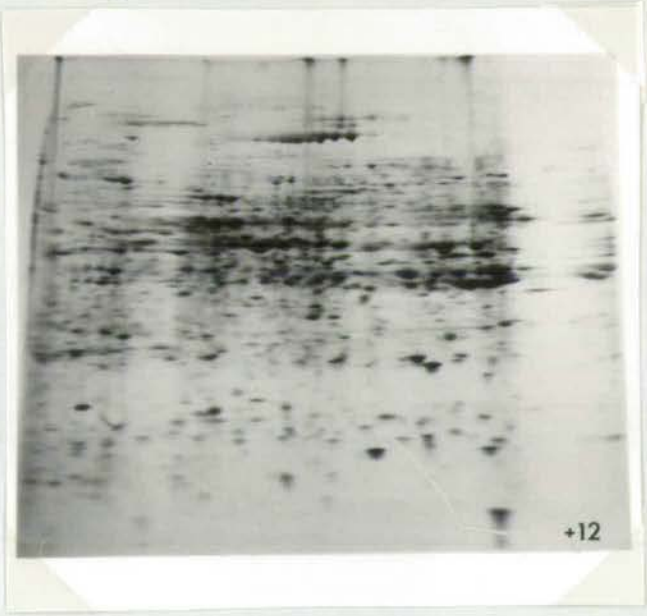


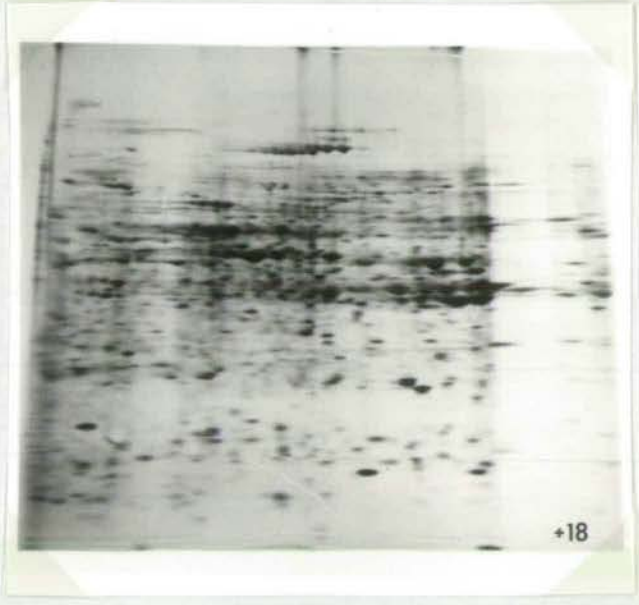
FIGURE 42 (continued)

C

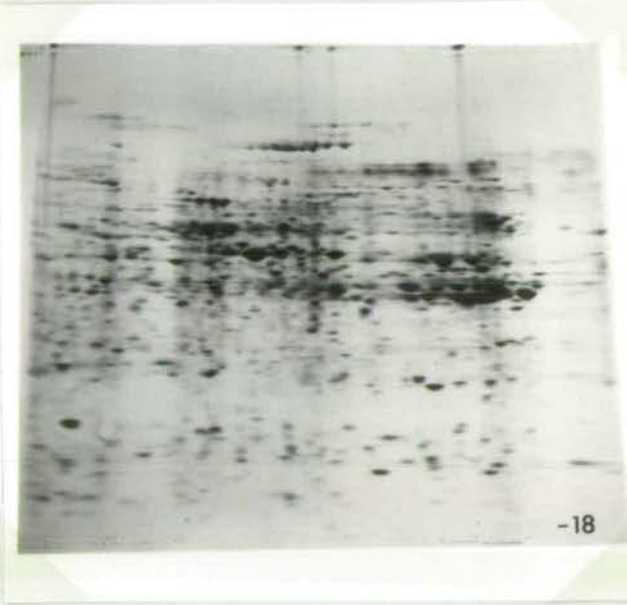


G

D



H



From Table (21), the activity on the 0 to 3 hour gels was very low but with the increased exposure time and greater energy of radiation the ^{35}S-labelled proteins were quite distinct.

SECTION B. Synthesis of nuclear proteins

The synthesis of the acidic proteins of the nucleus was monitored by the incorporation of ^{35}S-methionine. The progression of the auxin treated tissue through the pre-replication phase and the start of S was compared to the non-treated tissue during the same time period.

Experimental design

Artichoke discs (25/2.5 ml media) were incubated for 0, 6, 12, and 18 hours in media with or without auxin. The tissue was labelled as described in the previous section. The nuclei from 20 discs were isolated by chopping the tissue in Honda medium (Methods, C-4-d-ii) and the 10 M urea-soluble proteins (Methods, C-4-f) from half the sample were separated two-dimensionally (Methods, C-5-c) and the slab gels put on for autoradiography (Methods, B-4-b-iii). A duplicate set of gels were also run and these were put on for fluorography (Methods, B-4-b-iv) for the same length of time (5 weeks). The remaining 5 discs were extracted with TCA (Methods, C-4-c) for an estimate of the radioactivity incorporated into the acid-insoluble precipitate which contained nucleic acid and protein. The incorporation of the tritiated thymidine into DNA was discriminated from the incorporation of ^{35}S-methionine into protein by the double channels technique as previously described in PART V, CHAPTER 4, SECTION A.

Results

From Table (22), the auxin treated tissue demonstrated a 3.5 fold increase in the incorporation of tritiated thymidine into DNA over the non-treated tissue after 18 hour of culture, indicating that DNA replication had started by that time. The wound response was clearly evident at 6 to 9 hours in both cultural states, and the non-treated tissue also

TABLE 22.

Data for experiment on ^{35}S-methionine-labelled nuclear acidic proteins of auxin treated and non-treated tissue

In order to monitor possible changes in the synthesis of the nuclear acidic proteins in auxin treated and control tissue with culture, the discs were pulse labelled with ^{35}S-methionine at various times up to the onset of S-phase in the treated tissue. Tritiated thymidine was included to monitor the onset of DNA replication (experimental design). The nuclei were isolated (Methods, C-4-d-ii) and approximately 50 µg of urea-soluble proteins were loaded on each gel. An estimate of the amount of radioactivity on each gel was made from the radio-activity incorporated into an acid-insoluble precipitate.

TABLE 22.

Treatment	Cultural age at end of pulse	Estimate of total protein	Estimate of extracted nuclear protein	Ratio of total protein to nuclear protein	Incorporated of ^{35}S into total protein corrected uptake	Estimate of radio-activity on each gel	Incorporation of tritiated thymidine into DNA corrected uptake
	(hours)	(µg/disc)	(µg/disc)		(cpm/disc)	(cpm/10 discs)	cpm/disc
2,4-D	3	206	4.3	47.9	1.2×10^5	25052	250
	9	215	4.5	47.8	5.0×10^5	104603	860
	15	240	4.5	53.3	1.8×10^5	33771	560
	21	257	7.0	37.0	5.2×10^5	140541	3500
control	3	206	4.3	47.9	1.5×10^5	31315	140
	9	215	4.5	47.8	5.0×10^5	104603	940
	15	230	4.5	51.1	1.6×10^5	31311	330
	21	250	4.9	51.1	3.2×10^5	62745	990

FIGURE 43.

Photographs of autoradiograms of a 2-dimensional separation of ^{35}S-methionine-labelled nuclear acidic proteins of auxin treated and non-treated tissue

^{35}S-methionine-labelled nuclear urea-soluble proteins were prepared and separated by 2-dimensional gel electrophoresis (experimental design). The proteins were resolved according to their iso-electric point in the first dimension (horizontal direction) and according to their molecular weight by SLS discontinuous gel electrophoresis in the second dimension (vertical direction).

The gels are displayed on the next two pages with auxin treated and non-treated tissue being compared when the pulse was effected between 0 - 3, 6 - 9 (first page), 12 - 15, and 18 - 21 hours (second page) of cultural time.

2,4-D - A (0 - 3) ; B (6 - 9) ; C (12 - 15) ; D (18 - 21)
control - E ; F ; G ; H (the same)

FIGURE 43.

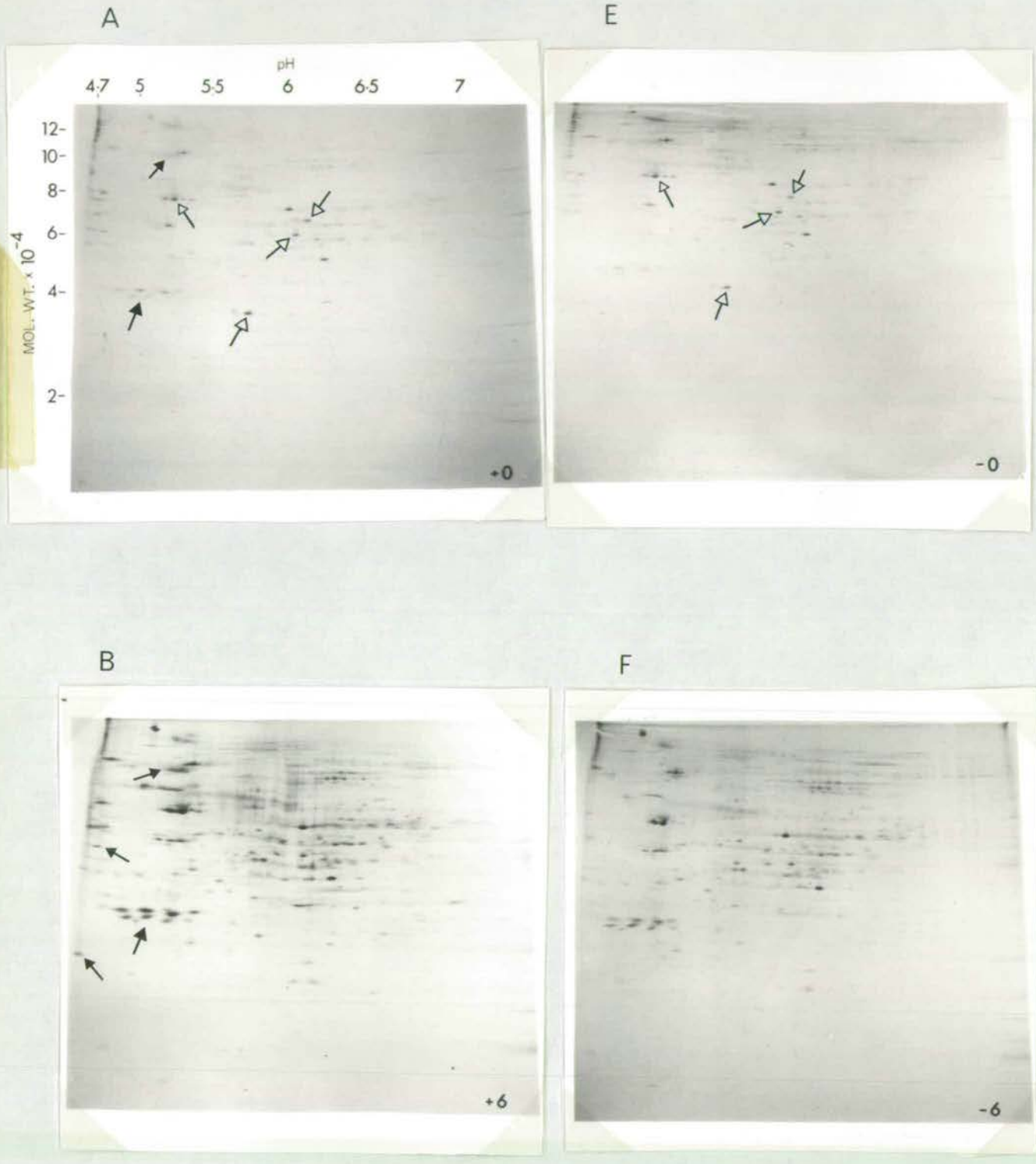


FIGURE 43 (continued)

C

G

D

H

FIGURE 44.

Photographs of fluorograms of a 2-dimensional separation of ^{35}S-methionine-labelled nuclear acidic proteins of auxin treated and non-treated tissue

A duplicate sample of the ^{35}S-methionine-labelled nuclear urea-soluble proteins used in the separation depicted in the previous figure was also resolved 2-dimensionally (see Figure 43). Following electrophoresis the gels were impregnated with PPO in Me_2SO, dried onto filter paper as before, and put on for fluorography at -100°C for 5 weeks (Methods, B-4-b-iv).

The gels are displayed on the next two pages with auxin treated and non-treated tissue being compared when the pulse was effected between 0 - 3, 6 - 9 (first page), 12 - 15, and 18 - 21 hours (second page) of cultural time.

2,4-D - A (0 - 3) ; B (6 - 9) ; C (12 - 15) ; D (18 - 21)

control - E ; F ; G ; H (the same)

FIGURE 44.

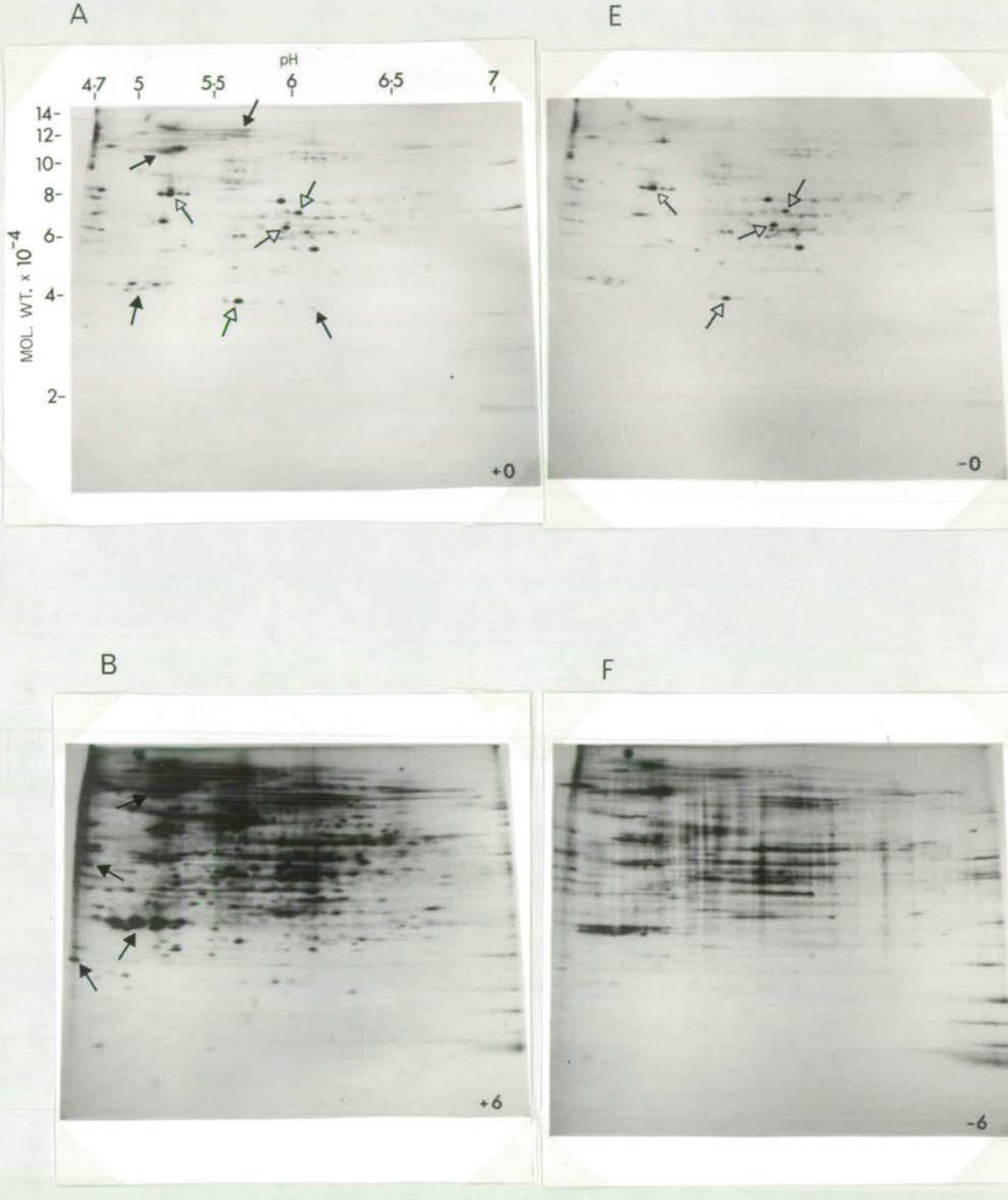


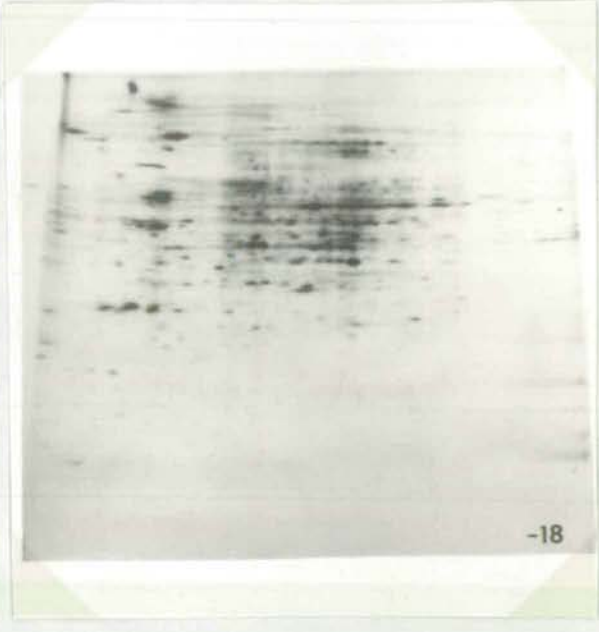
FIGURE 44 (continued)

C

G

D

H



demonstrated some synchrony with a small incorporation of radioactive thymidine at 18 hours. Owing to a lack of material a quantitative estimate of the amount of protein loaded on each gel was not possible. However, an estimate of the amount of nuclear protein expected from 10 discs after a 27% yield (PART V, CHAPTER 2.) is shown in Table (22). Using the incorporation of ^{35}S-methionine into an acid-insoluble precipitate expressed as cpm/disc and the ratio of total protein to nuclear protein extracted, an estimate of the amount of radioactivity on each gel was made. For the majority of samples this represented less than 20% of the optimal level recommended by O'Farrell, (1975), as discussed in PART V, CHAPTER 2, SECTION A.

Pictures of the autoradiograms are presented in Figure 43, (A,B,C,D) for auxin treated and (E,F,G,H) for non-treated tissue at the various cultural ages. Indeed, after a 5 week exposure the activity on some of the gels was very low, making an accurate comparison of the two cultural states difficult. To ensure the quantitative presence or absence of a spot it was decided to enhance the activity by fluorography (Bonner and Laskey, 1974 ; Laskey and Mills, 1975) as described in the Methods, (B-4-b-iv). The results are presented in Figure (44) for auxin treated (A,B,C,D) and non-treated (E,F,G,H) tissue after a 5 week exposure. It was estimated that the intensity of the spots was increased 4 fold.

For the analysis of nuclear protein synthesis in the two cultural states, the autoradiograms and fluorograms were combined in order that preparations giving a similar intensity of spots could be compared. The following comparisons were made :

	Time	+2,4-D compared to	-2,4-D
a.	0 - 3 hours	fluorography	fluorography
b.	6 - 9 hours	autoradiography	autoradiography
c.	12 - 15 hours	fluorography	fluorography
d.	18 - 21 hours	autoradiography	fluorography

a. 0 - 3 hours

Shortly after the addition of auxin there were several noticeable changes in the synthesis of the urea-soluble proteins of the nucleus. They are as follows :

1. 5.15-5.2/100
7. 5.5-5.7/120 (present as a streak)
8. 5.1/44
9. 5.15/44
10. 5.2/44
11. 5.0/44 (greatly enhanced)

] group of proteins

12. 6.15/37

Therefore, 6 additional proteins were present in the auxin treated as opposed to the non-treated tissue.

It might also be noted that in both cultural states, the protein (5.2/80) with 3 satellites at (5.15, 5.25, 5.3) was in common with the cytoplasm (previous section) minus the 4th satellite. With culture the two basic satellites disappeared as was the case in the cytoplasm.

b. 6 - 9 hours

Here, the protein profiles of the auxin treated and non-treated tissue are remarkably similar. The enhancement of incorporation of ^{35}S-methionine into the proteins is evident both in the radioactivity measurements (Table 22) and on the autoradiograms and is most likely

due to the wound response. There are, however, several changes :

1. 5.15-5.2/100 (absent in non-treated tissue)

13. 5.0-5.2/44 (same group as mentioned previously; number of proteins are the same, but the positions of 3 to 4 proteins are altered)

14. 4.7/30

15. 4.75/59

Therefore, after 9 hours of culture there were 6 to 7 different proteins in the auxin treated tissue.

c. 12 - 15 hours

Late in the pre-replication phase of auxin treated tissue several new proteins were synthesized and accumulated in the nucleus. The more obvious proteins are listed below :

1. 5.15-5.2/100

7. 5.5-5.7/120 (streaking)

8. 5.1/44

9. 5.15/44

16. 4.85/42

17. 4.75/42

18. 4.75/40

19. 5.9/100 (plus acidic satellites)

20. 5.75/85 (plus acidic satellites)

21. 6.1-6.2/95 (group of 6 proteins)

22. 5.6-5.7/60 (group of 3 as in inverted L shape)

23. 5.65/82 (plus 1 basic and 1 acidic satellite)

I would estimate that approximately 20 new proteins were synthesized and accumulated in the nucleus of auxin treated tissue in the period of time just prior to the onset of DNA synthesis.

d. 18 - 21 hours

During S-phase or DNA replication in the auxin treated tissue the synthesis of many additional proteins was noticed, especially those in the low molecular weight range. Two in particular were noted as being synthesized in fairly large amounts :

3. 5.8/30

4. 6.0/30

In addition, the synthesis of protein (1. 5.15-5.2/100), present from the start of culture, was enhanced several fold. There appeared to be an enhancement in the synthesis of other proteins in this high molecular weight zone, but the streaking, probably caused by the relative insolubility of some of these very large proteins, made an estimate of the number impossible. I would judge, however, that approximately, 40 additional proteins have been synthesized and accumulated in the nucleus of auxin treated tissue at this time.

e. Proteins which disappeared with culture

There are, in both cultural states, 3 prominent proteins which were lost through culturing :

24. 5.7/38

25. 6.05/59

26. 6.15/65

Discussion

It was possible to detect a number of similarities between the acidic proteins of the nucleus and those of the cytoplasm (see previous section). I did not, however, note any definitive shift of protein(s) from one compartment of the cell to the other at any phase in the culture.

In summary, the following trend in the synthesis of the urea-soluble

proteins of the nucleus occurred in tissue which was stimulated to divide by auxin. Within just 3 hours of cutting there were approximately 6 differences in the synthesis of the nuclear proteins. Although between 6 and 9 hours the enhancement of cellular activity due to excision in both cultural states makes interpretation of the gels difficult, there were still about 7 additional proteins in the auxin treated tissue. Then, after 12 hours of culture in media containing 2,4-D I would estimate that approximately 20 new proteins were synthesized and accumulated only in the tissue which was preparing to divide. Two of the more prominent were :

19. 5.9/100

20. 5.75/85

Finally, after 18 hours or during DNA replication there were 40 or more differences between the two cultural states. It is evident from this time course that cells preparing for DNA replication increase both the rate of synthesis and the number of specifically-nuclear proteins made.

CHAPTER 6. SITE OF SYNTHESIS OF NUCLEAR PROTEINS

SECTION A. Is there a cytoplasmic pool of nuclear proteins?

Introduction

Assuming from reports of other systems that nuclear proteins are synthesized in the cytoplasm, (Zetterberg, 1966 ; Robbins and Borun, 1967 ; Gallwitz and Mueller, 1969) it appears from my results that the nuclear proteins of the artichoke are synthesized and immediately accumulated in the nucleus. Although I could not detect a shift from the cytoplasm of the newly synthesized nuclear proteins mentioned in the previous section, it is still possible that some proteins may be synthesized considerably in advance of their appearance in the nucleus and only move into the nucleus on receipt of the appropriate signal. The presence of such a cytoplasmic pool of nuclear proteins may have remained undetected in the artichoke for the following reasons:

1. The nuclear proteins in the cytoplasm have been obscured by the vast background of cytoplasmic proteins. An effort was made to rectify this by loading not more than 2 discs worth of cytoplasmic proteins on each gel compared to about 4 discs worth (after correction for 27% nuclear yield) of nuclear proteins. But in µg amounts, there was still 2 fold more cytoplasmic proteins on each gel.
2. The proteins were present in the cytoplasm but in a modified form as caused by acetylation, methylation, etc...
3. The proteins were associated with a membranous component in the cytoplasm and were not extractable in sucrose buffer (Methods, C-4-e).

Experimental design

To test the possibility of a cytoplasmic pool of nuclear proteins,

a pulse/chase experiment was set up. The tissue was labelled at two time intervals prior to DNA synthesis. If the proteins were synthesized at this time yet remained in the cytoplasm until the onset of S, when they were moved into the nucleus, they would be detectable as highly labelled spots on a two-dimensional gel of nuclear proteins at the later time in culture when the isotope was absent.

After 0 and 9 hours of culture in media containing or lacking 2,4-D, 2 batches (23 discs/2 ml) each of the discs were given a 3 hour pulse with 200 μCi/ml ^{35}S-methionine plus 10 μCi/ml (CH_3-^{3}H) thymidine in 1 μM thymidine carrier. The discs were washed free of excess label with a large volume of sterile distilled water followed by the appropriate sterile media. The discs were placed in chase media containing 10^{-5}M methionine and all of the cultures were removed after a total culture time of 21 hours or when DNA replication was in progress in the auxin treated tissue. The nuclei from 40 discs were isolated by the usual procedure (Methods, C-4-c-ii) and the nuclear proteins soluble in 10 M urea (Methods, C-4-f) were separated two-dimensionally (Methods, C-5-c) and the slab gels put on for autoradiography (Methods, B-4-b-iii). Approximately half the sample, equalling the nuclear proteins extracted from 20 discs, was loaded on each first dimension gel. Total nucleic acids and protein were extracted from 5 discs with TCA (Methods, C-4-c) and estimates of the amounts were made as previously described (Methods, B-3 and B-2). An estimate of the radioactivity incorporated into each was also made by the double channels method (see PART V, CHAPTER 4, SECTION A and Methods, B-4-b-i).

Results

Estimations of DNA, total protein and radioactivity are presented in Table (23). Both auxin treated and non-treated tissue demonstrated

similar specific activities of DNA and protein after 21 hours of culture. This suggests that the chase has worked since S-phase had started at 21 hours in the auxin treated tissue and a large increase in the incorporation of radioactive thymidine into DNA should have occurred if there was sufficent label present in the tissue. For instance, in the previous experiment the incorporation of tritiated thymidine between 18 and 21 hours was 4 fold that of the non-treated tissue. It is also evident that approximately 3 times as much label was incorporated when the pulse was effected after 9 hours of culture than when label was added immediately after cutting.

Photographs of the autoradiograms of the proteins separated by two-dimensional gel electrophoresis are presented in Figure 45, (A,B) for auxin treated tissue and (C,D) for non-treated tissue. Discussion will be limited to the 0 - 3 hour pulse time since the 9 - 12 hour sample was over exposed and made comparison of the two cultural states difficult. Since an 18 hour chase period demonstrated approximately 3 fold less incorporation of label into DNA compared to the other sample with half the length of chase (9 hours), the earlier pulse was better suited to analysis. Also, the label may have been more easily removed from the tissue only 3 hours after excision. Therefore, the 0 to 3 hour gels were compared to those of the time sequence of the newly synthesized nuclear proteins in the previous section (CHAPTER 5, SECTION B). As it was not possible to accurately match these gels both positions are indicated. The presence or absence of each protein spot was determined solely on the basis of relative spot positions and patterns.

TABLE 23.

Experimental data: Testing for the presence of a cytoplasmic pool of nuclear proteins.

After 0 and 9 hours of culture in media containing or lacking 2,4-D, the tissue was given a 3 hour pulse with ^{35}S-methionine. Tritiated thymidine was added to monitor the onset of DNA synthesis. The discs were washed free of label and re-incubated in fresh media to a final cultural age of 21 hours when S-phase should be in progress in the treated tissue. The nuclei were isolated and the urea-soluble proteins were separated 2-dimensionally (Methods, C-5-c). Estimates of the radioactivity on the gels were made from the specific activity of total soluble proteins (experimental design).

TABLE 23.

Treatment	Cultural age at end of pulse	Total protein	DNA	Specific activity corrected for uptake cpm/µg		Amount of radioactivity on gel
	Hours	µg/disc	µg/disc	^{35}S-protein	^{3}H -DNA	cpm
2,4-D	3	208.0	3.9	1756.6	136.4	82956
	12	157.3	2.7	5490.8	406.0	260016
control	3	186.7	3.3	1954.0	109.4	62856
	12	174.7	2.6	5019.8	385.0	243156

Number	pulse/chase position	pulse position
24.	5.75/35	5.7/38
27.	6.25/30	6.15/33
28.	5.35, 5.4, 5.45 satellites of 5.25/75	5.25, 5.3 satellites of 5.2/80

These proteins were all highly labelled in the pulse/chase experiment after 21 hours of culture of both auxin treated and non-treated tissue. However, as is evident in the previous section, they were only synthesized early in culture (less than 9 hours). Therefore, the high specific activity relative to the other proteins indicates that the chase with 10^{-5}M methionine was successful as was concluded from the DNA data. In addition, these particular proteins must be fairly stable.

If there was a cytoplasmic pool of nuclear proteins which were synthesized early and moved into the nucleus at the onset of S it should be possible, after 21 hours, to detect these highly labelled proteins in the nuclei of the auxin treated tissue only. I did not, however, notice any differencies between the two cultural states. In conclusion, there is no evidence for the presence of a cytoplasmic pool of relatively stable nuclear proteins which are moved into the nucleus at the onset of DNA replication.

The gels were then investigated for the presence of those proteins which only appeared during late G-1 or S-phase of the auxin treated tissue. Two proteins :

3. 5.8/30
4. 6.0/30

which appeared in the nucleus only during S-phase, are just barely evident after the pulse/chase (Figure 45, A and C). Therefore, it is evident

FIGURE 45.

Photographs of the autoradiograms of ^{35}S-methionine-labelled nuclear acidic proteins when pulsed, then placed under chase conditions. Test for cytoplasmic pool of nuclear proteins

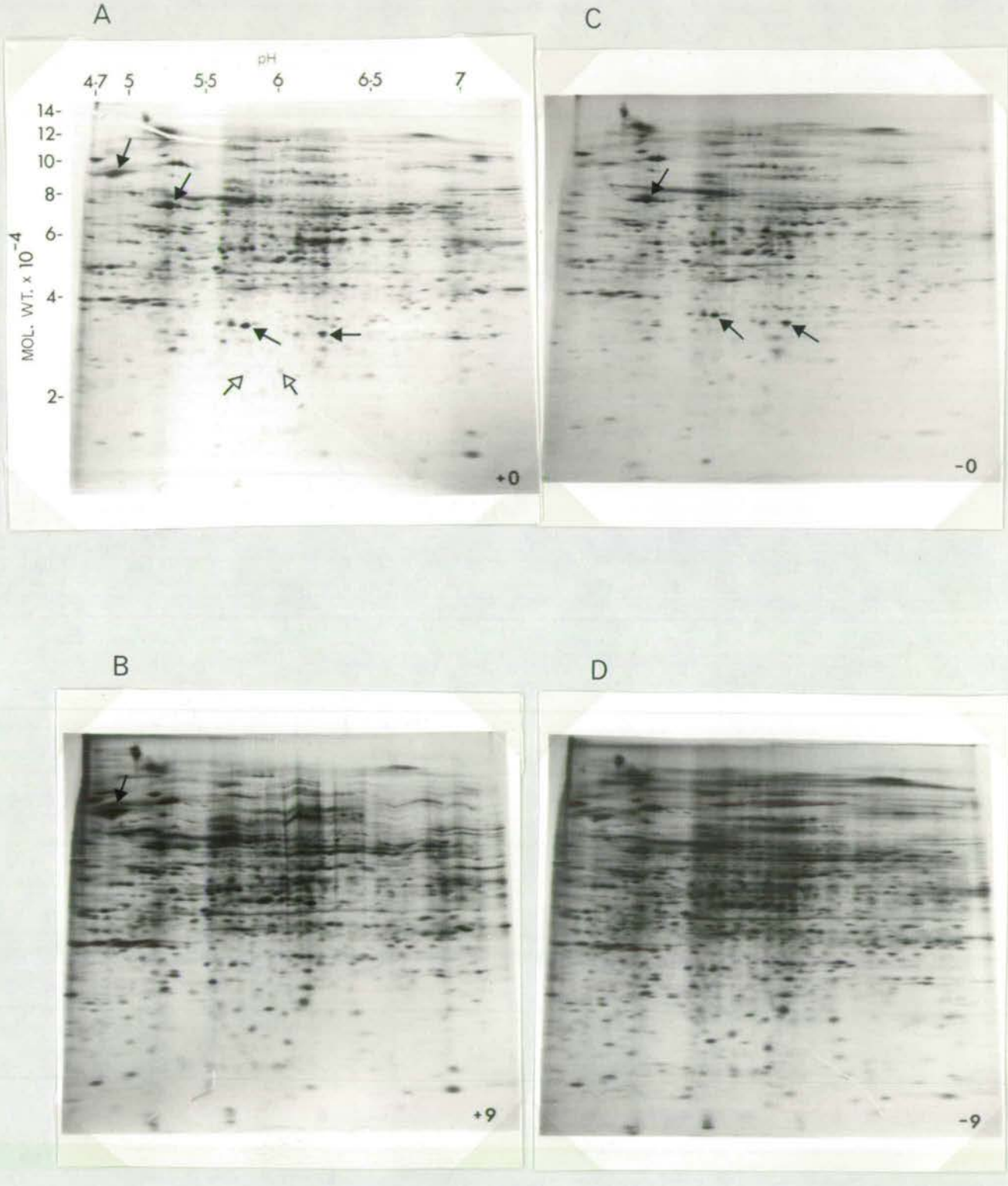
Following a 3 hour pulse with ^{35}S-methionine at 0 and 9 hours of culture and further incubation in cold (10^{-5}M) methionine until a final age of 21 hours, the urea-soluble nuclear proteins of auxin treated and control tissue were separated 2-dimensionally (Methods, C-5-c). The proteins were resolved according to their iso-electric point in the first dimension (horizontal direction) and according to their molecular weight by SLS discontinuous gel electrophoresis in the second dimension (vertical direction).

2,4-D - A (0-3) ; B (9 - 12) hours

control - C (0 - 3) ; D (9 - 12) hours

Design of the experiment: Assuming that the synthesis of proteins takes place in the cytoplasm, if there were any nuclear proteins which were synthesized early in culture and moved into the nucleus upon the receipt of the appropriate signal at some later time, they would be evident as highly labelled spots. No such proteins were detected.

FIGURE 45.



that these two proteins were synthesized and accumulated in the nucleus only at the onset of DNA replication. Similarly, other proteins which suddenly appeared in late G-1 or S were not present as labelled protein spots in the pulse/chase experiment. There was, however, one protein which was on the gel. Protein number (1.) recorded as 5.15-5.2/100 in the previous section was evident as 4.8-5.0/90 in the current pulse/chase experiment. From the gels of the previous experiment where a 3 hour pulse with ^{35}S-methionine was followed by immediate extraction and analysis, it is evident that this protein was synthesized in small amounts throughout culture of the auxin treated tissue, and at the onset of S it was enhanced several fold. In the pulse/chase situation presented here, its reduced specific activity relative to the other proteins is consistent with its low rate of synthesis throughout the pre-replication period.

Protein number (1.) 4.8-5.0/90 was normally associated with a protein at position 5.25/100 but here it had shifted to a more acidic iso-electric point by 0.2 pH units. Evidence that this protein and 5.15-5.2/100 are one and the same arises from the results of the $^{32}P_i$ experiments. After a 3 hour pulse with ^{32}P-orthophosphate was given to tissue treated with auxin for 18 hours (CHAPTER 5, SECTION B) protein number (1.) was highly phosphorylated and demonstrated a position of 5.25/100. In the next experiment (CHAPTER 5, SECTION C) where isolated nuclei from auxin treated and non-treated tissue were incubated in (γ -^{32}P)-ATP this protein was similarly phosphorylated in the 18 hour auxin treated sample. In this case, however, I also showed that it was shifted to a more acidic iso-electric point (4.8/100) due to a greater state of phosphorylation. This suggests that this protein was fairly stable and increasingly phosphorylated with time, which resulted in the observed

shift in the iso-electric point.

Discussion

The pulse/chase experiment was designed to investigate whether any nuclear proteins were specifically synthesized early in culture and then moved into the nucleus at a later stage. No such proteins were found. I did show a low level of synthesis of protein number (1.) 4.8-5.0/90 during the G-1 phase which was only enhanced at S. The results presented here do not support the notion that a pool of nuclear proteins resides in the cytoplasm and are then moved in at the onset of DNA replication as has been reported by some workers. Instead, they suggest that the burst of nuclear protein synthesis occurring at S produces proteins essential for DNA replication and that their movement to the nucleus depends upon their availability in the cytoplasm.

SECTION B. Evidence for differential synthesis of nuclear proteins

Introduction

The results of the previous experiment do not explain the complete absence of the nuclear proteins from the cytoplasm. Evidence thus far on the site of protein synthesis indicates that free polysomes produce intracellular proteins (Redman, 1969 : Ganzoa and Williams, 1969) and ribosomes bound to the endoplasmic reticulum (ER) produce extracellular proteins (Redman, 1969 ; Sherr and Uhr, 1970). But to which class does the synthesis of nuclear proteins belong? If they were synthesized on free polysomes, the polypeptides would be released directly into the cytoplasm, whereas synthesis on rough-ER seems to better favour the transport of proteins to an organelle, since the lumen of the endoplasmic reticulum is continuous with the peri-nuclear space. A particularly advantageous site for synthesis of the nuclear proteins would be on ribosomes associated with the outer nuclear envelope. Proteins synthesized on these membranes could, therefore, be discharged across the membrane and enter the nucleus without ever passing through the cytoplasm proper. The results of Kuehl and Sumsion, (1971) suggest that this might be the case. They found that the specific activity of nuclear proteins exceeded that of the cytoplasmic proteins after administration of puromycin. This would mean that the synthesis of nuclear proteins occurs independent of the manufacture of cytoplasmic proteins. Similarly I have investigated the possibility of compartmentalization of nuclear protein synthesis in the artichoke, but without inhibitors. Another problem concerned the diffusion of radioactive methionine, the isotope used in the pulse/chase type of experiment required here and in the previous section. In an auxin treated disc

there are 2 populations of cells, the outer dividing cells, and a central core which do not divide. It is important, therefore, to ensure that any differences observed between; (1.) the labelling patterns of the two cultural states and (2.) the synthesis of cytoplasmic and nuclear proteins, would not be due to a difference in the rate of diffusion of label through the 2 cell types.

Experimental design

A total of 100 discs were prepared for each sample (plus or minus 2,4-D) and 50 discs/ml media were labelled from 0 to 3 hours with 100 μCi/ml ^{35}S-methionine and 10 μCi/ml (CH_3-^{3}H) thymidine in 1 μM thymidine carrier. The discs were placed under chase conditions as described in the previous section and samples were removed after 3, 6, 9, 14, and 24 hours of culture. From each sample 4 discs were used for a total extraction of the TCA-insoluble nucleic acids and proteins (Methods, C-4-c). The amount of protein was estimated by the Lowry method (Methods, B-2) and the incorporation of ^{35}S-methionine into protein and radioactive thymidine into DNA was measured in the alkali hydrolysate (Methods, B-4-b-i) by the double channels technique (CHAPTER 4, SECTION A). The nuclei were isolated from 15 discs by the usual procedure (Methods, C-4-d-ii) and subsequently extracted with 0.3 ml cold 10% TCA in a glass-n-glass homogenizor and left in the cold overnight to precipitate the mamromolecules. The nuclear sample was washed 2 x with cold 10% TCA and dissolved in 1 ml 0.1 N NaOH. A volume of 0.75 ml was used for a Lowry estimation of protein (Methods, B-2) while the remaining 0.15 ml was counted for a measurement of radioactivity as above.

The remaining disc was tested for the diffusion of ^{35}S-methionine into the tissue. The disc was washed well with distilled water and

the centre, equal to ¼ the volume of the entire disc was removed with a cork borer. The uptake of label into the centre and the outside of the disc were compared by crushing the tissue in a small volume of distilled water and measuring the amount of ^{35}S-label in the supernatant (as above).

Results.

From Table (24), the similar incorporation of tritiated thymidine into an acid-insoluble precipitate after 24 hours of culture in media containing or lacking auxin, indicates that the chase conditions had worked, since the treated tissue would have been synthesizing DNA at this time. The two cultural states also demonstrated a similar increase in the accumulation of total protein up until 24 hours of culture, in keeping with previous results (PART III, CHAPTER 1, SECTION D). However, the nuclear proteins were increased in the auxin treated tissue only, which is in agreement with the results presented in PART V, CHAPTER 2. In addition, the yield of nuclear proteins corresponded to the usual yield of nuclei of 25 to 30%.

The specific activities of the nuclear and total proteins were calculated and after correction for uptake are presented in graphic form in Figure (46). Since approximately 90% of the TCA-extracted proteins would be of cytoplasmic origin they were regarded as such, the two terms being used synonymously. Within 6 hours of removal of the label, the specific activities of nuclear and cytoplasmic proteins in either cultural state were approximately equal. A steady state level of 700 cpm/µg was reached in the auxin treated tissue and 575 cpm/µg in the non-treated tissue. It appears, however, that the specific radioactivities of the nuclear proteins exceeded that of the cytoplasmic proteins at the early chase times, particularly in the

TABLE 24.

Data from a pulse/chase experiment with ^{35}S-methionine. Measurement of the specific activity of the nuclear and cytoplasmic proteins

Discs or artichoke tuber tissue were given a 3 hour pulse with ^{35}S-methionine and tritiated thymidine at 0 time and subsequently cultured a further 24 hours in media with or without auxin. Samples were removed after 3, 6, 9, 14 and 24 hours of culture time. Nuclei from each sample were isolated and the amount of total nuclear proteins as well as the level of total soluble proteins were measured. Following, the incorporation of radioactivity into each fraction was estimated (experimental design).

TABLE 24.

Treatment	Cultural age at end of pulse	Total protein	Nuclear protein extracted	Nuclear protein corrected 30% yield	Specific activity of protein-corrected uptake		Incorporation of tritiated thymidine corrected
					Total	Nuclear	
	Hours	µg/disc	µg/disc	µg/disc	cpm/µg	cpm/µg	cpm/disc
2,4-D	3	202.5	4.5	14.8	395.8	564.0	2694
	6	201.0	3.9	12.9	379.3	688.4	4118
	9	244.5	4.1	13.6	768.6	731.8	7040
	14	229.5	4.1	13.6	704.2	619.0	6068
	24	256.5	5.7	19.6	552.6	747.4	5437
control	3	208.5	4.1	13.6	332.9	385.3	1949
	6	223.5	4.0	13.2	484.5	583.4	3499
	9	214.5	3.6	11.8	710.0	565.6	5120
	14	255.0	3.9	12.9	579.6	570.1	5220
	24	268.5	3.0	10.1	595.1	382.9	5794

FIGURE 46.

Changes in the specific activity of the ^{35}S-methionine-labelled nuclear and cytoplasmic acidic proteins when placed under chase conditions

Following a 3 hour pulse with ^{35}S-methionine at 0 hours the tissue was incubated in auxin or control media containing cold (10^{-5}M) methionine until a final cultural age of 24 hours. Samples were removed at various intervals. The specific activity of the total nuclear and total tissue proteins after a TCA extraction were calculated.

A. 2,4-D ; B. control

nuclear proteins - (●)

cytoplasmic proteins - (○)

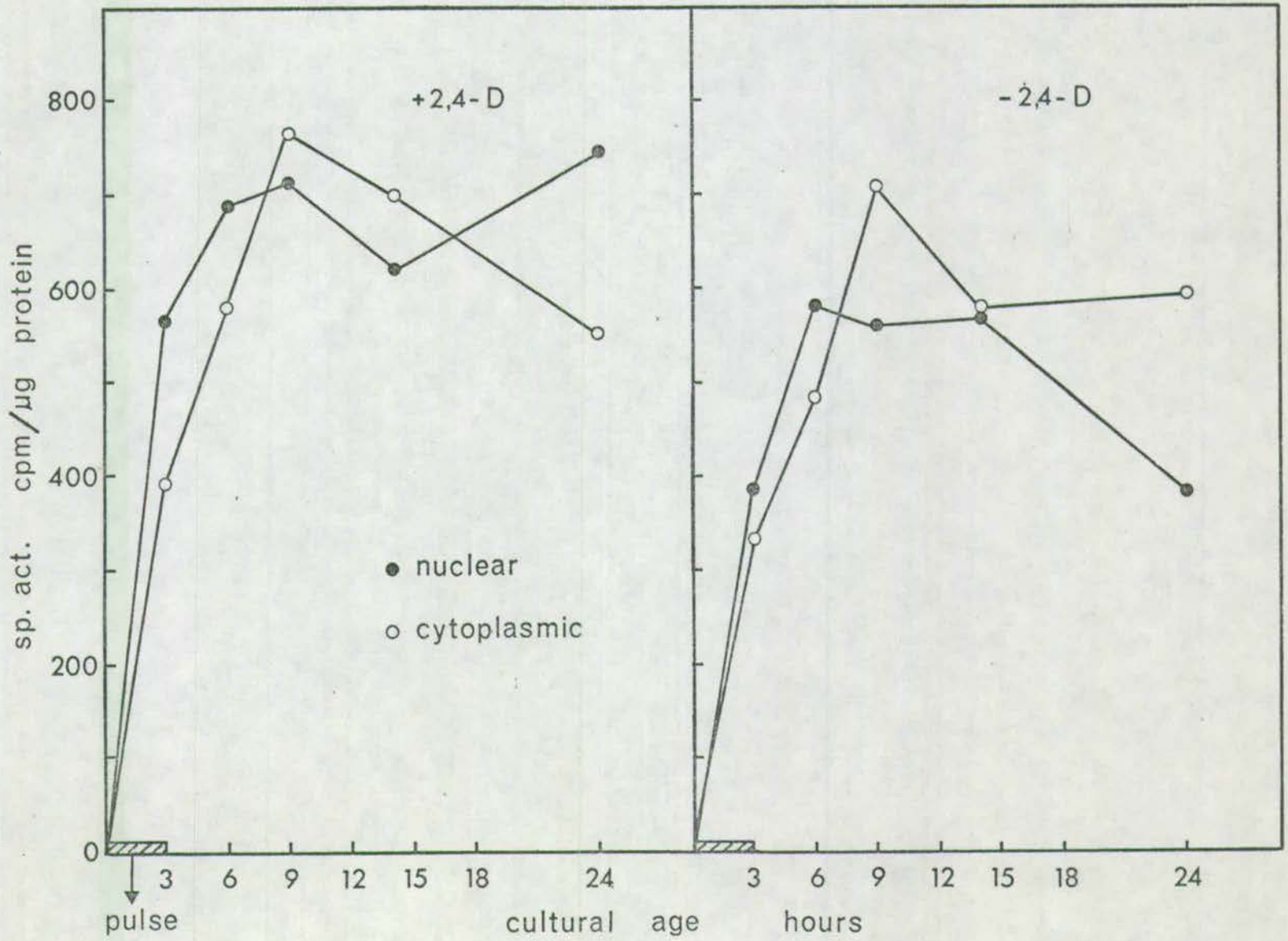

+2,4-D
-2,4-D
sp. act. cpm/µg protein
800
600
400
200
0
nuclear
cytoplasmic
3
6
9
12
15
18
24
pulse
cultural age
hours

FIG. 46

auxin treated tissue which was preparing to divide. This is good evidence in support of a differantial synthesis of nuclear and cytoplasmic proteins.

A measure of the diffusion of label was taken to ensure that, following the removal of the ^{35}S-methionine from the media, both the inside and the outside of the disc simultaneously stopped uptake of the isotope. If there was a continued slow diffusion of label through the tissue the difference in the labelling of the cytoplasmic and nuclear proteins could just be a reflection of this. The calculations and results are presented in Table (25), while Figure (47) is a graphic interpretation. It appears that both the inside and the outside parts of the disc ceased to take up label after the isotope was removed. Although the amount of label in the circumference of the disc was proportionally higher by a factor of 1.6 after removal, this differential was maintained throughout the chase period. In conclusion, any effect diffusion may have had on the results was negligible.

Discussion

It is apparent that, for this experiment and the previous one, a continued slow diffusion of radioactive methionine through the disc was not a serious problem and therefore, I have not been labelling different types of cells. The plateau level of label of the inside of an auxin treated disc was similar to the total level for the non-treated tissue. Since both are non-dividing cells any difference in the labelling pattern or extent of labelling would appear to be due to the dividing cells. It appears, then, that the increased specific activity of the nuclear proteins as compared to the cytoplasmic proteins at the early labelling times was a real event and is good evidence for the differential synthesis of nuclear proteins. Since this was

TABLE 25.

Measurement of the diffusion of radioactivity through discs of artichoke tuber tissue

As described for the previous Table (24) and Figure (46) the discs were pulse labelled with ^{35}S-methionine and tritiated thymidine at 0 time and subsequently transferred to auxin or control media containing 10^{-5} M methionine (unlabelled) to incubate a further 21 hours.

A single disc from each time sample was taken and the centre (=to $\frac{1}{4}$ the volume of the entire disc) was removed. The uptake of radioactivity into each piece, outside ring and the centre, were compared (experimental design).

TABLE 25.

Treatment	Cultural age when sample taken Hours	Radioactivity corrected for uptake inside = 4 x volume cpm/disc		Ratio of outside/inside	Ratio Total/inside
		Outside	Inside		
2,4-D	3	16.6×10^4	11.6×10^4	1.4	5.3
	6	17.8×10^4	11.5×10^4	1.6	5.7
	9	18.5×10^4	7.1×10^4	2.6	8.9
	14	15.5×10^4	11.1×10^4	1.4	5.2
	24	15.1×10^4	12.4×10^4	1.2	4.7
control	3	13.7×10^4	9.4×10^4	1.5	5.4
	6	11.9×10^4	6.8×10^4	1.8	6.3
	9	13.2×10^4	9.9×10^4	1.3	5.0
	14	9.2×10^4	8.5×10^4	1.1	4.3
	24	8.7×10^4	4.9×10^4	1.8	6.3

FIGURE 47.

Pattern of diffusion of radioactivity through discs of auxin treated and non-treated tissue

This is a graphic interpretation of the results presented in Table (25), (previous). After correction for volume differences, the uptake of radioactivity into each part of the disc was calculated for the various chase times.

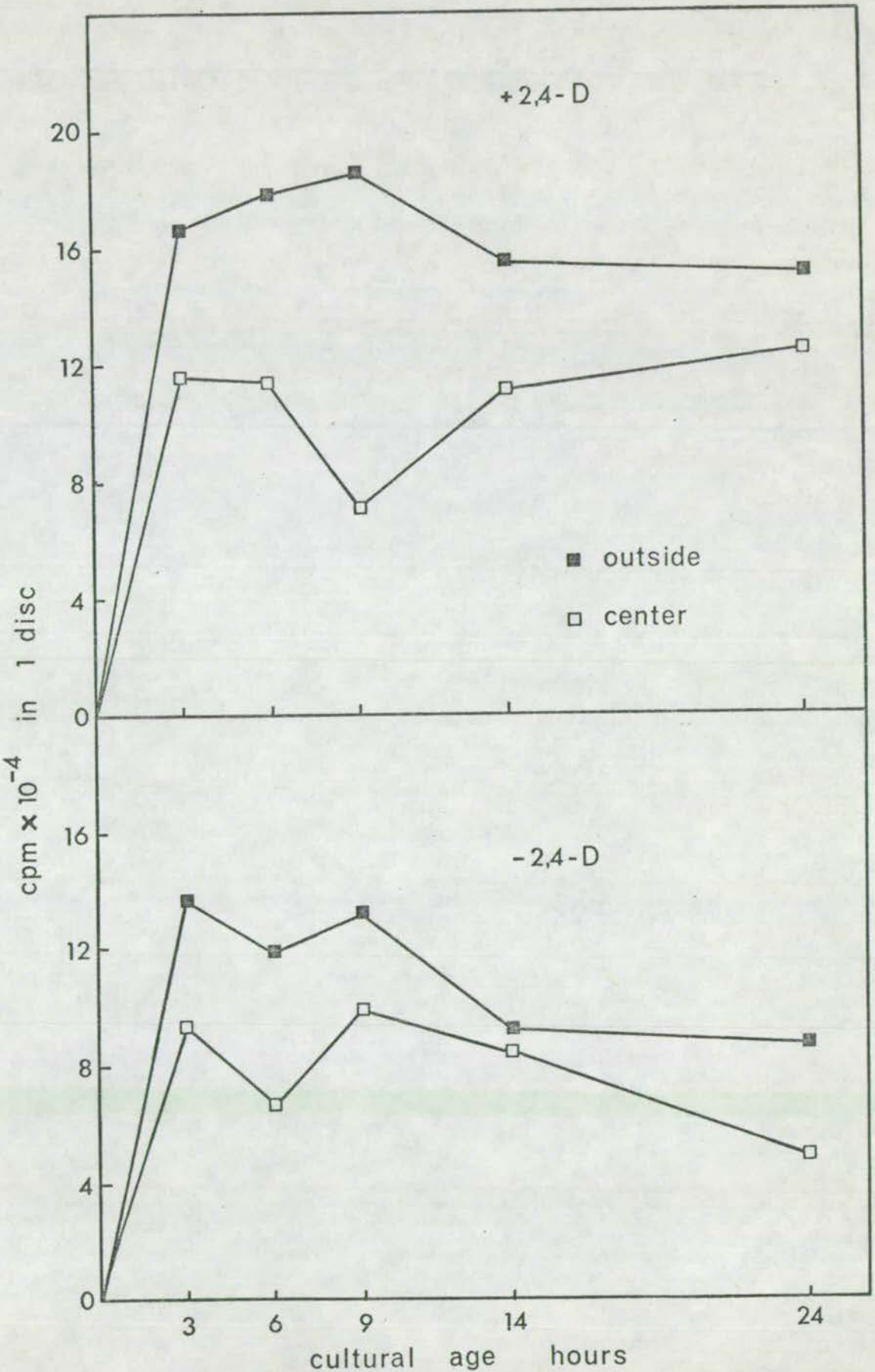
A. 2,4-D - top

B. control - bottom

outside ring of tissue - (■)

centre portion - (□)

FIG. 47


+2,4-D
-2,4-D
outside
center
cpm x 10⁻⁴ in 1 disc
0
4
8
12
16
20
3
6
9
14
24
cultural age hours

particularly associated with the auxin treated tissue it suggests that the synthesis of nuclear proteins may be critical to the onset of DNA synthesis in this tissue. The evidence for differential synthesis combined with the fact that I could not detect any specifically nuclear proteins in the cytoplasm indicates that the most probable site of synthesis for these proteins is on ribosomes associated with the endoplasmic reticulum or the nuclear envelope as suggested by other workers.

CHAPTER 7. SUMMARY

When 2,4-D is added to the resting tissue of the artichoke tuber, DNA synthesis is induced, followed by cell division. Since it appears that the timing of this first division cannot be altered it implies that a certain sequence of metabolic events must occur in preparation of the first S-phase. As DNA replication itself requires certain proteins and enzymes it is likely that a change in the synthesis of particular proteins is the major factor in the timing of the onset of DNA synthesis. Although my results showed very little difference in the accumulation of total cellular protein between the two cultural states up until the onset of S in the auxin treated tissue, there was a 1.5 fold difference in the levels of nuclear proteins at this time.

Indeed, from my analysis of the acidic proteins of the cytoplasm I found no differences between the two cultural states in the synthesis of proteins at any time in culture. I did, however, detect several changes in the synthesis of the acidic proteins of the nucleus in auxin treated tissue early in culture which were markedly increased in late G-1 and more in S. In addition, there was a general enhancement of synthesis in general. During late G-1 (12-15 hours) several newly synthesized proteins appeared in the nucleus. Two of special mention include 5.9/100 and 5.75/85, which are the most obvious changes. Then at 18 to 21 hours, or the start of DNA synthesis a great many additional proteins were synthesized and accumulated in the nucleus. Of particular mention is a high molecular weight protein (5.15-5.2/100) which, although synthesized in small amounts from the start of culture was greatly enhanced at the onset of S and also highly phosphorylated at this time. The synthesis of this protein was only detected and also phosphorylated

in the nucleus. Two other proteins, (5.8/30 and 6.0/30) were only synthesized at S and immediately accumulated in the nucleus where they were phosphorylated. Since I did not detect a definitive shift of any ^{35}S-labelled proteins from the cytoplasm to the nucleus there did not appear to be a cytoplasmic pool of nuclear proteins. Additional evidence in favour of synthesis and immediate acquisition by the nucleus comes from the observed greater specific activity of the nuclear proteins compared to the cytoplasmic proteins. This suggests that nuclear proteins are synthesized independent of cytoplasmic proteins.

The modification of the acidic proteins by phosphorylation did not play a significant role until the onset of S. At this time, a large population of the phosphorylated cytoplasmic proteins demonstrated a massive shift to the same and rather basic iso-electric point. The significance of such a gross alteration in charge is not known and I was unable to detect whether any additional proteins were phosphorylated at S. Several proteins in the nucleus were, however, specifically modified by this means but it did not appear as if any were in common with the cytoplasm. From the *in vitro* $^{32}P_i$ experiments, at least 4 of the nuclear proteins were phosphorylated in the nucleus, with ATP as the phosphate donor. One of these (1. 5.25/100) was highly phosphorylated at the onset of S-phase, and in addition, demonstrated a capacity to be phosphorylated to different extents. If this protein is functional in the replication of DNA the degree of phosphorylation could determine its activity. I did not, however, obtain any definite information on the relationship of the activity of protein kinase in the nucleus with the start of DNA synthesis. The activity only rose gradually throughout the pre-replication phase and did not demonstrate a sudden increase consequent with the observed enhancement of protein

phosphorylation at S.

In conclusion, it appears that the major controlling feature in the induction of DNA synthesis is the synthesis of new proteins, structural or enzymatic. My results indicate that this occurs in sequence with the synthesis of a few proteins regulating the synthesis of more until the cell is prepared for division. It also appears that the timing of the events in the progression to DNA replication cannot be altered. The final control in the actual onset of DNA synthesis may depend on the phosphorylation of particular proteins. Certain critical enzymes may be activated by this means or the phosphorylation of structural proteins associated with the chromatin may alter its availability in favour of replication.

The concepts that I have proposed for the initiation of DNA replication in the artichoke agree in general with the sequential progression through the cell cycle as suggested by Brown, (1976). In addition, the regulation of DNA synthesis by select nuclear protein synthesis and the use of phosphorylation as a specific switching mechanism have recently been reported in several animal systems.

PART VI

DISCUSSION

1. CRITIQUE OF THE METHODS AND RESULTS

Artichoke tubers are harvested in the winter, but as no account is taken of the amount of frost kill, the degree of dormancy of the tubers is not known. Therefore, the comparison of results from year to year may involve a certain degree of error resulting from the variation of the physiological state of each crop. In addition, since the tubers begin to break dormancy after 4 to 5 months of storage, the experimental period is seriously limited. The tissue also exhibits a changing response to auxin which is related to this seasonal activity in that the length of the lag phase prior to the induction of cell division, increases with the time of storage. Although the onset of S-phase was monitored in the majority of protein experiments this was not the case for the RNA work. Here, I had no record of the position in the cell cycle and had to assume that the cell cycle events changed very little during these experiments. In addition, each experiment was done with tubers of a single plant which imposes a further influence of background variation to which all the investigations were subjected.

Regardless of whether the tissue was prepared as an explant or a disc, the response to auxin in terms of increases in cell number was the same. But, because of the particular differences in the methods of culture, it may not be entirely correct to equate the two solely on this basis. First of all, the nutrient medium was continually flowing over the explants while in the second method of preparation the medium was allowed to cover the stationary discs by capillary action only. In addition, the discs were treated with a higher concentration of 2,4-D.

Only 50 to 60% of the cells of an auxin treated explant or a disc actually divide in the first, almost synchronous division. The inert core of undividing cells could, in fact, be masking certain division related changes and perhaps undergoing their own specific metabolic changes. The fact that the changes I noted in the auxin treated tissue did not occur in the non-treated tissue is not conclusive evidence to suggest a similarity between the inert central core of the treated tissue and the non-dividing cells of the control. I cannot, therefore, be certain whether the events which changed in the auxin treated tissue were actually occurring in the dividing cells.

The experiments on ribosomal RNA maturation were carried out during the second wave of cell division when many of the cells were out of phase in the cell cycle. Therefore, an estimate of the stability of rRNA under these conditions represents an average value for the different cell types. In addition, I have previously mentioned the problems of using a simple equation to describe the kinetics of a more complicated set of events.

Although it was proposed that the limitations in the maturation of the ribosomal RNAs was due to a deficit of proteins no attempt was made to investigate this. In view of the difficulty in obtaining measurable amounts of ribosomal protein, especially from the nucleus, and to be certain of their identity, I have assumed, along with other investigators, that the stability of rRNA depends on the presence of the appropriate proteins. Also, I did not attempt to measure ribonuclease or acid phosphatase activities, but as little degradation of the rRNA peaks or radioactivity profiles was evident this appeared unnecessary.

There are several specific problems in regard to the investigation

of the induction of DNA synthesis in the artichoke by auxin.

1. The entry of the cells into the first division is only semi-synchronous and due to this slight variation, the protein changes, which appear gradual may, in fact, be sudden and associated with one particular part of the pre-replication phase. In addition, sudden, yet minute, changes may have occurred which, in the background of the numerous other proteins, remained undetected.

2. One of the more serious problems to consider is the use of an inducer to stimulate the transition of a resting cell to a dividing cell. The nature of the action of 2,4-D is not known and it is not certain whether the progress of cell division is normal or identical to a natural situation. This is especially true for the first division but may also apply to the second and subsequent divisions.

3. The response of the tissue to excision itself, was not studied in detail, and consequently, its effect on the induction of DNA synthesis is not known.

4. A particular fault in the design of my experiments is that I never examined the changes beyond S and into G-2 and M. The experiments, however, take several weeks to complete and by the time it appeared that there were specific division related changes the tubers had progressed beyond the dormant stage and were unfit for cell cycle analysis.

5. The two-dimensional method for the separation of proteins is a powerful analytical tool for the detection of specific changes. It is, however, a degredative and purely descriptive technique which would be particularly useful in combination with other methods. For instance, as the proteins are denatured it is not possible to identify them as structural or enzymatic, nor is it possible to tell whether a single

protein spot is functional on its own or as part of a larger complex. The incorporation of label into the proteins has been used as a quantitative measure of specific synthesis although qualitative changes in the proteins may also determine their detection on the gels. Even if these specific changes are ignored, estimates of the amount of radioactivity in the protein spots, made by microdensitometer analysis of the exposed autoradiograms, would not give an accurate rate of synthesis. It would have to be assumed that each protein had the same proportion of methionine or the same amount of the phosphate acceptor amino acid, serine. It must also be remembered that the enhanced synthesis or phosphorylation of a particular protein is not necessarily an indication of its relative importance in a cell.

6. Light microscopy was used to estimate the purity of nuclei, free from cytoplasmic contaminants. A more thorough examination by the use of electron microscopy and various enzymatic and chemical tests for the presence of cytoplasmic components may have been advisable but did not appear essential in this initial investigation.

In the protein experiments correction of isotope incorporation data for the uptake of particular labels was made without an attempt to confirm the validity of the corrections used. As a detailed analysis was not being done their use probably had not resulted in any significant error in the interpretation of the results. In the ribosomal RNA experiments several attempts were made to correct for the specific activity of the precursor pool to the macromolecules but they were not particularly successful.

2. CONTROL OF RNA SYNTHESIS BY THE AVAILABILITY OF PROTEIN

My results showed that the accumulation of ribosomal RNA in artichoke tissue is dependent upon 2 factors; (1) the synthesis of rRNA precursors (transcription) and (2) the rate of processing of the precursors to the mature rRNAs (post-transcriptional regulation). A kinetic analysis of rRNA synthesis in auxin treated and non-treated tissue revealed a correlation between the amount of the initial rRNA precursor and the supposed level of RNA polymerase (Gore, 1972 ; Gore and Ingle, 1974). This difference in transcription was not, however, maintained in the mature rRNA product but was increased several fold. Since this enhancement in the final level of mature rRNA did not appear to be due to an increase in the processing of the precursor rRNAs in the auxin treated tissue, it was concluded that the stability of rRNA in the non-treated tissue was somehow decreased. The most obvious cause is an increase in specific nucleases but this did not appear likely in view of the similarity between the RNA gel scans of the two cultural states. Instead, it seemed highly probable that the apparent instability of the rRNA in the non-dividing cells of the untreated tissue was due to a deficit of proteins in the nucleolus. Most of the ribosomal structural proteins are associated with the initial rRNA precursor in the nucleus, (Kumar and Warner, 1972 ; Shepherd and Maden, 1972) but since no attempt was made to measure this I do not have any direct evidence of a decrease in the level of protein. It does appear, however, that the association of proteins with the RNA might protect against accidental cleavage or non-specific degradation. Indeed, Liau et al, (1968) and Winicov and Perry, (1974) have shown

that pre-ribosomal particles are only digested to a limited extent by nucleolar nucleases under conditions in which naked rRNA is almost totally destroyed. In addition, Roth and Dampier, (1972) by culturing yeast cells in media lacking essential amino acids or in the presence of a protein inhibitor, cycloheximide, have shown that RNA synthesis depends on the availability of protein. Maden, (1972) has shown a similar occurrence for HeLa cells. It may be inferred then, that the stability of rRNA in the artichoke and hense the final level of ribosomes in the cytoplasm depends on the synthesis of adequate amounts of ribosomal protein. Although the appropriate protein measurements were not conducted, a similar conclusion was reached by Leaver and Lovett, (1974) for Blastocladiella zoospores and by Rogers et al, (1970) for excised pea root tips. This post-transcriptional regulation of rRNA accumulation in the artichoke system is also similar to that demonstrated in resting lymphocytes (Cooper and Gibson, 1971 ; Cooper, 1973), in unfertilized Urechis eggs (Das et al, 1970), and in differentiating yolk sac erythroid cells (Fantoni et al, 1972). The results obtained in all these cases serve to emphasize the variation among cell types with regard to processing efficiency; quiescent or slow growing cells exhibiting substantial degradation of the rRNA precursor molecules without giving rise to mature rRNA components.

Since the association of proteins with rRNA appears to play such an important role in the stabilization of this macromolecule it suggested to me that this might serve as a model for the regulation of the synthesis of other RNA species. For instance, if it is accepted that processing of hnRNA (heterogeneous nuclear RNA) to mRNA (messenger RNA) is not on naked RNA but rather on RNA-protein complexes (RNP) called informefers (Lukanidin et al, 1972 ; Pederson, 1974 ;

Levner et al, 1975 ; Malcolm and Sommerville, 1977) then the synthesis of mRNA would be subject to an analogous and at least equally complex set of transformations as in the maturation of rRNA. Consequently this would mean that the synthesis of ribosomal protein is dependent on the production of protein which would stabilize the appropriate messenger RNA in cyclical fashion. Similarly, if the availability of sufficient amounts of protein is the determinant factor for mRNA stabilization this may explain why the auxin treated tissue continues to accumulate protein with culture while the non-treated tissue demonstrates a steady state level only. Since I found that the total amount of ATP in a cell is about the same in both cultural states it suggested to me that the production of stable messenger may determine the synthesis of new cellular components in response to auxin and therefore indicates that the availability of energy is not the limiting factor in the non-treated tissue. It appears, then, that the maturation pathway for RNA is exploited by the cells as a means of regulating rates of synthesis of the various cellular products.

For instance when assessing what type of RNA is being regulated it is important to know whether it places great demands on the cells. Ribosomes, themselves, are only indirectly responsible for changes in the constituent molecules of the cells, whereas mRNA is the immediate vector. Therefore, control of activation of the tissue and the developmental transition of cells from the non-dividing differentiated state to the dividing undifferentiated state would depend on the synthesis of stable mRNA. The quiescent tuber tissue itself contains appreciable amounts of messenger-like RNA and it appears to be the predominant species synthesized in the period of culture immediately following excision (Fraser, 1975 ; Byrne and Setterfield, 1977).

Fraser and Loening, (1974) have also demonstrated that the massive synthesis and accumulation of rRNA noted to occur after auxin treatment is not required for the induction of the first cell division. This result in combination with the previous observation suggests that sufficient ribosomes are present and that it is the availability of the appropriate mRNAs which is the determining factor in the induction of DNA synthesis. In support of this, Yasuda et al, (1974) have shown that protein and RNA synthesis are required in the induction of cell division in the artichoke by auxin.

Therefore, I feel that a better understanding of how the synthesis and degradation of macromolecules is regulated may provide a greater insight into the control of cell division itself. Consequently, the study of the kinetics of rRNA maturation in the artichoke presented in this thesis may represent the framework by which other events in the induction and maintenance of cell division are regulated.

3. PHOSPHORYLATION AS A SWITCHING MECHANISM

It has been contended, despite direct evidence, that the phosphorylation of certain acidic proteins in the nucleus may regulate the activity of certain genes (Allfrey, 1970 ; Allfrey et al, 1973 ; Stein et al, 1974). There are obvious difficulties in attempting to measure the transcription of a single messenger for a particular protein out of many. I have, therefore investigated the role of phosphorylation in one event, the induction of DNA replication. In the artichoke, my results show that the phosphorylation of the acidic proteins in both cultural states was similar until the start of S-phase or DNA synthesis in the auxin treated tissue. At this time the majority of phosphorylated proteins of the cytoplasm had shifted to a different position in the iso-electric focusing gel, indicating an alteration of charge. In the nucleus, however, there were several specific changes with about 6 new proteins being phosphorylated. Since the changes in phosphorylation only occur at the onset of S and not during the pre-replication period it suggests to me that it does not function as a regulator of transcription. For instance it seems plausible that the regulation of the production of certain proteins relevant to the mechanism of DNA synthesis would occur in the time prior to S. Although new proteins did appear in the nucleus at this time there was no change in the phosphorylation pattern. In addition, I cannot believe that the specificity of phosphorylation is such that it would preferentially modify a particular part of the genome for transcription. Schmidt and Goodman, (1976) have recently supplied autoradiographic evidence in support of the non-specific phosphorylation of non-histone proteins of the chromosomes. Also, in view of the

multitude of control points on each chromosome, phosphorylation as a control mechanism appears highly unlikely. Furthermore, the majority of protein kinases appear highly non-specific, phosphorylating a range of proteins (Silberstein and August, 1973 ; Trewavas, 1976b). Although there are certainly specific protein kinases such as phosphorylase b kinase, (see review by Trewavas, 1976b) and histone phosphokinase, (Bradbury et al, 1974b) the activity of kinase in the majority of cases is probably governed by the availability of substrate, and this does not appear to be an accurate method of control. Also, the requirement for the phosphorylation of the 2 proteins quoted above is more or less predictable by the cell whereas the control by phosphorylation of the transcription of a certain protein in response to an unknown external factor would be practically impossible.

I would like to propose, therefore, that phosphorylation serves to modulate the function of the genome by altering the structure of the chromatin itself, and does not regulate the activity of specific genes. In view of the particular association of phosphorylation with S-phase in the artichoke and the large amounts of energy employed in the reaction it suggests to me that it is an essential feature of the replication of DNA. It is conceivable that phosphorylation may do this in two ways:

1. By the modification of certain structural proteins of the chromatin and thereby serving to maintain the DNA in an extended conformation while replication proceeds.
2. By altering the activity of the polymerase itself.

Regarding the first hypothesis, there are a class of proteins called the unwinding proteins which are credited with this function (Gefter, 1975). Although I have no evidence that phosphorylation

modulates their activity it would seem to be an attractive possibility as a mechanism of control. For instance, these proteins could be phosphorylated by a specific kinase at the onset of S. There is, however, better evidence that phosphorylation behaves as a switching device to change the chromatin from one structural form to another. This is the phosphorylation of histone F 1 which apparently serves to initiate the condensation of chromatin before metaphase, (Bradbury et al, 1974a). Recently, Hotta et al, (1977) and Stern and Hotta, (1977) have shown the transient appearance of a protein (r-protein) which facilitates the re-annealing of DNA. In this case, although its activity is destroyed by phosphorylation, it still indicates a form of control of the chromatin structure. Another isolated case where dephosphorylation may be the controlling factor has been reported by Otto et al, (1977). Here, the phosphorylation of an unwinding protein in mouse ascites cells results in a marked reduction in polymerizing activity.

In the second hypothesis, since phosphorylation is known to modify the activity of many enzymes (Trewavas, 1976b) it suggests to me that DNA polymerase in artichoke tissue could be activated in this way, either by direct modification or by its association with a pilot protein which is phosphorylated. I am able to cite two examples where the activity of DNA polymerase appears to be enhanced by phosphorylation. Tsiapalis, (1977) has reported the presence of a small acidic phosphate acceptor protein in association with purified avian myeloblastosis virus DNA polymerase after in vitro phosphorylation. In the phosphorylated form it resulted in a 10 fold increase in the rate of DNA synthesis, while in the dephosphorylated form there was no effect on the rate. Also, Reisher et al, (1975) found that

phosphorylation of E. coli DNA polymerase with or without cAMP (cyclic AMP) stimulated the activity 2 fold. Although a detailed analysis has not yet been done Dunham and Yunhans, (1977) have suggested that the stimulation of DNA polymerase activity in soya bean may also be mediated through phosphorylated acidic proteins. In view of this evidence it suggests that DNA polymerase in artichoke tissue may also be activated by phosphorylation.

Although phosphorylation may initiate and enhance the replication of DNA in the artichoke there must be an analogous mechanism to stop the reaction. For instance, what prevents the re-association of polymerase and the re-initiation of chain formation. As a working hypothesis I would like to propose the following set of events for the initiation and termination of DNA replication. In the initial transcript containing the gene coding for DNA polymerase there is also a gene coding for a specific replication terminator. DNA replication is initiated when the chromatin has been altered to an extended conformation by phosphorylation and / or unwinding proteins and when DNA polymerase has been activated by phosphorylation. After the initial reaction the specific replication terminator is deposited on the initiation site and prevents further attachment of polymerase. As synthesis comes close to termination it would be advantageous for the cell to reduce the level of phosphorylated proteins. Although the re-association of DNA polymerase is prevented it would be an additional assurance and also allow the chromatin to revert to its native state. This could occur most readily by a balanced activity of protein kinase and phosphoprotein phosphatase, as suggested by Maller et al, (1977). In some cases the presence or absence of a phosphoprotein may be regulated solely on the synthesis of the

particular kinase as suggested by Bradbury et al, (1974b) and therefore the decay of the phosphorylated form is related to the half-life of the enzyme. This does not appear to be a very accurate method of control. Since certain protein phosphates may have a fairly long half-life, control by a fast acting phosphatase would seem to better regulate their amounts in the cell. It is still possible, however, that the activity of certain enzymes may be regulated by the degree of modification. For example, RNA polymerase (Bell et al, 1976) and DNA polymerase (Tsiapalis, 1977) may only be functional with a specified number of phosphorylated serine residues and additional modification may serve to inactivate the enzymes. The extent of the dependence by a cell on the activities of protein kinases and phosphatases has yet to be investigated but it could turn out to be quite substantial. For instance, in the artichoke it appeared that the nucleus was starting to acquire increased levels of alkaline phosphatases at the onset of S-phase. In the cytoplasm at this time I noticed a massive shift in charge of the majority of proteins to a more basic value, which I have attributed to dephosphorylation. It is possible that most of the phosphatase activity was still compartmentalized in the cytoplasm after translation and I had, inadvertently, co-isolated this enzyme with the normal cytoplasmic flora. Therefore, it is possible that increased levels of phosphatase are required for the completion of S-phase.

In view of the evidence that specific acidic proteins of the cytoplasm move to the nucleus (Goldstein, 1974 ; Feldherr, 1975 ; Bonner, 1975b) the question did occur to me that dephosphorylation might be required for this migration. Since I was unable to detect the acquisition from the cytoplasm of any proteins by the nucleus at

the start of S this does not appear to be the case. In addition Maller et al, (1977) noted that the phosphorylated cytoplasmic proteins of Xenopus oocytes were totally different from the nuclear forms. Therefore, the alteration in charge of the cytoplasmic proteins at the onset of S just appears to be a manifestation of the metabolic state of the cell.

In conclusion, although I do not have definitive proof certain things point to the highly phosphorylated, protein of 110,000 molecular weight as being a DNA polymerase. Firstly, the protein is synthesized in large amounts at S and is particularly associated with the nucleus. Harland et al, (1973) have demonstrated an increase in the activity of DNA polymerase activity concomitant with S, which was not due to the activation of pre-existing enzyme or to the removal of inhibitors. Also, this protein is similar in size to the α-polymerase of animal systems (Weissbach, 1977) and wheat germ (Tarragó-Litvak et al, 1975).

4. MECHANISM OF AUXIN ACTION

When the auxin, 2,4-D is added to artichoke tissue in culture, DNA replication and subsequent cell division is induced after a certain period of time. In addition, I found that if 2,4-D is removed from the culture medium fewer cells divide indicating that the auxin must be present continuously in order for DNA replication to occur. This suggests that there is probably a threshold value for the control of cell division. A similar observation was made by Leguay and Guern, (1977) for Acer cells.

Although the mode of action is not known, 2,4-D must interact with a cellular component. Generally it is assumed that plant growth substances, like animal hormones react with a protein since only proteins are able to recognise subtle structural differences between small molecules (Kende and Gardener, 1976). In addition, it is the general contention (Davies, 1973 ; Kende and Gardener, 1976 ; Trewavas, 1976c) that the site of this interaction is the cell membrane. I would, therefore, like to suggest that the decrease in the number of dividing cells upon removal of 2,4-D reflects the loss of the auxin from its respective 'receptor' on the membrane. A possible model for this auxin-receptor complex would be via a plasma membrane-bound ion pump to regulate ion fluxes. Both Hager et al, (1971) and Hertal et al, (1972) have proposed the existance of an ATPase-proton pump which is activated by auxin. Indeed, the influence of ions on various activities of the cell is a well known phenomenon. For instance, depending on the ion concentration of the media, the nuclei can be made to swell or contract. In low concentrations of potassium ions swelling is accompanied by a dispersal of the chromatin and

increased synthesis of RNA (Trewavas, 1976c). In the artichoke I noted that both auxin treated and non-treated tissue accumulated rRNA and protein at the same rate for the first few hours of culture. Similarly, Rose et al, (1972) showed that the nucleoli of artichoke tissue swelled and incorporated RNA precursors when 'aged' in media without the growth substance. Since auxin is also a known agent of swelling and reactivation of the nucleus the initial mode of action would appear to involve ion shifts. All told, this suggests to me that the similarities in the behaviour of auxin treated and non-treated tissue during the first 24 hours of culture were probably the result of permeability changes and general reactivation of the tissue. It is evident, however, that auxin has an effect over and above this reactivation response. First of all, the auxin treated tissue is in the process of DNA replication after culture for 24 hours, whereas the non-treated tissue did not proceed into S-phase at all. Secondly, I noticed a number of changes in nuclear proteins within a few hours of auxin addition which increased progressively to the onset of S. This suggests to me that they are specifically division related changes and therefore, the early events leading to DNA replication are not linked to the permeability effects. Similar to the auxin induced changes in nuclear proteins, Peterson and McConkey, (1976) found that Me_2SO specifically induces hemoglobin production in Friend leukemia cells. In this case too, the mode of action of the inducing substance is not known. Since 2,4-D does not apparently act as the initial entity of a fixed sequence of events, but behaves more like a signal or master switch, I would like to propose that it serves to maintain a suitable ionic or molecular environment for the induction of cell division which is not related to the general reactivation of the tissue.

There appear to be 2 Principal Control Points (Van't Hof and Kovacs, 1972) in the cell cycle, G-1 and G-2. It is at these points of 'arrest' that the commitment to mitosis is made. It has been suggested that G-1 phase is preceeded by a G-0 phase where the cells are in a state not 'committed' to progression through the division cycle (Smith and Martin, 1973). The idea of G-0 is that the initiation of cell replication is at random. Therefore, the increase in population is determined by the probability of the cells leaving G-0 and entering G-1, where the cells are committed to divide. 2,4-D could serve to lower the energy of transition and thereby act as a switch to allow cell passage to the second Principal Control Point. It would be interesting to find out whether 2,4-D is required for the second control since Mitchell, (1967) has shown that artichoke cells which increase their DNA always divide.

In general, we can suppose that the cells are 'arrested' because the metabolic conditions required for the cell cycle to proceed have not yet been achieved. But, in most cases, the reason for the block has been attributed to the lack of proteins for the G-1→S or the G-2→M transitions. Since the synthesis of proteins requires a certain length of time this could account for the observed lag period between the addition of auxin to the artichoke cells and the onset of DNA replication. In addition, since I have shown that the length of this lag phase cannot be altered it suggests to me that the pre-replication period involves an obligatory sequence of events. These events probably involve proteins.

The mechanism of DNA replication itself would require various enzymes and their co-factors and thus far, the cell appears to go through a set of synthetic reactions which occur in a certain temporal

order. For instance, the successive appearance of 3 enzymes specific to G-1 phase has been demonstrated in artichoke tissue (Yeoman and Aitchison, (1973). Similarly, Harland et al, (1973) have shown the synthesis of 3 biosynthetic enzymes which are dependent entirely upon DNA replication. I would assume that the production of these enzymes would have to be regulated and therefore be under some form of genetic control. Since it has been implied that specific gene regulation is a property of the non-histone or acidic proteins (Paul and Gilmour, 1968 ; Wang, 1970 ; Stein et al, 1974). I have investigated the changes in this protein fraction in the artichoke during the pre-replication phase and on into S. This was accomplished by the high resolution two dimensional gel electrophoresis technique developed by O'Farrell, (1975) for the separation of proteins. Since this method will resolve most individual proteins, it has greatly enhanced prospects for the identification of specific gene regulators.

Analysis of the acidic proteins of the artichoke revealed several changes which increased in number as the tissue progressed through the pre-replication period and into S. These changes were, however, in the nucleus only. In particular, late in G-1 phase I noticed the specific accumulation of 2 high molecular weight proteins in the nucleus of auxin treated tissue which, because of their large amount are probably not regulators but, may be involved in the mechanism of DNA replication itself. It was also evident that the protein which I had previously called DNA polymerase was synthesized in low amounts throughout culture but was greatly enhanced at the onset of S. I cannot be certain that no new cytoplasmic proteins of particular relevance to the first division are synthesized in response to auxin but at least there were no obvious changes.

In addition, I noticed that the majority of cytoplasmic acidic proteins were less than 50,000 molecular weight while those of the nucleus were greater than 40,000. Regarding this obvious boundary, Bonner, (1975a) has demonstrated that the size of a protein is the predominant factor affecting the entry of a protein into the nucleus, those proteins greater than 69,000 molecular weight being preferentially excluded. On the other hand, the histones with varying molecular weights of less than 20,000 demonstrated selective accumulation. This illustrates that the nucleus is highly selective in the types of protein it acquires. It also implies that those proteins which do migrate between the cytoplasm and the nucleus are involved in control processes important for cellular maintenance, growth, and differentiation. For instance, nuclear transplantation and cell fusion techniques have shown that the signal for the initiation of DNA synthesis comes from the cytoplasm. Specifically, Gurdon and Woodland, (1968) found that following transplantation of nuclei from mature brain tissue, which is no longer synthesizing DNA, to an enucleated amphibian egg DNA synthesis started within 1 to 2 hours. Similarly, mature chick erythrocyte nuclei introduced into HeLa cells also re-initiated the synthesis of DNA. In my experience, where the inducing agent is added externally, I know that the signal for DNA replication must come through the cytoplasm., but this would not be clear in the normal closed system of the cell cycle. In addition, since nucleoplasmic synthesis of proteins is still under dispute I have assumed that all protein synthesis occurs in the cytoplasm (Gordstein, 1974). Therefore, if a series of protein changes, relevant to the induction of DNA replication, are dependent on genetic control, then the cytoplasmic/nuclear rapport is of the utmost importance

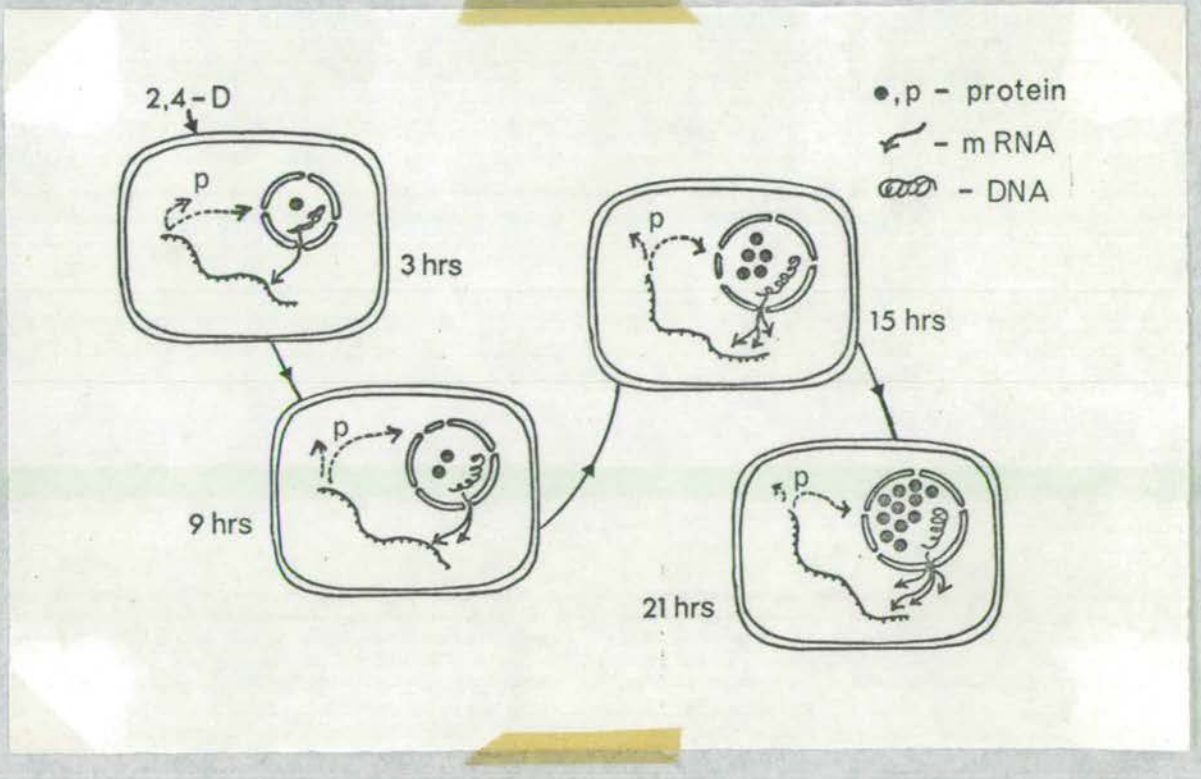
to the artichoke system as well.

Cytoplasmic control of gene expression by the nucleus has been summarised by Gurdon, (1974) and apparently is involved in many phases of animal and plant development. In one case in particular, Seale and Aronson, (1975) have shown that during the development of sea urchin embryos there is a constant rate of synthesis of non-histone proteins which remain in a cytoplasmic pool and enter the nucleus only at specific developmental stages. This implies, not only the presence of a specific entry mechanism for moving proteins from the site of synthesis in the cytoplasm into the nucleus, but also the existence of a cytoplasmic pool of nuclear proteins. Similarly, Fransler and Loeb, (1972) have reported that in each successive cell cycle of dividing sea urchin embryos DNA polymerase is translocated to the nucleus at the start of S-phase and is moved back to the cytoplasm at the termination of DNA replication. I also investigated the possibility that specific proteins, required for DNA synthesis in the artichoke, were synthesized considerably in advance of their appearance in the nucleus and only moved to the nucleus upon receipt of the appropriate signal. I did not, however, find any evidence of a cytoplasmic pool of nuclear proteins which might be required for the replication of DNA. This suggests that the proteins which I noted to accumulate in the nuclei during the pre-replication and S phases, were synthesized de novo and immediately translocated to the nucleus. If this is indeed the case, it suggests to me that the synthesis of nuclear proteins may be compartmentalized from the cytoplasmic proteins. I did, in fact, note that at the early labelling times the specific activity of the nuclear proteins was greater than that of the cytoplasm and therefore suggests the independent manufacture of proteins for the

nucleus. A similar conclusion was reached by Kuehl and Sumsion, (1971) for rat liver and by Gorovsky, (1969) for Tetrahymena. Although more definitive experiments must be done I would like to propose that a probable site of synthesis of the nuclear proteins would be on ribosomes either attached to the endoplasmic reticulum or to the nuclear envelope itself. In this way the proteins could be immediately discharged across the membrane without ever being free in the cytoplasm and would, therefore, account for the fact that I did not detect any nuclear proteins in the cytoplasm.

In conclusion, I would like to emphasize that the induction of DNA synthesis is dependent on a sequence of events, the timing of which cannot be altered. Similarly, Hartwell et al, (1974) on studying mitotic cell division in yeast concluded that the cell cycle is under genetic control as an ordered pathway. In the artichoke too, this apparently occurs by the sequential synthesis of a number of proteins which are subsequently accumulated in the nucleus.

I would, therefore, propose the following simplified model for the induction of DNA synthesis in artichoke tissue :



2,4-D enters the cell and in some way alters the cellular environment allowing the entry of a few specific proteins into the nucleus. These proteins induce the transcription of new messenger RNA which leaves the nucleus to be translated in the cytoplasm. The resultant proteins are immediately translocated back into the nucleus where they induce the synthesis of new forms of mRNA coding for other proteins, which likewise enter the nucleus. This process continues in cyclic fashion until the cell is prepared for the replication of DNA. It is not known whether these proteins are directly involved in the replication of DNA as regulators of some sort or enzymes; or whether they are utilized in other ways; for example, stabilization of messenger RNA. I feel certain, however, that these protein changes are specifically related to the induction of DNA synthesis in one form or another. It is likely from the evidence presented here, and that presented by other workers (Harland et al, 1973) that one of the last proteins to be synthesized prior to S is the polymerase itself.

This serial release suggests that the basic mechanism in the division cycle is the sequential transcription of the genome. I would also like to propose that the appropriate genes are assembled in groups, each being required for a specific phase of the cycle. For example, I have already suggested that the polymerase enzyme might be accompanied by a specific replication terminator. Fitting into this scheme, then, the protein kinases are possibly located on a previous gene whereas phosphoprotein phosphatase is induced in sequence after the polymerase.

This model agrees with the master gene theory proposed by Brown, (1976). In the artichoke the master gene is probably switched on by 2,4-D and subsequently a set of operator genes are activated in timed

sequence. At some point a depressor is generated which would deactivate the unit. Since in my experiments 2,4-D is constantly present, the cycle repeats itself again and again until the auxin is exhausted or a competative substance is built up as suggested by Leguay and Guern, (1977).

It is obvious that this model is inadequate in that it does not provide for feedback mechanisms or the enhancement of certain catalytic activities concomitant with the preparation for replication. It does, however, serve to illustrate that the visible succession of phases in mitosis is developed from a corresponding condition of molecular events which were set up in G-1.

BIBLIOGRAPHY

BIBLIOGRAPHY

ADAMSON, D., (1962) Can. J. Bot. 40, 719.

ALLFREY, V.G, LITTAU, V.C., MIRSKY, A.E., (1963) Proc. Nat. Acad. Sci. USA 49, 414.

ALLFREY, V.G., (1970) Fred. Proc. 29, 1447.

ALLFREY, V.G, JOHNSON, E.M., KARN, J., VIDALI, G., (1973) in 'Protein Phosphorylation in Control Mechanisms', 217-244, ed. F. Huijing and E.Y.C. Lee, New York, Academic.

ATTARDI, G., and AMALDI, F., (1970) Ann. Rev. Biochem 39, 183.

BARRITAULT, D., EXPERT-BEZANCON, A., GUERIN, M-F., HAYES, D., (1976) Eur. J. Biochem 63, 131.

BELL, G.I., VALENZUELA, P., and RUTTER, W.J., (1976) Nature 261, 429.

BHORJEE, J., and PEDERSON, T., (1972) Proc. Nat. Acad. Sci. USA 69, 3345.

BIRNSTIEL, M., SPIERS, J., PURDOM, I., JONES, K., LOENING, V.E., (1968) Nature 219, 454.

BISHOP, D.H.L., CLAYBROOK, J.R., and SPIEGELMAN, S., (1967) J. Mol. Biol. 26, 373

BLANCHARD, J-M., BRUNEL, C., and JEANTEUR, P., (1977) Biochem. Soc. Trans. 5, 670.

BOBB, D., and HOFSTER, B.H.J., (1971) Anal. Biochem. 46, 209.

BONNER, J., DAHMUS, M.E., FAMBROUGH, D., HUANG, R.C., MARUSHIGE, K., TUAN, D.Y.H., (1968) Science, 159, 47.

BONNER, W.M., (1975a) J. Cell Biol. 64, 421.

BONNER, W.M., (1975b) J. Cell Biol. 64, 431.

BONNER, W.M., and LASKEY, R.A., (1974) Eur. J. Biochem, 46, 83.

BRADBURY, E.M., INGLIS, R.J., and MATTHEWS, H.R., (1974a) Nature 247, 257

BRADBURY, E.M., INGLIS, R.J., MATTHEWS, H.R., and LANGAN, T.A., (1974b) Nature 249, 533.

BRADY, T., and CLUTTER, M.E., (1972) J. Cell Biol. 53, 827.

BRANDHORST, B.P., and HUMPHREYS, T., (1971) Biochem, 10, 877.

BRANDHORST, B.P., and McCONKEY, E.H., (1974) J. Mol. Biol. 85, 451.

BRAWERMAN, G., (1974) Ann. Rev. Biochem, 43, 621.

BROWN, R., (1976) in 'Cell Division in Higher Plants', 3-46. ed. M.M. Yeoman. Academic, London.

BROWN, R., and RICKLESS, P., (1949) Proc. Roy. Soc. Series B, 136, 110.

BRYANT, J.A., (1976) in 'Molecular Aspects of Gene Expression in Plants', 1-51. ed. J.A. Bryant, Academic, London.

BURTON, K., (1956) Biochem, J. 62, 315.

BYRNE, H., and SETTERFIELD, G., (1977) Planta 136, 203.

CAMERON, I.L., and JETER, J.R., (1974) ed. 'Acidic Proteins of the Nucleus', Academic, London.

CECCHINI, J.P., MIASSOD, P., RICARD, J., (1972) FEBS Lett., 28, 183.

CHAMBON, P., (1975) Ann. Rev. Biochem, 44, 613.

CHAPMAN, K.S.R., and INGLE, J., (1976) Biochem. J. 159, 585.

CHEN, Y., LIN, C., CHANG, H., GUILFOYLE, T.J., and KEY, J.L., (1975) Plant Physiol. 56, 78.

COOPER, H.L., (1973) J. Cell Biol. 59, 250.

COOPER, H.L., and GIBSON, E.M., (1971) J. Biol. Chem. 246, 5059.

COX, B.J., and TURNCOCK, G., (1973) Eur. J. Biochem, 37, 367.

CRAIG, N.C., and PERRY, R.P., (1970) J. Cell Biol. 45, 554.

DARNELL, J.E., (1968) Bact. Rev. 32, 262.

DAS, N.K., MICOU-EASTWOOD, J., RAMAMURTHY, G., ALFERT, M., (1970) Proc. Nat. Acad. Sci. USA 67, 968.

DAVIDSON, A.W., (1971) Ph.D. Thesis, University of Edinburgh, Scotland.

DAVIES, P.J., (1973) The Bot. Rev. 39, 139

DAVIS, B.J., (1964) Ann. N.Y. Acad. Sci. 121, 404.

DUDA, C.T., and CHERRY, J.H., (1971) Plant Physiol, 47, 262.

DUNHAM, V.L., and YUNGHANS, W.N., (1977) Biochem and Biophys. Res. Commun. 75, 987.

EIKHOM, T.S., ABRAHAM, K.A., and DOWBEN, R.M., (1975) Exp. Cell Res. 91, 301.

EMERSON, C.P., and HUMPHREYS, T., (1971) Anal. Biochem. 40, 254.

EVANS, P.K., (1967) Ph.D. Thesis, University of Edinburgh, Scotland.

FAIZ-ur-RAHMAN, A.T.M., (1969) Ph.D. Thesis, University of East Anglia, Norwich, England.

FAMBROUGH, D.M., FUJIMURA, F., and BONNER, J., (1968) Biochem. 7, 575.

FANTONI, A., BORDIN, S., and LUNADEI, M., (1972) Cell Diff. 1, 219.

FELDHERR, C.M., (1975) Exp. Cell Res. 93, 411.

FRANSLER, B., and LOEB, L.A., (1972) Exp. Cell Res. 75, 433.

FRASER, R.S.S., (1968) Ph.D. Thesis, University of Edinburgh, Scotland.

FRASER, R.S.S., (1975) Eur. J. Biochem. 50, 529.

FRASER, R.S.S., and LOENING, U.E., (1974) J. Exp. Bot. 25, 847.

FRENSTER, J.H., (1969) in 'Handbook of Molecular Cytology', 251-276, ed. A. Lima-de-Faria, North-Holland, London.

FRENSTER, J.H., (1974) in 'The Cell Nucleus' Vol. I, 565-580, ed. H. Busch, Academic, London.

FRENSTER, J.H., ALLFREY, V.G., MIRSKY, A.E., (1963) Proc. Nat. Acad. Sci. USA 50, 1026.

GALLOWITZ, D., and MUELLER, G.C., (1969) Science, 163, 1351.

GANZOA, M.C., and WILLIAMS, C.A., (1969) Proc. Nat. Acad. Sci. USA 63, 1370.

GEFTER, M.L., (1975) Ann. Rev. Biochem. 44, 45.

GILMOUR, R.S., and PAUL, J., (1975) in 'Chromosomal Proteins and their Role in the Regulation of Gene Expression', 67-92, eds. G.S. Stein and L.J. Kleinsmith, Academic, London.

GOLDSTEIN, L., (1974) in 'The Cell Nucleus', Vol. I, 387-438. ed. Harris Busch, Academic, London.

GORE, J.R., (1972) Ph.D. Thesis, University of Edinburgh, Scotland.

GORE, J.R., and INGLE, J., (1974) Biochem, J. 143, 107.

GOROVSKY, MA., (1969) J. Cell Biol. 43, 46a.

GREENBERG, J.R., (1972) Nature. 24, 102.

GRIERSON, D., and LOENING, U., (1974) Eur. J. Biochem. 44, 501.

GRIERSON, D., ROGERS, M.E., SARTIRANA, M.L., and LOENING, V.E., (1970) Cold Spring Harbour Symp. Quant. Biol. 35, 589.

GRONOW, M., and GRIFFITHS, G., (1971) FEBS Lett. 15, 340.

GRUMMT, I., and GRUMMT, F., (1976) Cell, 7, 447.

GUILFOYLE, T.J., and HANSON, J.B., (1974) Plant Physiol, 53, 110.

GUILFOYLE, T.J., LIN, C.Y., CHEN, Y.M., NAGAO, R.T., and KEY, J.L., (1975) Proc. Nat. Acad. Sci. USA 72, 69.

GURDON, J.B., (1974) The Control of Gene Expression in Animal Development, Oxford U.P.

GURDON, J.B., and WOODLAND, H.R., (1968) Biol. Rev. 43, 233.

HAGER, A., MANZEL, H., and KRAUSS, A., (1971) Planta 100, 47.

HARDIN, J.W., CHERRY, J.H., MORRÉ, D.J., and LEMBI, C.A., (1972) Proc. Nat. Acad. Sci. USA 69, 3146.

HARLAND, J., JACKSON, J.E., and YEOMAN, MM., (1973) J. Cell Sci. 13, 121.

HARRIS, S.E., ROSEN, J.M., MEANS, A.R., and O'MALLEY, B.W., (1975) Biochem. 14, 2072.

HARRIS, S.E., SCHWARTZ, R.J., TSAI, M-J., O'MALLEY, B.W., and ROY, A.K., (1976) J. Biol. Chem. 251, 524.

HEPBURN, A.G., (1974) Ph.D. Thesis, Univ. of Edinburgh, Scotland.

HEPBURN, A.G., and INGLE, J., (1975) Phytochem. 14, 1157.

HERSHKO, A., MAMONT, P., SHIELDS, R., and TOMKINS, G.M., (1971) Nature NB. 232, 206.

HERTEL, R., THOMSON, K-ST. and RUSSO, V.E.A., (1972) Planta, 107, 325.

HNILICA, L.S., JOHNS, E.W., and BUTLER, J.A.V., (1962) Biochem, J. 82, 123.

HOLM, R.E., and KEY, J.L., (1971) Plant Physiol 47, 606.

HOLM, R.E., O'BRIEN, T.J., KEY, J.L., CHERRY, J.H., (1970) Plant Physiol. 45, 41.

HONDA, S.I., HONGLADAROM, T., LATIES, G.G., (1966) J. Exp. Bot. 17, 460.

HOTTA, Y., CHANDLEY, A.C., and STERN, H., (1977) Nature, 269, 240.

HOTTA, Y., and STERN, H.S., (1965) J. Cell Biol. 25, (3), 99.

HUIJING, F., and LEE, E.Y.C., (1973) eds. in 'Protein Phosphorylation in Control Mechanisms', 321 p.p. Academic, London.

HUMPHREYS, T., (1973) in 'Molecular Techniques and Approaches in Developmental Biology', 141-164. ed. Maartin J. Chrispeels. John Wiley and Sons, London.

HUTCHISON, W.C., and MONRO, H.N., (1961) The Analyst 86, 768.

INGLE, J., and SINCLAIR, J., (1972) Nature, 235, 30.

INGLE, J., TIMMIS, J.N., and GORE, J.R., (1976) Perspect. Exp. Biol. 2, 273.

INGLE, J., TIMMIS, J.N., and SINCLAIR, J., (1975) Plant Physiol. 55, 496.

ISHERWOOD, F.A., and BARRETT, F.C., (1967) Biochem. J. 104, 922.

JACKSON, M., and INGLE, J., (1973) Plant Physiol. 51, 412.

JACOB, F., and MONOD, J., (1961) J. Mol. Biol. 3, 318.

JACOBSEN, J.V., (1977) Ann. Rev. Plant Physiol. 28, 537.

JETER, J.R., and CAMERON, I.L., (1974) in 'Acidic Proteins of the Nucleus', 213-245. eds. I.L. Cameron and J.R. Jeter, Jr. Academic, London.

KAHL, G., (1974) Bot. Rev. 40, 236.

KARN, J., JOHNSON, E.M., VIDALI, G., and ALLFREY, V.G., (1974) J. Biol. Chem. 249, 667.

KEATES, R.A.B., (1973) Biochem. Biophys, Res. Commun. 54, 655.

KEATES, R.A.B., and TREWAVAS, A.J., (1974) Plant Physiol. 54, 95.

KENDE, H., and GARDNER, G., (1976) Ann. Rev. Plant Physiol. 27, 267.

KEY, J.L., (1969) Ann. Rev. Plant Physiol. 20, 449.

KEY, J.L., and INGLE, J., (1968) in 'Biochemistry and Physiology of Plant Growth Substances', 711-722. eds. F. Wightman and G. Setterfield. Runge, Ottawa.

KING, P.J., COX, B.J., FOWLER, M.W., and STREET, H.E., (1974) Planta 117, 109.

KLEINSMITH, L.J., and ALLFREY, V.G., (1969) Biochem. Biophys. Acta 175, 123.

KLEINSMITH, L.J., STEIN, J., and STEIN, G., (1975) in 'Chromosomal Proteins and Their Role in the Regulation of Gene Expression', 59-66. eds. G.S. Stein and L.J. Kleinsmith. Academic, London.

KOVACS, C.J., and VAN'T HOF, J., (1970) J. Cell Biol. 47, 536.

KUEHL, L., and SUMSION, E.N., (1971) J. Cell Biol. 50, 1.

KUMAR, A., and WARNER, J.R., (1972) J. Mol. Biol. 63, 233.

LAEMMLI, U.K., (1970) Nature 227, 680.

LANGAN, T.A., (1967) in 'Regulation of Nucleic Acid and Protein Biosynthesis', 233-43. ed. U.V. Koningsberger and L. Bosch, B.B.A. 10, Library Ser. Amsterdam, Elsevier.

LASKEY, R.A., and MILLS, A.D., (1975) Eur. J. Biochem. 56, 335.

LEAVER, C.J., and KEY, J.L., (1970) J. Mol. Biol. 49, 671.

LEAVER, C.J., and Lovett, J.S., (1974) Cell Diff. 3, 165.

LEGUAY, J-J., and GUERN, J., (1977) Plant Physiol. 60, 265.

LEVNER, M.H., FROMSON, D., RICKLIS, S., GRAHAM, M., NEMER, M., (1975) Arch. Biochem. Biophys. 169, 638.

LEWIN, B., (1975) Cell 4, 11.

LIAU, M.C., CRAIG, N.C., and PERRY, R.P., (1968) Biochem. Biophys. Acta 169, 196.

LIN, C.Y., CHEN, Y.M., GUILFOYLE, T.J., and KEY, J.L., (1976) Plant Physiol. 58, 614.

LODISH, H.F., (1976) Ann. Rev. Biochem. 45, 39.

LOENING, U.E., (1967) Biochem. J. 102, 251.

LOENING, U.E., (1969) Biochem. J. 113, 131.

LOOMAX, M.I.S., and GREENBERG, G.R., (1967) J. Biol. Chem. 242, 109.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L., and RANDALL, R.J., (1951) J. Biol. Chem. 193, 265.

LUKANIDIN, E.M., ZALMANZON, E.S., KOMAROMI, L., SAMARINA, O.P., and GEORGIEV, G.P., (1972) Nature NB. 238, 193.

MACGILLIVRAY, A.J., CAMERON, A., KRAUZE, R.J., RICKWOOD, D., and PAUL, J., (1972) Biochem. Biophys. Acta 277, 384.

MACGILLIVRAY, A.J., and RICKWOOD, D., (1974) Eur. J. Biochem. 41, 181.

MADEN, B.E.H., (1972) Biochem. Biophys. Acta 281, 396.

MALCOLM, D.B., and SOMMERVILLE, J. (1977) J. Cell Sci. 24, 143.

MALLER, J., WU, M., and GERHART, J.C., (1977) Devel. Biol. 58, 295.

MARKS, D.B., PAIK, W.K., and BORUN, T.W., (1973) J. Biol. Chem. 248, 5660.

MARTELO, O.J., WOOD, S.L.C., and DAVIE, E.W., (1974) J. Mol. Biol. 87, 685.

MATTHYSSE, A.G., and PHILLIPS, C., (1969) Proc. Nat. Acad. Sci. USA 63, 897.

MASUDA, Y., (1966) Plant and Cell Physiol. 7, 75.

MITCHELL, J.P., (1967) Ann. Botany (London) 31, 427.

MITCHELL, J.P., (1969) Ann. Botany (London) 33, 25.

MONDAL, N., MANDAL, R.K., and BISWAS, B.B., (1972) Nature, NB. 240, 111.

NAGL, W., (1976) Ann. Rev. Plant Physiol. 27, 39.

NEWTON, B.A., (1965) Ann. Rev. Microbiol. 19, 209.

NIESSING, J., and SEKERIS, C.E., (1973) Nature NB. 243, 9.

O'BRIEN, T.J., JARVIS, B.C., CHERRY, J.H., and HANSON, J.B., (1968) Biochem. Biophys. Acta 169, 35.

O'FARRELL, P.H., (1975) J. Biol. Chem. 250, 4007.

O'MALLEY, B.W., and MEANS, A.R., (1976) Progr, Nucl. Acid Res. Molec. Biol. 19, 403.

ORNSTEIN, L., (1964) Ann. N.Y. Acad. Sci. 121, 321.

OTTO, B., BAYNES, M., and KNIPPERS, R., (1977) Eur. J. Biochem. 73, 17.

PARDEE, A.B., (1974) Proc. Nat. Acad. Sci. USA 71, 1286.

PARISH, J.H., and KIRBY, K.S., (1966) Biochem. Biophys. Acta 129, 554.

PATEL, G.L., and THOMAS, T.L., (1975) in 'Chromosomal Proteins and Their Role in the Regulation of Gene Expression', 249-264. eds. G.S. Stein and L.J. Kleinsmith. Academic, London.

PAUL, J., and GILMOUR, S., (1968) J. Mol. Biol. 34, 305.

PEDERSON, T., (1972) Proc. Nat. Acad. Sci. USA 69, 2224.

PEDERSON, T., (1974) J. Mol. Biol. 83, 163.

PERRY, R.P., (1976) Ann. Rev. Biochem. 45, 605.

PETERSON, J.L., and McCONKEY, E.H., (1976) J. Biol. Chem. 251, 555.

PLATZ, R.D., GRIMES, S.R., HORD, G., MEISTRICH, M.L., and HNILICA, L.S., (1975) in 'Chromosomal Proteins and Their Role in the Regulation of Gene Expression', 67-92, eds. G.S. Stein and L.J. Kleinsmith. Academic, London.

PLATZ, R.D., KISH, V.M., and KLEINSMITH, L.J., (1970) FEBS Lett. 12, 38.

PLATZ, R.D., STEIN, G.S., KLEINSMITH, L.J., (1973) Biochem. Biophys. Res. Commun. 51, 735.

POLYA, G.M., and JAGENDORF, A.T., (1971) Arch. Biochem. Biophys. 146. 635.

REDMAN, C.M., (1969) J. Biol. Chem. 244, 4308.

REEDER, R.H., (1974) in 'Ribosomes' 489-518. eds. M. Nomura, A. Tissières, and P. Lengyel. Cold Spring Harbor Laboratory.

REIMANN, E.M., WALSH, D.A., KREBS, E.G., (1971) J. Biol. Chem. 246, 1986.

REINER, J.M., (1953) Arch. Biochem. Biophys. 46, 53.

REISHER, S., RUTMAN, R.J., and ERHAN, S., (1975) Cytobios 12, 57.

RICKWOOD, D., RICHES, P.G., and MACGILLIVRAY, A.J., (1973) Biochem Biophys. Acta 299, 162.

RIZZO, P.J., PEDERSON, K., and CHERRY, J.H., (1977) Plant Sci. Lett. 8, 205.

ROBBINS, E., and BORUN, T.W., (1967) Proc. Nat. Acad. Sci. USA 57, 409.

ROEDER, R.G., and RUTTER, W.J., (1970) Proc. Nat. Acad. Sci. USA 65, 675.

ROGERS, ME., LOENING, U.E., and FRASER, R.S.S., (1970) J. Mol. Biol. 49, 681.

ROSE, R.J., SETTERFIELD, G., and FOWKE, L.C., (1972) Exp. Cell Res. 71, 1.

ROTH, R.M., and DAMPIER, C., (1972) J. Bact. 109, 773.

SEALE, R.L., and ARONSON, A.I., (1975) J. Mol. Biol. 75, 633.

SETTERFIELD, G., (1963) Symp. Soc. Exp. Biol. 17, 98.

SCHMIDT, E., and GOODMAN, R.M., (1976) Diff. 6, 177.

SCHMIDT, G., and THANNHAUSER, S.H., (1945) J. Biol. Chem. 161, 83.

SHAW, L.M.J., and HUANG, R.C.C., (1970) Biochem. 9, 4530.

SHELTON, K.R., and CLARK, JR, J.M., (1967) Biochem. 6, 2735.

SHELTON, K.R., and NEELIN, J.M., (1971) Biochem. 10, 2342.

SHELTON, K.R., SELIGY, V.L., and NEELIN, J.M., (1972) Arch. Biochem. Biophys. 153, 375.

SHEPHERD, J., and MADEN, B.E.H., (1972) Nature, 236, 211.

SHERR, C.J., and UHR, J.W., (1970) Proc. Nat. Acad. Sci. USA 66, 1183.

SILBERSTEIN, H. and AUGUST, J.T., (1973) J. Virol. 12, 511.

SMITH, J.A., and MARTIN, L., (1973) Proc. Nat. Acad. Sci. USA 70, 1263.

SPENCER, D., and WILDMAN, S.G., (1964) Biochem. 3, 954.

STANLEY, P.E., and WILLIAMS, S.G., (1969) Anal. Biochem. 29, 381.

STAYNOV, D.Z., PINDER, J.C., and GRATZER, W.B., (1972) Nature NB. 235, 108.

STEDMAN, E., and STEDMAN, E., (1951) Phil. Trans. Roy. Soc. London, Series B. 234, 565.

STEIN, G.S., and BORUN, T.W., (1972) J. Cell Biol. 52, 292.

STEIN, G.S., SPELSBERG, T., KLEINSMITH, L.J., (1974) Science, 183, 817.

STEIN, G.S., STEIN, J., THRALL, C., and PARK, W., (1975) in 'Chromosomal Proteins and Their Role in the Regulation of Gene Expression', 67-92. eds. G.S. Stein and L.J. Kleinsmith, Academic, London.

STERN, H., and HOTTA, Y. (1977) Phil. Trans. Roy. Soc. London, Series B. 277, 183.

STRAIN, G.C., MULLINIX, K.P., and BOGORAD, L., (1971) Proc. Nat. Acad. Sci. 68, 2647.

SZESZÁK, F., (1976) Acta Biochem et Biophys. Acad. Sci. USA 66, 1183.

TABER, JR. R.L., and VINCENT, W.S., (1969) Biochem. Biophys. Acta. 186, 317.

TARRAGÓ-LITVAK, L., CASTROVIEJO, M., and LITVAK, S., (1975) FEBS Lett. 59, 125.

TEISSERE, M., PENON, P., RICARD, J., (1973) FEBS Lett. 30, 65.

TEISSERE, M., PENON, P., VAN HUYSTEE, R.B., AZOU, Y., RICARD, J., (1975) Biochem. Biophys. Acta 402, 391.

TENG, C.S., and HAMILTON, T.H., (1970) Biochem. Biophys. Res. Commun. 40, 1231.

TENG, C.S., TENG, C.T., ALLFREY, V.G., (1971) J. Biol. Chem. 246, 3597.

TOWILL, L.E., and NOODEN, L.D., (1973) Plant and Cell Physiol. 14, 851.

TRENDELENBURG, M.F., SPRING, H., SCHEER, U., and FRANKE, W.W., (1974) Proc. Nat. Acad. Sci. USA 71, 3626.

TREWAVAS, A.J., (1968) Prog. Phytochem. 1, 113.

TREWAVAS, A., (1970) Plant Physiol. 45, 742.

TREWAVAS, A., (1976a) Phytochem. 15, 363.

TREWAVAS, A., (1976b) Ann. Rev. Plant. Physiol. 27, 349.

TREWAVAS, A.J., (1976c) in 'Molecular Aspects of Gene Expression in Plants', 249-297. ed. J.A. Bryant. Academic, London.

TREWAVAS, A., and STRATTON, B.R., (1977) in 'Nucleic Acids and Protein Synthesis in Plants', 309-319. eds. L. Bogorad and J.H. Weil. Nato Series, Plenum, London.

TS'O, P.O.P., HELMKAMP, G.K., and SANDER, C., (1962) Biochim, Biophys. Acta 55, 584.

TSUBOI, K.K., and PRICE, T.D., (1959) Arch. Biochem. Biophys. 81, 223.

VAN'T HOF, J., and KOVACS, C.J., (1972) in 'The Dynamics of Meristem Cell Populations', 15-30. ed. M.W. Miller and C.C. Kuehnert, Plenum, New York.

VENIS, M.A., (1971) Proc. Nat. Acad. Sci. USA 68, 1824.

VERMA, D.P.S., MACLACHLAN, G.A., BYRNE, H., and EWINGS, H., (1975) J. Biol. Chem. 250, 1019.

WALBOT, V., BRADY, T., CLUTTER, M., and SUSSEX, I., (1972) Devel. Biol. 29, 104.

WANG, T.Y., (1970) Exp. Cell Res. 61, 455.

WANKA, F., and MOORS, J., (1970) Biochem. Biophys. Res. Common. 41, 85.

WARNER, J.R., (1974) in 'Ribosomes', 461-488. eds. M. Nomura, A. Tissières and P. Lengyel. Cold Spring Harbor, Laboratory.

WEISSBACH, A., (1977) Ann. Rev. Biochem. 46, 25.

WIEGERS, U., KRAMER, G., KLAPPROTH, K., and HILZ, H., (1976) Eur. J. Biochem. 64, 535.

WILLEMS, M., PENMAN, M., and PENMAN, S., (1969) J. Cell. Biol. 41, 177.

WILLIAMS, D.E., and REISFELD, R.A., (1964) Ann. N.Y. Acad. Sci. 121, 373.

WINICOV, I., and PERRY, R.P., (1974) Biochem, 13, 2908.

YASUDA, T., YAJIMA, Y., and YAMADA, Y., (1974) Plant and Cell Physiol. 15, 321.

YEOMAN, M.M., (1970) Inter. Rev. Cytology 29, 383.

YEOMAN, M.M. and AITCHISON, P.A., (1973) in 'The Cell Cycle in Development and Differentiation', 185-201. eds. M. Balls and F.S. Billet, Cambridge, U.P.

YEOMAN, M.M. and DAVIDSON, A.W., (1971) Ann. Botany (London) 35, 1081.

YEOMAN, M.M., DYER, A.F., and ROBERTSON, A.I., (1965) Ann. Botany (London) 29, 265.

YEOMAN, M.M., EVANS, P.K., NAIK, G.G., (1966) Nature, 209, 1115.

YEOMAN, M.M., and MITCHEL, J.P., (1970) Ann. Botany (London) 34, 799.

YEOMAN, M.M., NAIK, G.G., and ROBERTSON, A.I., (1968) Ann. Botany (London) 32, 301.

YOMO, H., and VARNER, J.E., (1971) in 'Current Topics in Developmental Biology', Vol. 6, 111-144. eds. A.A. Moscona and A. Monroy, Academic, London.

ZETTERBERG, A., (1966) Exp. Cell Res. 42, 500.

APPENDIX

PUBLICATIONS

D.L. MELANSON and J. INGLE.

Regulation of ribosomal RNA accumulation by auxin in artichoke tissue (manuscript accepted; Plant Physiology (1978), 61, -.)

D.L. MELANSON and A.J. TREWAVAS.

Changes in nuclear proteins preceding the onset of DNA replication in artichoke. (in preparation).