PHOSPHORYLATION OF HISTONE H.1 IN JERUSALEM ARTICHOKE

by

B. R. STRATTON

Doctor of Philosophy.
University of Edinburgh, 1976.
Would you that splangle of Existence spend
About THE SECRET - quick about it, Friend!

A Hair perhaps divides the False and True -
And upon what, prithee, may life depend?

A Hair perhaps divides the False and True;
Yes; and a single Alif were the clue -

Could you but find it - to the Treasure-house,
And peradventure to THE MASTER too;

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

December, 1976.
CONTENTS.

ACKNOWLEDGEMENTS.

ABBREVIATIONS.

ABSTRACT.

CHAPTER 1. INTRODUCTION.

1. CHROMATIN.

2. GENERAL PROPERTIES OF HISTONES.

3. FUNCTIONS OF HISTONES
   A. Functions of unmodified histones.
   B. Functions of modified histones.
   C. Conclusions.

4. THE NATURE OF THE INVESTIGATION.

CHAPTER 2. MATERIALS.

1. PLANT MATERIAL.

2. RADIOCHEMICALS.

3. ENZYMES.

4. CHEMICALS.

CHAPTER 3. METHODS.

1. DECONTAMINATION AND CLEANING OF EQUIPMENT.
   A. Cleaning of glassware.
   B. Decontamination and cleaning of apparatus used in work with radioisotopes.
   C. Siliconisation of glass gel tubes.
CHAPTER 2. METHODS (Contd)

D. Sterilisation methods.

2. ANALYTICAL METHODS.

A. Cell number determination.

B. Protein determination by the Folin and Ciocalteus reagent.

C. DNA determination.
   (i) By the Diphenylamine reagent.
   (ii) By absorbance at 260 nm.

D. Radioactive techniques.
   (i) Preparation of scintillant.
   (ii) Determination of radioactivity.

3. EXPERIMENTAL TECHNIQUES.

A. Pea shoot tissue.
   (i) Treatment and growth of peas.
   (ii) Extraction of pea shoot chromatin.
   (iii) Extraction of histones from pea shoot chromatin.

B. Jerusalem artichoke tuber tissue.
   (i) Extraction of tuber chromatin.
   (ii) Extraction of histones from tuber chromatin.
   (iii) Extraction of tuber total nucleic acid.

C. Jerusalem artichoke tuber explants.
   (i) Preparation of explant culture media.
   (ii) Preparation and culture of explants.
### CHAPTER 3. METHODS. (Contd)

| iii. | (iii) Labelling of explants with radioisotopes. | 46 |
|      | (iv) Extraction of explant chromatin. | 47 |
|      | (v) Extraction of histones from explant chromatin. | 49 |
|      | (vi) Extraction of labelled DNA and acidic proteins from explant chromatin. | 49 |
|      | (vii) Extraction of explant DNA. | 49 |
|      | (viii) Extraction of explant total nucleic acid. | 50 |
|      | (ix) Hydrolysis of labelled histone fractions from explants, and paper electrophoresis for serine and threonine phosphates. | 51 |
|      |   - Hydrolysis of labelled histone fractions with HCl. | 51 |
|      |   - Enzymatic hydrolysis of labelled histone fractions. | 51 |
|      |   - Paper electrophoresis for serine and threonine phosphates. | 52 |

### D. General.

| (i) | Phosphatase incubation of histones. | 54 |
| (ii) | Electrophoresis of histones on urea - acetic acid - 15% acrylamide gels. | 54 |
| (iii) | Determination of histone molecular weights by electrophoresis on SLS - 15% acrylamide gels. | 57 |
|      |   - Sample preparation. | 57 |
CHAPTER 3. METHODS. (Contd)

- Gel electrophoresis.  
  (iv) Electrophoresis of nucleic acids on SLS - 2.5% acrylamide gels.

CHAPTER 4. RESULTS.

1. THE DEVELOPMENT OF ADEQUATE CHROMATIN ISOLATION PROCEDURES.

A. Introduction.  
  (i) Criteria for estimating yield and purity of chromatin.  
  (ii) Correction for light scattering in a chromatin solution.

B. Chromatin isolation and purification from pea shoot tissue. (Plumilar hook.)  
  (i) Initial preparations.  
  (ii) Final preparations.

C. Chromatin isolation and purification from artichoke tuber tissue.  
  (i) Initial preparations.  
  (ii) Final preparations.

D. Chromatin isolation and purification from artichoke explant tissue.  

E. Summary and discussion.
CHAPTER 4. RESULTS. (Contd)

2. ISOLATION AND CHARACTERIZATION OF HISTONES.
   A. Pea shoot histones.
      (i) Extraction. 75
      (ii) Gel electrophoresis. 75
   B. Artichoke tuber and explant histones.
      (i) Extraction. 77
      (ii) Gel electrophoresis. 77
      (iii) Histone molecular weights. 80
   C. Summary and discussion.

3. HISTONE H.1 - PHOSPHATE. ENZYMATIC DATA FROM NON-LABELLED ARTICHOKE TUBER AND EXPLANT TISSUES.
   A. Introduction. 85
   B. Enzymatic dephosphorylation of tuber histone H.1. 86
   C. Enzymatic dephosphorylation of explant histone H.1. 89
   D. Breakdown of histones on incubation. 91
   E. Summary and discussion. 93

4. HISTONE H.1 - PHOSPHATE. LABELLING DATA FROM EXPLANTS.
   GENERAL INTRODUCTION. 95
   SUB-SECTION 1. EXPERIMENTS ON ASYNCHRONOUSLY-DIVIDING TISSUE.
   A. Histone synthesis and phosphorylation in asynchronously-dividing tissue. 96
   B. Effect of alkaline phosphatase incubation on incorporated $^{32}$P-phosphate. 99
C. Isolation and determination of serine phosphate and threonine phosphate residues from labelled histones.

D. Summary and discussion of labelling experiments on asynchronously-dividing tissue.

SUB-SECTION 2. EXPERIMENTS ON SYNCHRONOUSLY-DIVIDING TISSUE. (CELL-CYCLE EXPERIMENTS.)

A. Determination of phases in the first division cycle.

B. Histone synthesis and phosphorylation during the first division cycle.
   (i) Introduction.
   (ii) Experiments performed.
   (iii) Uptake of $^{32}$P-phosphate and $^3$H-lysine by the explants.
   (iv) Synthesis of DNA and acidic proteins.
   (v) Synthesis and phosphorylation of explant histone H.1.
   (vi) Synthesis and phosphorylation of a protein within the explant histone H.2 + 3 region.

C. Summary and discussion of labelling experiments on synchronously-dividing tissue.
## CHAPTER 5. DISCUSSION.

1. **THE JERUSALEM ARTICHOKE EXPLANT SYSTEM.**
   - Interpretation of results and suggestions for further experimentation.
     - Page 122
   - A critique of the methods and results.
     - Page 131

2. **PROPOSED INVESTIGATIONS ON OTHER PLANT TISSUES.**
   - Page 134

3. **THE MECHANISM OF CHROMATIN CONDENSATION.**
   - Page 138

4. **CONTROL POINTS WITHIN THE CELL-CYCLE.**
   - Page 142

5. **CONCLUDING REMARKS.**
   - Page 147

### BIBLIOGRAPHY.

- Page 148

### APPENDIX - PUBLICATIONS.

- Page 162
I gratefully acknowledge the advice and encouragement of my supervisor, Dr. A. J. Trewayas. Thanks are also due to Mrs E. Mills for drawing the figures in this thesis, and to my mother for many weeks of patient typing.

I am indebted to members of the Botany Department workshop staff for technical assistance, and to the Science Research Council for providing me with a Studentship.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>A.R.</td>
<td>Analytical Reagent</td>
</tr>
<tr>
<td>6-BAP</td>
<td>6-Benzylaminopurine</td>
</tr>
<tr>
<td>bis-acrylamide</td>
<td>N.N'-methylene bisacrylamide</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees centigrade</td>
</tr>
<tr>
<td>c.</td>
<td>circa</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie(s)</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre(s)</td>
</tr>
<tr>
<td>Coomassie Blue</td>
<td>Coomassie Brilliant Blue R, (Trisodium 4'-anilino-8-hydroxy-1,1'-azonaphthalene-3,6,5'-trisulfonate)</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute. (≡ dpm, disintegrations per minute)</td>
</tr>
<tr>
<td>c-AMP</td>
<td>cyclic-AMP, (cyclic-Adenosine monophosphate)</td>
</tr>
<tr>
<td>2,4-D</td>
<td>2,4-Dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>FuDR</td>
<td>5-Fluorodeoxyuridine</td>
</tr>
<tr>
<td>g</td>
<td>gramme(s), or unit of gravitational force</td>
</tr>
<tr>
<td>H.2 + 3</td>
<td>refers to the region on histone gels comprising histones H.2A, H.2B and H.3</td>
</tr>
<tr>
<td>IAA</td>
<td>Indoleacetic acid</td>
</tr>
<tr>
<td>L (1)</td>
<td>Litre(s)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>( \log_{10} )</td>
<td>logarithm to the base 10</td>
</tr>
<tr>
<td>( \log_{e} )</td>
<td>natural logarithm</td>
</tr>
<tr>
<td>M</td>
<td>Molar, (Molarity)</td>
</tr>
<tr>
<td>mA</td>
<td>milliamp(s)</td>
</tr>
<tr>
<td>Methylene Blue</td>
<td>Methylthionine chloride, (3,7-Bis(dimethylamino)-phenazathionium chloride)</td>
</tr>
<tr>
<td>mg</td>
<td>milligramme(s)</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre(s)</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre(s)</td>
</tr>
<tr>
<td>N</td>
<td>Normal, (Normality)</td>
</tr>
<tr>
<td>nm</td>
<td>nanometre(s)</td>
</tr>
<tr>
<td>O.D.</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PAS</td>
<td>p-Aminosalicylic acid</td>
</tr>
<tr>
<td>PCA</td>
<td>Perchloric acid</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethysulphonyl fluoride</td>
</tr>
<tr>
<td>p.s.i.</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>PVP (40,000)</td>
<td>Polyvinylpyrrolidone, (average molecular weight, 40,000 daltons)</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SLS</td>
<td>Sodium lauryl sulphate, (( \equiv ) SDS, Sodium dodecyl sulphate)</td>
</tr>
<tr>
<td>SSC</td>
<td>Standard Saline Citrate, (0.15M NaCl, 0.015M sodium citrate, ( \text{pH} ) 7.2)</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TEMED</td>
<td>(N,N',N',N'-\text{tetramethylethylenediamine})</td>
</tr>
<tr>
<td>TNS</td>
<td>(\text{Triisopropyl naphthalene sulphonate})</td>
</tr>
<tr>
<td>Tris</td>
<td>(\text{Tris(hydroxymethyl) aminomethane})</td>
</tr>
<tr>
<td>(\mu\text{Ci})</td>
<td>(\mu\text{curie(s)})</td>
</tr>
<tr>
<td>(\mu\text{g})</td>
<td>(\mu\text{gramme(s)})</td>
</tr>
<tr>
<td>(\mu\text{l})</td>
<td>(\mu\text{litrer(s)})</td>
</tr>
<tr>
<td>U.V.</td>
<td>(\text{Ultra-Violet})</td>
</tr>
<tr>
<td>V</td>
<td>(\text{Volt(s)})</td>
</tr>
<tr>
<td>(\text{var.})</td>
<td>(\text{variety})</td>
</tr>
</tbody>
</table>
Chromatin was extracted and purified from tuber tissue of Jerusalem artichoke, *Helianthus tuberosus* L. (cultivar Bunyard's Round). The nuclear histone proteins were acid-extracted from the chromatin and fractionated on polyacrylamide gels. The molecular weight of histone H1 was shown to be c. 43,000 daltons, approximately twice the value for the corresponding histone from animal tissues. The remaining histones had molecular weights very similar to those reported for animal histones.

Histone H1 from tuber and cultured tuber explant material showed heterogeneity after polyacrylamide gel electrophoresis. Alkaline phosphatase incubations indicated that this heterogeneity was, at least in part, due to phosphorylation of some of the molecules. Dividing, (auxin-cultured), explants showed slightly greater phosphatase-lability of histone H1 than non-dividing explants.

Synthesis and phosphorylation of explant histones was next studied using the radioisotopes $^3$H-lysine and $^{32}$P-phosphate respectively. Both parameters were higher in dividing explants. Histone H1 and a fraction within the H2+3 histone region both showed significant $^{32}$P-phosphate incorporation in dividing tissue. These 'phosphorylations' were largely phosphatase-labile. Acid-hydrolysis of the phosphorylated fractions indicated that approximately half of the incorporated phosphate was in the form
of serine and threonine phosphate residues, whilst the remainder was probably in acid-labile linkages.

The timing of phases in the first division cycle of auxin-cultured explants was determined using 2 parameters, mean cell number per explant and specific activity of DNA when pulse-labelled with $^3$H-thymidine. Synthesis of histone H$_{1.1}$ occurred in late G$_1$ and S, whereas phosphorylation occurred in S and G$_{2/M}$. The ratio of histone H$_{1.1}$ phosphorylation : synthesis increased 3-fold as the cells passed from S to G$_{2/M}$. I have interpreted these results as indicating a large G$_{2/M}$-related phosphorylation of histone H$_{1.1}$, similar to that observed in synchronously-dividing animal cells and in synchronized plasmodia of the slime mold Physarum polycephalum. I support the hypothesis that this phosphorylation may represent passage through a Control Point in the cell-cycle resulting in chromosome condensation and entry into mitotic cell division.
CHAPTER 1

INTRODUCTION
The existence and maintenance of Life is, to the Biologist, a continual source of wonderment and intrigue.

It is now widely believed that DNA, (deoxyribonucleic acid), is the primary genetic material in most living systems. The transcriptional activity of DNA produces RNA, (ribonucleic acid), and thence, indirectly, the cellular proteins. A specific group of proteins, the enzymes, is responsible for the complex functional and regulatory machinery of the living system. Consequently, the basis of Regulation within a cell, (and indeed the underlying principle of Life itself), is the control of DNA transcriptional activity, and its interactions with the other cellular processes.

This thesis describes investigations into one particular aspect of eukaryotic cellular regulation. Namely, the belief that a 'bulk' phosphorylation of histone H4 may be concerned with the control of mitotic cell division, via the initiation of chromatin condensation.

The purpose of this Introduction will be to describe the structure and properties of chromatin, and then to continue with a presentation of the general properties of the histone components of chromatin. The functions of histones will next be considered, and phosphorylation, (as well as other forms of modification), will be introduced and discussed. Finally, the nature of the investigation will be described.
2. CHROMATIN

Eukaryotic cells typically restrict most of their primary genetic information to organelles known as nuclei. There are 2 fractions within nuclei, a soluble disperse fraction, (nucleoplasm), and a denser insoluble fraction known as chromatin. Distinction between the soluble and insoluble fractions is on purely physical grounds, and tends to be rather subjective. When nuclei are lysed in a buffer solution the fraction which pellets after centrifugation is termed chromatin, and that which remains in the supernatant is the nucleoplasm. It is the chromatin which contains the primary genetic information of the cell, in the form of DNA. The nucleoplasm contains various protein and RNA species. It is probably responsible for the post-transcriptional binding of RNA to proteins prior to transfer through the nuclear membrane to the cytoplasm.

In the interphase nucleus chromatin can be stained with 'nuclear dyes'. These dyes bind to the DNA regions. It was this property which gave rise to the name 'chromatin', (Greek, Khroma - colour). By staining and by selective extraction procedures it was discovered that chromatin was heterogeneous in structure. The more diffuse regions were called euchromatin, and the denser regions heterochromatin, (De Robertis, 1954). Heterochromatin may appear to be granular in stained nuclei; the Barr bodies in most mammalian cell lines are a particular example of this. (Each Barr body is a 'redundant' sex chromosome.) In general, there is very little transcription of heterochromatin DNA, most of the new RNA species being produced in euchromatin, (Frenster et al, 1963; Fukasawa, and Hamada,
Possibly the DNA sequences within heterochromatin are more closely associated with proteins and are therefore less accessible to RNA polymerase activity. The finding that heterochromatin has a higher histone content, and lower acidic protein content, than euchromatin, (Gottesfeld et al, 1974), may offer possible support for this suggestion since the histone proteins bind to DNA. Other work has indicated that euchromatin contains higher levels of phosphorylated acidic proteins and acetylated histones than heterochromatin, (Frenster, 1965; Allfrey et al, 1973). However, a 'cause and effect' relationship is difficult to establish, and it may be that these differences reflect the activities of the euchromatin rather than its maintenance.

In mitotic or meiotic nuclei the chromatin condenses to more organised structures known as chromosomes. These are responsible for the equal distribution of nuclear DNA to daughter nuclei. This type of condensation probably differs in nature and/or complexity from that occurring in heterochromatin, since in the latter the condensed regions show no chromosome organisation.

In association with the DNA in chromatin are histone proteins, nuclear acidic proteins, (otherwise known as non-histone chromosomal proteins), some RNA and various enzymic components, such as RNA polymerase. Dense ribosomal-RNA-containing bodies, known as nucleoli, are loosely attached to the chromatin. Typically, each nucleus contains one or more nucleolus. Nucleoli probably contribute significantly to the RNA component of purified chromatin. If the method of chromatin preparation is vigorous then the nucleoli are
broken down and to a large extent dispersed into the soluble phase. In general, chromatin contains approximately equal amounts by weight of DNA and histones. The acidic protein component varies from 0.2 to 3.0 times the amount of DNA, (depending on the tissue source), and the remaining fractions are present only in very small quantities.
Histones are usually defined as the basic protein constituents of chromatin. They were discovered at the beginning of this century by Albrecht Kossel. He and his students collected a great deal of information on histones, (and protamines), which Kossel later published as a monograph, (Kossel, 1923). Histones contain an excess of the basic amino acids lysine and arginine, such that the basic : acidic amino acid ratio is significantly greater than unity.

Extraction of these proteins from chromatin is best achieved by mixing with dilute solutions of strong acids. The basic proteins will dissolve, leaving an insoluble residue of DNA and acidic proteins. There is usually no selective extraction of particular histones here, and the low pH inhibits proteolytic activity in the suspension. Salt dissociation methods can be employed to break the DNA - histone attraction forces, (primarily electrostatic forces), prior to separation of the components by centrifugation or gel filtration of the solution, or by precipitation of the DNA. This method of extraction, however, has disadvantages. Histones tend to aggregate above pH 4.0, and there may also be a degree of selective histone extraction.

Fractionation of histones can be by column chromatography or gel electrophoresis. At present the most satisfactory method is electrophoresis on polyacrylamide disc gels.

With few exceptions chromatin from all euakaryote cells contains just 5 types of histone, (Hnilica et al, 1962; Fambrough et al, 1968; Towill and Noodén, 1973). The major characterization of
these fractions has been from mammalian tissue preparations. Table A shows the main properties of each of the 5 histones, as well as the most commonly used systems of nomenclature. These different naming systems are very confusing and have probably resulted in many errors of description or interpretation in the literature. Throughout this presentation I shall use the system classifying histones as H.1, H.2A, etc.

Histone H.1 is the largest of the histones, comprising approximately 212 amino acid residues. It has a molecular weight of c. 20,000 daltons. Of the histones, H.1 has by far the highest basic : acidic amino acid ratio, (5.5), and, because of the excess of lysine to arginine residues in the molecule, it is frequently termed 'very lysine-rich'. Histones H.2A, ('slightly lysine-rich'), and H.2B, ('moderately lysine-rich'), contain approximately 140 and 125 amino acids respectively. Histones H.3 and H.4 are 'arginine-rich' and contain approximately 135 and 102 amino acids respectively. Histone H.3 is the only histone to contain a cysteine residue. Under non-reducing conditions dimerisation of this molecule can occur across a cysteine linkage. The basic : acidic amino acid ratio for histones other than histone H.1 is within the range 1.8 - 2.4.

The amino acid sequence of each histone has been determined from various tissues. In general, histones H.2A, H.2B, H.3 and H.4 vary between tissues and species by only a few amino acids. Extreme conservation of primary structure is shown by histone H.4. There are only 2 amino acid differences between calf thymus histone H.4 and pea shoot histone H.4; and these are simply conservative
### TABLE A.

The most commonly used systems of nomenclature, and major properties of the histone species.

Details are for animal histones. Plant histones are less well documented.
<table>
<thead>
<tr>
<th></th>
<th>H.1</th>
<th>H.2A</th>
<th>H.2B</th>
<th>H.3</th>
<th>H.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common system</td>
<td>F.1</td>
<td>F.2A</td>
<td>F.2B</td>
<td>F.3</td>
<td>F.2M</td>
</tr>
<tr>
<td>of Histon</td>
<td>F.1</td>
<td>F.2A</td>
<td>F.2B</td>
<td>F.3</td>
<td>F.2M</td>
</tr>
<tr>
<td>nomenclature</td>
<td>I</td>
<td>IIa</td>
<td>IIb</td>
<td>III</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>IIa</td>
<td>IIb</td>
<td>III</td>
<td>IV</td>
</tr>
<tr>
<td>Description based on composition</td>
<td>Very lysine-rich</td>
<td>Slightly lysine-rich</td>
<td>Moderately lysine-rich</td>
<td>Arginine-rich</td>
<td>Arginine-rich</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>12,000-32,000</td>
<td>14,000-15,000</td>
<td>19,000-14,000</td>
<td>14,000-15,000</td>
<td>11,000-12,000</td>
</tr>
<tr>
<td>Approximate number of amino acid residues</td>
<td>212</td>
<td>140</td>
<td>125</td>
<td>130-140</td>
<td>102</td>
</tr>
<tr>
<td>Moles lysine (%)</td>
<td>23.7</td>
<td>12.5</td>
<td>16.7</td>
<td>19.4</td>
<td>9.8</td>
</tr>
<tr>
<td>Moles arginine (%)</td>
<td>1.7</td>
<td>9.3</td>
<td>6.4</td>
<td>13.6</td>
<td>13.9</td>
</tr>
<tr>
<td>Lysine : Arginine ratio</td>
<td>16.88 : 1</td>
<td>1.34 : 1</td>
<td>2.51 : 1</td>
<td>0.74 : 1</td>
<td>0.71 : 1</td>
</tr>
<tr>
<td>Basic : Acidic amino acid ratio</td>
<td>5.6</td>
<td>1.8</td>
<td>2.0</td>
<td>1.8</td>
<td>2.4</td>
</tr>
</tbody>
</table>
substitutions of valine and lysine by isoleucine and arginine, (De Lange et al., 1969). According to Dayhoff, (1969), this molecule has a mutation rate of c. 0.06 residues per 100 million years, making it the most evolutionary stable protein known. The conservation of primary structure during evolution indicates some very specific function of these 4 histones; a function not directly related to tissue or species, but perhaps related, in some way, to the 'eukaryotic state'. Histone H1, on the other hand, shows small differences in composition between species, (Rall and Cole, 1971). It also exhibits heterogeneity in molecular components, (Bustin and Cole, 1968); polyacrylamide gels frequently showing this protein as 2 or more distinct bands. This type of heterogeneity is largely species-specific, (Kinkade, 1969), although it can also be tissue-specific, (Ruderman and Gross, 1974; Ruderman et al., 1974). The situation is complicated, however, by a further heterogeneity imposed by tissue-specific phosphorylation or acetylation of some of the histone H1 molecules, (Bustin and Cole, 1968; Panyim and Chalkley, 1971a, 1971b). Variation in histone H1 structure and modification is therefore species-specific, and to a large extent tissue-specific. Thus, in contrast to the other histones, it would appear that histone H1 has certain tissue-related functions, and may or may not have functions related to the 'eukaryotic state'.

Histone synthesis occurs on cytoplasmic ribosomes, (Robbins and Borun, 1967; Borun et al., 1967; Gallwitz and Mueller, 1969a, 1969b). The newly formed histones rapidly enter the nucleus and are incorporated into the nucleohistone complex, (Borun et al., 1967;
Bloch et al., 1967). Work on asynchronously-dividing tissues has shown an approximate 'one to one' relationship in the rates of total histone synthesis and DNA synthesis, (Bloch and Godman, 1955; Alfert, 1955). From synchronized tissues it has been shown that the periods of histone synthesis and DNA synthesis closely coincide, (Bloch et al., 1967; Briggs et al., 1976). This restriction of histone synthesis to the S-phase is what one might expect if histones are to be considered as DNA-binding proteins. Further, the histone genes have been identified and shown to be active in messenger-RNA production only during S-phase, (Stein et al., 1975). Studies of the effects of puromycin and fluorodeoxyuridine, (FuDR), on the synthesis of DNA and histone suggest that continuation of DNA synthesis is dependent on a concurrent protein synthesis, (Bloch et al., 1967). Histone synthesis, however, can proceed at a normal or slightly reduced rate under conditions in which DNA synthesis is inhibited by 60-80%, (Bloch et al., 1967; Briggs et al., 1976).

All the histone fractions appear to be conserved over long periods of time, with turnover rates similar to that of DNA, (Piha et al., 1966). There are, however, considerable discrepancies between reports concerning the relative rates of turnover of the individual histone fractions. These are difficult to explain simply on the basis of contamination with non-histone proteins, and it has been proposed that there may be extrachromatin pools of histones within the nucleus, (Curley and Hardin, 1970). Variations in the occurrence and/or size of these pools could explain many of the apparent discrepancies in the literature.
In addition to the 5 fractions common to most eukaryotes, a limited number of tissue-specific histones have been described. The best documented occurrence is that of an erythrocyte-specific histone, (H5). This 'serine- and lysine-rich' histone has been found in a few species, including chicken, (Moss, 1974; Gautière et al., 1975), and turtle, (Tsai and Mullica, 1975). Branson et al., (1975), have isolated 3 new histone fractions from rat testis. In both these examples the new histones do not appear to be replacing other fractions, but are present in addition to the 5 common histones.

One of the most extreme examples of tissue-specificity of basic proteins is the presence of protamines in the maturing spermatozoa of a great many fish species, (Moav et al., 1974). Protamines are typically 'very arginine-rich' peptides, (50% or more of the amino acid residues are arginines), with an average molecular weight of 5,000 daltons. These basic proteins have also been identified in the maturing spermatozoa of various mammals, (Kumaroo et al., 1975; Loïr and Lanneau, 1975), and in the house cricket, (McMaster-Kaye and Kaye, 1976). When protamines occur they appear to at least partially replace the histones. In fact, during certain stages in spermatogenesis there is probably complete replacement of histones by protamines.

A very interesting point is raised here. Why is it necessary to replace one set of basic proteins by another in this specific type of cellular differentiation? The protamines, being smaller and more basic than histones, may enable the chromatin to condense.
to a denser, (and less transcriptionally active), structure than histones could allow, (Marushige and Dixon, 1969), and possibly only if this is achieved can sperm head maturation proceed to completion. Alternatively, protamines may play some role in the organisation of the syngotic chromatin after penetration of the egg nucleus by the sperm head. A further possibility is that for conformational or regulatory reasons it may be necessary to have a purely maternal inheritance of histones.

The existence of protamines necessitates a more rigorous definition of histones than that given at the beginning of this section. Histones should more correctly be defined as the basic protein constituents of chromatin having a molecular weight of greater than 10,000 daltons and an arginine content of no higher than 20%.
A. Functions of unmodified histones

In 1950 Stedman and Stedman first proposed that histones may, in some way, be involved in 'gene masking' in the nucleus. From work on animal tissues they found that there were chemical differences between total histone preparations from different species. These they attributed to species-specific histones. Some cases of tissue-specificity were also proposed. We now know that these interpretations are probably incorrect, since differences of the magnitude found could simply be due to quantitative variations in the 5 main histone fractions. There are many reports of quantitative variations in the proportions of histones in chromatin: the histone : DNA ratio may vary from one organ to another, (Sporn and Dingman, 1963; Dingman and Sporn, 1964; Bonner et al., 1968), and there is variation in the amounts of individual histone components present in different tissues, (Hnilica et al., 1966; Fambrough et al., 1968; Kusanagi and Yanagi, 1970). The work by Stedman and Stedman, however, brought to light the possible regulatory importance of histones and the other chromatin proteins. In the 1960's and early 1970's investigations on this topic were continued, making use of the newly developed techniques for studying chromatin reconstitution and DNA-directed RNA synthesis. It was found that different animal tissues produced different RNA species, (Paul and Gilmour, 1966, 1968; Smith et al., 1969; Tan and Miyagi, 1970), and that in each case the amount of DNA actually involved in RNA synthesis was in the
order of 5-10% of the total; as compared to an activity of 40-50% when purified DNA was used. Paul and Gilmour, (1968), further found that this organ-specific repression of DNA transcription was preserved in isolated chromatin, and could be produced in reconstitution experiments. Histones from different tissues were shown to prevent all transcription when added to purified DNA, but when added to dehistoned chromatin the transcriptional activity was the same as that of the original intact chromatin. It was concluded, therefore, that the histones acted as non-specific repressors of transcription, but that specificity of transcription was conferred by a component of the dehistoned chromatin, probably the acidic proteins, (Paul and Gilmour, 1969; Spelsberg and Hnilica, 1970). Further evidence for this suggestion came in 1970 when Gilmour and Paul found that, in reconstituted chromatin, the transcription product depended on the tissue-source of the acidic proteins, not of the histones.

It is difficult to reconcile this proposed non-specific function of histones with the knowledge that 4 of the 5 histone fractions have highly conserved primary structures. It could be suggested that quantitative variations in the histone fractions might confer a degree of specificity to the repression of transcription. However, this seems unlikely. A further suggestion is that specificity may be given to a histone molecule by association with an RNA fraction, the nucleoprotein formed being a specific repressor, (Sypherd and Strauss, 1963). There is little evidence for this hypothesis or for a specific histone-RNA recognition system. Therefore, the problem of the specific functions of histones still remains.
During the past few years evidence has accumulated for the existence of a repeating subunit structure in chromatin, (Olins and Olins, 1974; Noll, 1974; Kornberg and Thomas, 1974; Kornberg, 1974). Micrococcal nuclease digestion of chromatin has shown that approximately 50% of the DNA is protected from extensive digestion, (Clark and Felsenfeld, 1971), with the products of digestion first appearing as multiples of a unit some 200 base-pairs in length, (Hewish and Burgoyne, 1973; Noll, 1974). These nuclease-resistant particles have been isolated and called v-bodies, (or nucleosomes). Each is a globular structure 80-100 Å in diameter, containing c. 100,000 daltons of protein, (Sahaarabuddhe et al., 1974). The size of the 'protected' DNA fragment within these particles is 130-140 base-pairs, (Shaw et al., 1974). It has been suggested by Van Holde et al., (1974), that the difference between the 200 base-pair subunit and the 140 base-pair particle may be accounted for by the presence of 'spacer' DNA. This DNA would link adjacent particles and would therefore be more accessible to nuclease activity than the 'protected' DNA. The proteins in v-bodies have been shown to be histones. 2 molecules of each of the major histone fractions, except histone $H_{4}$, are present in each v-body, (Thomas and Kornberg, 1975a, 1975b). Van Holde et al., (1974), proposed a model for the structure of v-bodies wherein the 'protected' DNA is wrapped around a histone core.

Chromatin, therefore, is considered to be made up of a series of threads, (or nucleofilaments), each consisting of a chain of nucleohistone particles. Histones $H_{2A}$, $H_{2B}$, $H_{3}$ and $H_{4}$ appear to have specific functions in this organisation. This may explain
the high degree of conservation of primary structure shown by these histones. Presumably, the 3-dimensional configuration of the v-bodies would depend upon considerable retention of primary structure by the proteins within the histone core. Wright and Olins, (1975), have reported differences in the molar ratios of histones within v-bodies. If this is indeed the case then the model presented above is a simplified version of the true 'state of affairs'. These differences between v-bodies, if confirmed, would indicate a more complex and specific role of the nucleosomal histones than previously thought.

The positioning of histone H1 in chromatin is uncertain, but it has been suggested that this protein may assist in the maintenance of nucleohistone secondary structure, (Lindigcoit et al, 1974; Renz, 1975), perhaps acting as a cross-linking agent between DNA strands, (Smythies et al, 1974). More recent papers have expressed the view that the highly basic C-terminal end of histone H1 is probably the main site of interaction with DNA, whilst the central apolar region may be involved in interactions with other histone H1 molecules, (Chapman et al, 1976; Barrett, 1976).

Bostock et al, (1976), have shown that the structure, size and distribution of v-bodies is similar in both extended and condensed chromatin. This leads to the conclusion that condensation of chromatin probably does not arise by greater coverage of DNA by histones, as was first thought when heterochromatin and euchromatin were compared. Other workers have shown that nucleofilaments may be condensed into a supercoil or solenoidal structure in chromatin,
(Finch and Klug, 1976), and it could be that this is the method by which chromatin condensation occurs. Histone H1 has been shown to be required for the stabilisation of this supercoil structure, and may therefore have a role in the maintenance of chromatin condensation. Thus far, there is no evidence for species- or tissue-specific functions of histone H1.

B. Functions of modified histones

In recent years investigations have revealed that histones can be modified in vivo by phosphorylation, acetylation, methylation, thiolation and ribosylation. Induction of tyrosine aminotransferase with insulin and hydrocortisone results in enzymatic modifications of the histones, including phosphorylation, acetylation and methylation, (De Villiers Graaff and Von Holt, 1973). During spermatogenesis in trout there is extensive phosphorylation and acetylation of histones, in particular histone H4, (Sung and Dixon, 1970; Louie and Dixon, 1972). Histone H1 phosphorylation has been found in rat liver during regeneration, (Balhorn et al, 1971), and following administration of glucagon and insulin, (Langan, 1969). Increase in total histone phosphorylation has been found during rat pancreas regeneration, (Fitzgerald et al, 1970). Many similar examples of animal histone modification have been reported in the literature.

These modifications of histone molecules may change their net
charge. For example, when histones are electrophoresed on acrylamide gels at pH 3–4 the phosphorylated molecules migrate slower than unphosphorylated molecules since they are more negatively charged. According to Chalkley et al. (1973), each phosphate group will decrease the mobility of a histone H1 molecule, under these conditions, by c. 1%.

Turnover of histone modifications is much more rapid than that of the histones. (Half-lives of one to a few hours have been reported.) This observation opens up the possibility that specific modifications of histones could have regulatory functions. As yet, though, the significance of these modifications remains uncertain. Acetylation of the N-terminal and basic amino acids in histones may assist in DNA-histone binding, (Zukowska-Ładzińska and Tomko, 1973; Grimes et al., 1975), and could also be involved in gene activation, (Burdick and Taylor, 1975). Methylation of the basic amino acids in histones occurs mainly during chromatin condensation, (Shepherd et al., 1974; Borun et al., 1974). However, any suggestion that methylation is directly related to the condensation process would be purely speculative. Thiolation and ribosylation of histone fractions have been only sparsely reported. Their significance is unknown. The phosphorylation of serine and threonine residues of histones H2a, (Louie et al., 1973a; Gurley et al., 1974), H2b, (Louie et al., 1973a; Parago et al., 1975), H3, (Gurley et al., 1973), and H4, (Louie and Dixon, 1972), have all been reported, but their significance is unknown. Louie and Dixon, (1973), have proposed that the interconversion of euchromatin and heterochromatin may be
regulated by histone phosphorylation and dephosphorylation, in the same way that dephosphorylation of protamines appears to aid condensation of chromatin during maturation of trout spermatozoa, (Louie et al., 1973b). The evidence for this suggestion has been strongly criticised, (Sherod et al., 1970; Balhorn et al., 1971; Smith and Stocken, 1973), and further work has indicated that it is probably untenable, (Bradbury et al., 1974a).

This capacity for localised modification indicates further specificity of function of the nucleosomal histones. Extrapolating from the report of Wright and Olins, (1975), that there are differences in composition between the v-bodies we can envisage a further heterogeneity imposed by the enzymatic modification of various histone molecules. Not only would the nucleosomal histones be responsible for binding the DNA into an organised 3-dimensional structure, but modifications of particular molecules may be responsible for localised conformational changes in the chromatin. These changes in the histone - DNA interactions could be involved in the initiation or modification of transcription of specific DNA sequences.

Much work has now been done on phosphorylation of histone H1. Data from work on animal tissues has shown that as cells are stimulated to divide the phosphate content of histone H1 increases. Systems such as in vivo rat liver regeneration, (Balhorn et al., 1971; Chalkley et al., 1973), and calf thymus regeneration, (Fitzgerald et al., 1970), have been used here. It has further been shown that the faster the rate of cell division, (or the greater the proportion
of cells dividing), the greater is the extent of phosphorylation, (Sherod et al., 1970; Balhorn et al., 1972). This work would suggest a correlation between histone H1 phosphorylation and the process of cell division.

Using synchronized mammalian cell cultures Shepherd et al. (1971), showed that the phosphate content of histone H1 rose steadily from G1 to early mitosis. They were unable to pin-point the exact time of phosphorylation, nor was any dephosphorylation observed. However, work by Lake et al. (1972), and Marks et al. (1973), using synchronous Chinese hamster and HeLa cells respectively, has shown that the main increase in histone H1 phosphorylation occurs in late G2; after the DNA has been replicated but before the onset of mitosis. An identical trend has since been shown for the slime mold Physarum polycephalum, (Bradbury et al., 1974a, 1974b). As mitosis proceeds the histone H1 phosphate content falls back to its original level. These changes coincide with similar changes in the activity of a specific histone H1 phosphokinase, (Lake and Salzman, 1972; Lake, 1973; Bradbury et al., 1974a, 1974b). This kinase is cyclic-AMP-independent and has been termed the 'growth-associated' kinase. More recent investigations have indicated that the 'growth-associated' kinase from Physarum consists of 2 components, (Hardie et al., 1976). It is suggested that these two enzymes phosphorylate different sites on the histone H1 molecule, and that they have different times of appearance during the G2-phase.

In Physarum there is a low level of histone H1 phosphorylation

* Labelling of the phases in the division cycle is as in Howard and Pelc, 1953).
in the S-phase, (where the kinase activity is still low). Its function appears to be to maintain a 'resting level' of phosphorylation during synthesis of new histone, (Bradbury et al., 1973, 1974a, 1974b), since the ratio of phosphorylated to total histone H.1 is constant through the G.1, S and Early G.2 periods. During Middle and Late G.2, where the kinase activity peaks, the level of phosphorylated histone H.1 increases 6-fold, (Bradbury et al., 1973, 1974a, 1974b). There is no histone synthesis at this time, therefore the peak of phosphorylation does in fact represent a net large-scale phosphorylation of histone H.1.

It is now considered by many researchers that the activity of a specific histone H.1 phosphokinase in G.2 may indirectly initiate chromosome condensation and that this, in turn, may cause the onset of mitosis, (Bradbury et al., 1974b). Thus, this kinase has been suggested to be a 'mitotic trigger' in the G.2-phase.

In further support of this hypothesis Bradbury et al., (1974b), have shown that by adding an heterologous preparation of the histone H.1 phosphokinase to Physarum they can bring forward the onset of mitosis by up to 40 minutes.

Gorovsky et al., (1974), have criticised this theory since it has been found that the amitotic macronucleus of Tetrahymena pyriformis, which undergoes no chromosome condensation during the cell-cycle, has a high level of histone H.1 phosphorylation in rapidly dividing cells. However, there are 2 or more types of histone H.1 phosphokinase, (see later), only one of which appears to be concerned with chromosome condensation. It is not known which kinase is responsible for the phosphorylation in this particular
example, Gorovsky and Keever, (1975), have further shown that the Tetrahymena micronucleus, which shows normal chromosome condensation followed by mitosis, contains no detectable histone H1. A report that histone H1 from avian erythrocytes cannot be phosphorylated in vivo or in vitro, (Tsuiki and Loeb, 1974), also raises a problem; as does the finding of Tanphaichitr et al, (1975), that chromatin decondensation at the M - G1 boundary in synchronized cells is not prevented by the inhibition of histone H1 dephosphorylation with ZnCl2. Therefore, although there is now substantial evidence for a 'bulk' phosphorylation of histone H1 during the Late G2-phase of the cell-cycle, the exceptions to this indicate that interpretation must remain speculative, and consequently much more work in this area should be done.

This is not, however, the full story as far as histone H1 phosphorylation is concerned. There appears to be a further type of phosphorylation associated with the response by animal tissues to hormonal stimuli. Langan (1969, 1973), has shown that administration of glucagon to adult rats causes a marked increase in the phosphorylation of serine 37, (near to the N-terminal end of the molecule), in liver histone H1. This phosphorylation is mediated by a cyclic-AMP-dependent kinase. Langan, (1969), proposes that this is the mechanism by which hormones whose actions are mediated by cyclic-AMP induce RNA synthesis in target tissues. More recent work has indicated the presence of a cyclic-AMP-independent protein kinase responsible for phosphorylating another serine residue, near to the C-terminal end of the molecule, (Langan, 1971a, 1971b).
Therefore, we can conclude that histone H1 exhibits 3 distinct types of phosphorylation. The first is a synthesis-related phosphorylation occurring during late G1 and S-phase. This maintains the proportion of phosphorylated molecules at a steady level during histone synthesis. The second is a large-scale net phosphorylation during the G2-phase of the cell-cycle. This phosphorylation is cyclic-AMP-independent and is probably related, in some way, to the condensation of chromatin prior to mitosis. If this is the case, it represents a non-gene-specific manipulation of the entire genome. The third type of phosphorylation is a response to hormonal stimuli and occurs on specific serine residues. The kinase involved here may be cyclic-AMP-dependent or independent. This type of phosphorylation is more in keeping with a selective mechanism of gene activation, (Langan, 1973).

The work mentioned above has been concerned with phosphorylation of serine, and to a lesser extent threonine, residues in the histone molecules. However, a few recent publications have indicated that there may also be phosphorylated derivatives of the amino acids lysine and histidine, (Smith et al, 1973, 1974; Chen et al, 1974). Since the linkages here are acid-labile most researchers would have failed to isolate them.

It should be pointed out that protein phosphorylation is not restricted to histones, but rather is a widespread occurrence. Activation or inactivation of enzymes, and the regulation of specific
gene activity by the nuclear acidic proteins are just 2 of the suggested roles of protein phosphorylation. For a discussion of these and other functions of protein phosphorylation see the reviews by Rubin and Rosen, (1975), and Trewavas, (1976). To my mind, protein phosphorylation is one of the most important regulatory mechanisms to be found in eukaryotic systems.

C. Conclusions

The nucleosomal histones, H2A, H2B, H3 and H4, are required for the maintenance of chromatin ultrastructure. These histones may, in addition, have specific regulatory properties conferred by uneven distribution to the nucleosomes or by enzymatic modification of particular molecules.

The non-nucleosomal histone, H1, when highly phosphorylated, may play some role in the condensation of chromatin prior to mitosis. Local, and specific, phosphorylations of histone H1 are probably concerned with activation of specific genes, in response to hormonal or other stimuli.
The main aim of this work was to study synthesis and phosphorylation of histone H1 through the division cycle in a cultured plant tissue. No investigations of this nature had previously been carried out on plant material. It was hoped, therefore, that the results of this investigation would contribute to the already increasing evidence, based on work from animal tissues, for the presence of a 'bulk' phosphorylation of histone H1 during the G2-phase, (Lake et al, 1972; Bradbury et al, 1974a, 1974b). If, as suggested, this phosphorylation is the 'trigger' for chromatin condensation prior to mitosis, (Bradbury et al, 1974a, 1974b), then it would be expected to occur in all eukaryotic cells, whether of animal or plant origin. If plant tissues exhibit no such phosphorylation of this histone then a more intriguing problem would present itself. Evolutionary differences between animal and plant mitoses would then have to be considered.

Jerusalem artichoke, (Helianthus tuberosus, L), tuber tissue was used throughout this investigation. If explants of tuber tissue are prepared and cultured in the presence of the auxin 2,4-dichlorophenoxyacetic acid, (2,4-D), then approximately 50% of the cells will divide. The first division is fairly synchronous, but the ensuing divisions rapidly become asynchronous. It is this initial division which was used for cell-cycle data. Explants cultured in the absence of auxin do not divide, and were therefore used as 'controls'. This system has been well characterised by Yeoman and co-workers in this Department; (for a brief review see
Yeoman, 1974). Both the preparation and culturing of explants are under strictly defined conditions.

The initial research was, however, not concerned with the cell-cycle changes, but rather with developing adequate isolation procedures for artichoke chromatin and histones. In addition, histone H1 phosphorylation was investigated in both the tuber tissue and in asynchronously-dividing explant material.
1. **PLANT MATERIAL**

Seeds of a dwarf garden pea, *Pisum sativum* L. (var. Peltham First), were obtained from Lawson Donaldson Seeds Ltd and were stored at 4°C until required.

Mature stem tubers of Jerusalem artichoke, *Helianthus tuberosus* L. (cultivar Bunyard’s Round), were obtained from plants grown in the garden of Edinburgh University Botany Department. Tubers were planted in March and the crop harvested the following December or January. The tubers were placed in polythene bags containing damp sand and stored at 4°C.

2. **RADIOCHEMICALS**

These were all obtained from The Radiochemical Centre, Amersham.

\[ ^3 \text{H} \_\text{DL-}(4.5(n)-\text{Lysine monohydrochloride)} \]  
Specific activity 10 Ci/mM, was obtained as an aqueous solution and stored at -20°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.

\[ ^3 \text{H} \_\text{(6-}\text{P-Thymidine)} \]  
Specific activity 5 Ci/mM, was obtained as an aqueous solution and stored at -20°C.

The radiochemicals were diluted with distilled water prior to
addition to culture media.

3. **ENZYMES**

**Acid phosphatase**, (Orthophosphoric monoester phosphohydrolase), from Potatoes. Obtained as a lyophilised powder. pH optimum 4.8. Sigma Chemical Company.

**Alkaline phosphatase**, (Orthophosphoric monoester phosphohydrolase), from *E. coli*. Obtained as a suspension in 3.2M ammonium sulphate solution, pH 6. pH optimum 8.0. Boehringer Mannheim.

**Alkaline phosphatase**, (Orthophosphoric monoester phosphohydrolase), from *E. coli*. Obtained as a suspension in 2.5M ammonium sulphate solution, pH optimum 10.4. Sigma Chemical Company.

**Leucine aminopeptidase**, from Hog kidney. Obtained as a chromatographically purified suspension in 0.75 saturated ammonium sulphate, 0.1M Tris and 0.005M magnesium chloride, pH 8. Sigma Chemical Company.

**Pronase**, from *Streptomyces griseus*. A purified non-specific protease in powder form. Sigma Chemical Company.
4. **CHEMICALS**

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

Acrylamide and N,N'-Methylene bisacrylamide were recrystallised from chloroform and acetone respectively when required for SLS - acrylamide gels. (Loening, 1967.)

Glass distilled water was used throughout this work.
CHAPTER 3

METHODS
1. DECONTAMINATION AND CLEANING OF EQUIPMENT

A. Cleaning of glassware

All glassware was washed in hot water containing detergent, then rinsed in tap water. After a final rinse in glass distilled water it was dried in an oven.

B. Decontamination and cleaning of apparatus used in work with radioisotopes

After pouring away the scintillation fluid glass bottles and tops were soaked in methanol for at least 2 hours, then rinsed in warm water for 2 hours. After a final rinse in glass distilled water they were dried in a warm oven.

All other contaminated apparatus was soaked overnight in 2% Decon-75, (Decon Laboratories Ltd), then washed by the method described in (A), above.

C. Silicisation of glass gel tubes

Approximately 5g sodium dichromate was dissolved in 10ml distilled water. Concentrated chromic acid was prepared by the addition of 90ml concentrated sulphuric acid to this solution. The gel tubes were immersed in the chromic acid for 2 days then washed very thoroughly in tap water, followed by glass distilled water. They were then dried in a hot oven, cooled, and immersed in a 2% solution of 2,4-dimethyldichlorosilane in carbon tetrachloride. After a few minutes the liquid was poured away and the tubes left
to dry at room temperature.

D. Sterilisation methods

When necessary, glassware was sterilized in an autoclave at 15 p.s.i. for 15 minutes. Instruments used for the preparation of artichoke explants were wrapped in silver foil and placed in a tin box, and sterilized by heating in an oven at 150°C for 3 hours.
2. **ANALYTICAL METHODS**

A. **Cell number determination**

Determination of cell number in artichoke tuber explants was by the method of Brown and Rickless, (1949). Samples of 5 explants were each placed in 2.0 ml of 5\% chromium trioxide. These were left for one day at room temperature and the cells counted. (Storage for longer periods was at 4°C.) Maceration of the samples was achieved by squirting rapidly in and out of a Pasteur pipette. Aliquots were taken with a glass capillary tube and the cells counted with the aid of a haemocytometer slide.

B. **Protein determination by the Folin and Ciocalteu reagent**

This was by the method of Lowry et al, (1951). The following solutions were prepared:

1. Alkaline copper tartrate, freshly prepared by mixing 2% CuSO₄·5H₂O, 4% Na-K tartrate and 4% Na₂CO₃·10H₂O in 0.1N NaOH in the ratio 1:1:100.
2. Commercial Folin and Ciocalteu reagent diluted 1:1 with water.
3. 0.1N NaOH.
4. A standard solution of Bovine Serum Albumin, (BSA), in 0.1N NaOH, containing 200μg/ml. (This stock solution was kept at 4°C and re-prepared every 2 months.) When histone extracts were assayed Calf Tymus Histone was used as the standard.

Duplicate tubes for a standard curve were prepared containing 0, 50, 100, 150 and 200μg BSA. Suitable samples of the tissue
extracts in 0.1N NaOH were taken, and all tubes were made to 1.0ml with 0.1N NaOH. 5.0ml of the alkaline copper tartrate was added to each tube and the contents mixed vigorously. The tubes were left approximately 10 minutes for the complete formation of the protein-copper complex. Then 0.5ml of the diluted Folin reagent was added to each tube and the contents again mixed vigorously. 20 minutes after the addition of the Folin reagent the optical density of the blue colour which developed was read on an Eel colorimeter, (Evans electroselemium Ltd), using a red, (608nm), filter. A standard calibration curve was drawn, from which the protein content of the sample tubes was determined. A typical standard curve is shown in Figure 1.

The Folin reagent reacts with the ring systems of the amino acids tyrosine and tryptophan.
FIGURE 1.

Lowry standard curve for protein determination.

(Method as in Lowry et al., 1951.)
FIG. 1.

LOWRY STANDARD CURVE.

calf thymus histone.
C. DNA determination

(i) By the Diphenylamine reagent

This was by the method of Burton, (1955). Unless otherwise indicated all steps were carried out at 4°C.

The tissue was homogenised in a pestle and mortar in an equal volume of 5% Trichloroacetic acid, (TCA). The homogenate was transferred to a 15ml plastic centrifuge tube, and the pestle and mortar rinsed with a small volume of 5% TCA. This was added to the homogenate. The sample was left overnight at 4°C then spun for 30 minutes at 20,000g on an MSE "Highspeed 18" centrifuge. The pellet was washed twice in 5% TCA and re-spun. It was then well-drained and suspended in a known volume of 0.5N Perchloric acid, (PCA), and hydrolysed at 70°C for 30 minutes. A standard calf thymus DNA, (200μg/ml), was diluted with an equal volume of 1.0N PCA and hydrolysed with the sample. (The DNA stock solution was kept at 4°C and re-prepared every 2 months.) After hydrolysis the tubes were cooled in water and the sample was spun for 10 minutes at top speed in a bench centrifuge. Suitable aliquots of the tissue extract were taken, as well as duplicate samples for a calibration curve of 0, 10, 20, 30, 40 and 50μg DNA. All tubes were made up to 1.0ml with 0.5N PCA and mixed with 2.0ml of diphenylamine reagent.

(Diphenylamine reagent: 1.5g diphenylamine in 100ml acetic acid plus 1.5ml concentrated sulphuric acid, to which 0.5ml of 16mg/ml aqueous acetaldehyde is added immediately prior to use.)
The optical density of the blue colour produced after 18 hours at 25°C, (or 4 hours at 40°C), was measured at 600nm in an SP.500 spectrophotometer, (Unicam Instruments Ltd), using quartz cells of 1cm path length. A standard calibration curve was drawn, from which the DNA content of the sample tubes was determined. A typical standard curve is shown in Figure 2.

The diphenylamine reagent reacts with the deoxyribose fraction of the hydrolysed DNA.

(ii) By absorbance at 260nm

The amount of DNA in a chromatin sample was determined in the following manner. The chromatin was suspended in a small volume of 1% sodium lauryl sulphate, (SLS), at room temperature. The suspension was homogenised in a glass homogeniser and left for at least one hour. It was then spun for 20 minutes at 15,000g on an MSE "Superspeed 50" centrifuge. The supernatant, (dissolved chromatin), was carefully removed from the pellet, (mainly cell wall debris), and its U.V. absorption spectrum determined by scanning from 400 - 200nm in an SP.800 Ultraviolet Spectrophotometer, (Unicam Instruments Ltd). Quartz cells of 1cm path length were used here, and 1% SLS was the absorption 'blank'. The amount of DNA in the sample was determined from the absorbance at 260nm, using the conversion; 1mg DNA/ml gives c. 18 O.D. Therefore 1 O.D. is equivalent to about 55μg DNA/ml.
FIGURE 2.

Diphenylamine standard curve for DNA determination.

(Method as in Burton, 1955.)
FIG. 2.

DIPHENYLAMINE STANDARD CURVE.

calf thymus DNA.
D. **Radioactive techniques**

(i) **Preparation of scintillant**

A standard scintillation fluid was used: (a 2:1 mixture of toluene: triton X-100, containing 5.0g/litre butyl-PBD). 667ml toluene was filtered and 5.0g butyl-PBD dissolved in it. 333ml triton X-100 was slowly added to the mixture with continuous stirring.

(ii) **Determination of radioactivity**

For liquid samples, a small volume of sample (usually less than 50µl), was added to 5.0ml of the scintillation fluid in a glass scintillation bottle. The contents were mixed vigorously and the radioactivity determined in an SL-30 Liquid Scintillation Spectrometer, (Intertechnique Ltd).

For polyacrylamide gels the following method was used. The gels were frozen in an aluminium trough on dry ice, (solid CO₂). They were then cut into 1.0mm or 0.5mm slices using a gel-slicer, (The Mickle Laboratory Engineering Co.), each slice being put into a separate glass bottle. The slices were dissolved by the addition of 100-volume hydrogen peroxide. 0.5ml of hydrogen peroxide was added to each bottle and the top loosely screwed on. The tray of bottles was placed in an oven at 70°C. After 15 minutes the tops were quickly tightened and the bottles returned to the oven and left for 4 hours. They were then cooled and 5.0ml of the standard scintillation fluid was added to each. The contents were
mixed vigorously and the radioactivity determined in the Liquid Scintillation Spectrometer.

For samples subjected to paper electrophoresis, the paper was cut into 5.0mm strips and each was placed in a separate scintillation bottle. 5.0ml of a modified scintillation fluid, (toluene containing 5.0g/L butyl-PBD), was added to each bottle and the radioactivity determined in the Liquid Scintillation Spectrometer.
3. EXPERIMENTAL TECHNIQUES

A. Pea shoot tissue

(i) Treatment and growth of peas

The pea seeds were selected by hand, and any which were visibly damaged or infected were discarded. They were then soaked in absolute alcohol for 5 minutes and in 2\% available chlorine, (as sodium hypoohlorite solution), for 20 minutes. After this sterilisation procedure the seeds were washed 4 times in sterile distilled water and imbibed for 4 hours in running sterile distilled water. They were planted in autoclaved moist vermiculite in pyrex dishes, and grown in total darkness at 25°C. Watering was with sterile distilled water.

(ii) Extraction of pea shoot chromatin, (From plumular hooks)

This was by the method of Bonner et al., (1967); with some modifications. Unless otherwise specified the entire procedure was carried out at 4°C.

1. After 4 - 5 days of growth the top 1.0cm was cut off each pea shoot. (10 - 40g fresh weight was used.)

2. Grinding medium was added to the tissue and the suspension homogenised at full speed for 10 seconds in a polytron homogeniser. (Grinding medium consisted of 0.3M sucrose, 0.01M magnesium chloride, 0.05M Tris-HCl, pH8.0, and a few drops of octanol to prevent frothing.)
3. 20% Triton X-100 was added to a final concentration of 1%, and the mixture shaken.

4. The solution was then filtered through 2 layers of muslin and 4 layers of mira cloth, (Calbiochem). The filters were washed with grinding medium and the muslin carefully squeezed out.

5. The filtrate was spun at 4,000g for 30 minutes on an MSE "Highspeed 18" centrifuge and the supernatant discarded.

6. The darker pellet material was removed from the pellet of starch and hand-homogenised in 0.01M Tris-HCl, pH7.8.

7. The homogenate was spun at 10,000g for 10 minutes and the supernatant discarded.

8. The grey-white gelatinous chromatin was scraped off the pelleted starch and darker cell wall debris, and washed twice more in 0.01M Tris-HCl, pH7.8.

9. The chromatin was washed once in 0.01M Na₂EDTA containing 0.15M NaCl, pH7.2, and spun at 16,000g for 15 minutes.

10. Finally the chromatin was washed in 0.01M Tris-HCl, pH7.8.

11. The chromatin was resuspended in 0.01M Tris-HCl, pH7.8, and 5.0ml aliquots were spun through 15.0ml 1.7M sucrose. This was a 3 hour spin at 73,000g in an MSE "Superspeed 50" centrifuge.

12. The supernatant was carefully poured away and a clear gelatinous pellet of purified chromatin was left at the bottom of
(iii) **Extraction of histones from pea shoot chromatin**

This was by the method of Bonner et al., (1967), with some modifications.

The entire procedure was carried out at 4°C.

1. The chromatin pellet was suspended in a small volume of 0.1 x SSC by repeatedly filling and ejecting from a 1.0ml syringe. (SSC is Standard Saline Citrate; 0.15M NaCl, 0.015M sodium citrate, pH7.2)

2. 1.0N H$_2$SO$_4$ was added to the suspension to a final concentration of 0.2N.

3. The mixture was stirred for 30 minutes, then spun for 15 minutes at 28,000g on an MSE "Superspeed 50" centrifuge.

4. The supernatant was retained and the pellet extracted with 0.4N H$_2$SO$_4$.

5. After a further 28,000g spin the supernatant was removed and added to the first supernatant.

6. Two volumes of cold ethanol were added to this 'acid-extract' and the tube contents mixed and stored overnight at 4°C.

7. The histone precipitate was spun down at 28,000g for 1 hour.

8. The pellet was drained and dissolved in a small volume of 7% acetic acid/1% 2-mercaptoethanol/10% sucrose, and stored at 4°C until required for electrophoresis on urea - acetic acid - 15% acrylamide gels.
B. Jerusalem artichoke tuber tissue

(1) Extraction of tuber chromatin

This was by the method of Bonner et al. (1967), with some modifications.

The extraction procedure was carried out at 4°C.

1. The tubers were scrubbed in cold water to remove soil. Then the outer layers were scraped off and the tissue sliced into 2.0mm discs.

2. The discs were broken by hand and weighed. (40 - 70g fresh weight was used.)

3. Grinding medium was added and the tissue homogenised in a polytron homogeniser at high speed for 10 - 15 seconds. ('Grinding medium' consisted of 0.3M sucrose, 0.04M magnesium chloride, 0.05M Tris-HCl, pH 8.0, 1.0mM sodium metabisulphite, 0.1% PVP 40,000, and 0.5% 2-mercaptoethanol.)

4. The homogenate was made to 1% Triton X-100 by the addition of a 20% solution, and was filtered through 2 layers of muslin and 4 layers of mira cloth, (Calbiochem). The filters were washed with 'grinding medium' and then the muslin only was squeezed out.

5. The filtrate was spun at 5,000g for 30 minutes on an MSE "Highspeed 18" centrifuge, and the supernatant discarded.

6. The whole pellet material was removed and hand-homogenised in
40.

0.01 M Tris-HCl, pH 7.8, containing 1.0 mM sodium metabisulphite.

7. The homogenate was spun at 10,000 g for 10 minutes, and the supernatant discarded.

8. The whole pellet material was again washed in Tris-HCl/metabisulphite.

9. The pellet was homogenised in 0.01 M Na₂EDTA, 0.15 M NaCl, pH 7.2, containing 1.0 mM sodium metabisulphite, and spun at 13,000 g for 15 minutes.

10. The final chromatin pellet wash was in Tris-HCl/metabisulphite. This was followed by a 10 minute spin at 10,000 g.

11. The chromatin was resuspended in Tris-HCl/metabisulphite and 5.0 ml aliquots were spun through 15 ml 1.7 M sucrose using a 3 x 25 ml 'swing-out' rotor. This was a 3-hour spin at 75,000 g on an MSE "Superspeed 50" centrifuge.

12. The supernatant was carefully poured away and a pellet of purified chromatin was left at the bottom of each tube.

(ii) Extraction of histones from tuber chromatin

This was exactly as described in section 3.4.(iii).

(iii) Extraction of tuber total nucleic acid

The extraction was carried out at room temperature unless otherwise specified.
1. A tuber was scrubbed in cold water, then the outer layers were scraped off and the tissue sliced into 2.0mm discs.

2. 4 - 6g of the tissue was broken by hand and homogenised in a pestle and mortar in a small volume of grinding medium, (Grinding medium was a detergent mix of 1% TNS, 6% PAA, 50mM NaCl, 10mM Tris-HCl, pH7.4, and 6% phenol, (Parish and Kirby, 1966).)

3. The homogenate was transferred to a 15ml glass centrifuge tube and the pestle and mortar were rinsed with a small volume of grinding medium. This was added to the homogenate.

4. An equal volume of phenol-cresol mixture was added and the tube contents mixed thoroughly. (The phenol-cresol mixture was a deproteinising solution consisting of phenol containing 15% redistilled m-cresol, 0.1% 8-hydroxyquinoline, saturated with 0.01M Tris-HCl, pH7.4.)

5. The homogenate was spun for 10 minutes at 1,200g on an MSE "Mistral 4L" centrifuge and the aqueous layer removed.

6. 5.0M NaCl was added to the aqueous fraction, to a final concentration of 0.5M NaCl.

7. One volume of phenol-cresol was added to the mixture and the tube vigorously shaken.

8. The mixture was spun for 10 minutes at 1,200g and the aqueous phase removed.
9. One volume of phenol-cresol was added to the aqueous phase and the tube contents mixed thoroughly and spun down for 10 minutes at 1,200g.

10. The aqueous phase was removed and 2 volumes of cold ethanol were added.

11. The tube contents were mixed and the nucleic acids allowed to precipitate overnight at 4°C.

12. The precipitate was spun down, drained and resuspended in 80% ethanol containing 0.2% SLS.

13. The precipitate was spun down, drained and dissolved in 0.15M sodium acetate containing 0.5% SLS, (pH 6.0).

14. The solution was mixed with 2 volumes of cold ethanol and the nucleic acids allowed to precipitate overnight at 4°C.

15. The precipitate was spun down, drained and resuspended in 80% ethanol containing 0.2% SLS.

16. After a final 10 minute spin at 1,200g the pellet was drained and dissolved in a small volume of "E" running buffer. ("E" running buffer is described in section 3.D.(iv).)

17. The U.V. absorbance spectrum of the solution was determined in an SP,800 Ultraviolet Spectrophotometer, using "E" running buffer as the blank.

18. Sucrose was added to the solution to a final concentration of
12% and the nucleic acids were fractionated by electrophoresis of aliquots on 3L3 - 2.5% acrylamide gels.
C. Jerusalem artichoke tuber explants

(i) Preparation of explant culture media

The following stock solutions were prepared and stored at 4°C until required, (Yeoman et al., 1965).

**Solution A.** (In a total volume of 250ml)
- Potassium nitrate, ($\text{KNO}_3$): 2.03g. ($8.0 \times 10^{-3}$M).
- Potassium chloride, ($\text{KCl}$): 1.63g. ($8.8 \times 10^{-3}$M).
- Magnesium sulphate, ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$): 0.99g. ($1.5 \times 10^{-2}$M).

**Solution B.** (In a total volume of 250ml)
- Potassium dihydrogen phosphate, ($\text{KH}_2\text{PO}_4$): 0.3g. ($8.8 \times 10^{-3}$M).

**Solution C.** (In a total volume of 250ml)
- Calcium nitrate, ($\text{Ca(NO}_3)_2$): 5.9g. ($1.4 \times 10^{-1}$M).
- Ferric chloride, ($\text{FeCl}_3$): 0.025g. ($6.2 \times 10^{-4}$M).

2.2mg 2,4-dichlorophenoxyacetic acid, (2,4-D), in 10ml ethanol, ($10^{-3}$M).

In 1.0 litre of culture medium there were 10ml of each of the solutions A, B, and C, 1.0ml of the 2,4-D solution and 40g sucrose. When the explants were to be labelled with $^{32}$P-phosphate, solution B was excluded from the culture medium. The volume of medium added to each culture flask was usually 15ml, but for the cell-cycle experiments this was reduced to 10ml.
Molecular concentrations in the 'complete' culture medium were:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO$_3$</td>
<td>$8.0 \times 10^{-4}$ M</td>
</tr>
<tr>
<td>KCl</td>
<td>$8.8 \times 10^{-4}$ M</td>
</tr>
<tr>
<td>MgSO$_4 \cdot 7$H$_2$O</td>
<td>$1.5 \times 10^{-4}$ M</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>$8.8 \times 10^{-5}$ M</td>
</tr>
<tr>
<td>Ca(NO$_3$)$_2$</td>
<td>$1.4 \times 10^{-3}$ M</td>
</tr>
<tr>
<td>FeCl$_3$</td>
<td>$6.2 \times 10^{-6}$ M</td>
</tr>
<tr>
<td>Sucrose</td>
<td>$1.17 \times 10^{-1}$ M (≈ 4% sucrose)</td>
</tr>
<tr>
<td>2,4-D</td>
<td>$10^{-6}$ M</td>
</tr>
</tbody>
</table>

(ii) Preparation and culture of explants

Explants were prepared and cultured by the method of Yeoman et al., (1965).

Artichoke tubers were washed free of soil and scrubbed with a hard brush to remove the outer layers. They were then immersed in 20% sodium hypochlorite solution, (c. 2.5% available chlorine), for 30 minutes. The tubers were rinsed twice with sterile distilled water, immersed in sterile distilled water and taken into a 'sterile' room. The remainder of the procedure was carried out in sterile conditions under a green 'safe' light. The water was poured away, and each tuber was dried with tissue paper and the ends cut off. The cut tubers were placed separately into glass petri dishes. A cannula of 2.0mm diameter was then used to cut bores of the tuber tissue from one cut end to the other. These were placed in a glass petri dish. Using a specially designed cutter the bores of tuber tissue were sliced into 2.4mm lengths. These small segments were
the explants. One operation of the cutter produced 100 explants.
The products of each cutting were added to separate 100ml conical
flasks, each containing culture medium and a small magnetic
stirring bar. The explants were grown, (with continuous stirring),
in total darkness at a temperature of 25°C.

The culture flasks and distilled water had been sterilized by
autoclaving. The instruments used in explant preparation had been
sterilized by heating in an oven and, wherever possible, were dipped
into ethanol and flamed at intervals throughout the procedure.

(iii) Labelling of explants with radioisotopes

Dual labelling with $^{32}$P-orthophosphate and $^3$H-lysine

When 3-day old explants were labelled, 750 µCi $^{32}$P-
orthophosphate and 25 µCi $^3$H-lysine were added to each flask. After
4 hours of labelling the explants were removed from culture, washed
and the chromatin extracted. Each radioisotope was added in
distilled water, as 0.5ml aliquots. No 'carrier' phosphate or
lysine was used. The volume of culture medium in each flask was
15ml, (before radioisotope addition). $^{32}$P-orthophosphate labelling
concentration was c. 4.7 µCi/ml. $^3$H-lysine labelling concentration
was c. 1.56 µCi/ml.

When explants were labelled during the first division cycle,
(i.e. labelled during the first c.28 hours of culture), 400 µCi
$^{32}$P-orthophosphate and 20 µCi $^3$H-lysine were added to each flask.
Again, labelling was for 4 hours, and each radioisotope was added in
distilled water, without 'carrier', as 0.5 ml aliquots. The volume of culture medium in each flask was 10 ml, (before radioisotope addition). 

\[ 32^P \text{-orthophosphate labelling concentration was } c. \ 36.4 \mu\text{Ci/ml}. \]

\[ ^3\text{H-lysine labelling concentration was } c. \ 1.82 \mu\text{Ci/ml}. \]

**Labelling with $^3\text{H}$-thymidine**

When $^3\text{H}$-thymidine was used in the cell-cycle phase determination experiments, 10 \( \mu\text{Ci} \) was added to each flask. Labelling was for 1.5 hours, and the radioisotope was added in distilled water, without 'carrier', as 0.4 ml aliquots. The volume of culture medium in each flask was 10 ml. 

\[ ^3\text{H-thymidine labelling concentration was } c. \ 0.96 \mu\text{Ci/ml}. \]

Histone or nuclear acidic protein synthesis was determined by the incorporation of $^3\text{H}$-lysine, and histone phosphorylation was determined by incorporation of $^{32}\text{P}$-orthophosphate. DNA synthesis was measured by the incorporation of $^3\text{H}$-thymidine or, in some experiments, by $^{32}\text{P}$-orthophosphate incorporation. In each case, uptake of the radioisotope by the tissue was taken into account.

(iv) *Extraction of explant chromatin*

This was by the method of Bonner et al, (1967), with some modifications.

The extraction procedure was carried out at \( 4^\circ\text{C} \).

1. The explants were removed from the culture flasks and washed thoroughly in cold distilled water.
2. They were homogenised in 'grinding medium' in a pestle and mortar, and then at high speed in a polytron homogeniser for 10 - 15 seconds. ('Grinding medium' was as for artichoke tuber chromatin preparation.)

3. The homogenate was made to 1% Triton X-100 and filtered through 1 layer of muslin and 2 layers of mira cloth, (Calbiochem). The filters were washed with 'grinding medium' and then both were carefully squeezed out.

4. The solution was spun at 8,000g for 30 minutes on an MSE "Highspeed 18" centrifuge, and the supernatant discarded.

5. The whole pellet material was removed and hand-homogenised in 0.01M Na₂EDTA, 0.15M NaCl, pH7.2, containing 1.0mM sodium metabisulphite, and spun at 18,000g for 20 minutes.

6. The pellet was resuspended in 14ml 0.01M Tris-HCl, pH7.8, containing 1.0mM sodium metabisulphite and the entire sample was spun through 6.0ml 1.7M sucrose, using a 3 x 25ml 'swing-out' rotor. This was a 3-hour spin at 73,000g on an MSE "Superspeed 50" centrifuge.

7. The supernatant was carefully poured away and a pellet of chromatin was left at the bottom of the tube.

When 'labelled' explants were used the supernatant from the first centrifugation was retained for scintillation counting. This gave data on uptake of radioisotopes by the tissue.
49.

(v) Extraction of histones from explant chromatin

The method was as described in section 2.A.(iii), with the exception that there was only one acid-extraction. This was with 0.4N H$_2$SO$_4$.

(vi) Extraction of labelled DNA and acidic proteins from explant chromatin

After acid-extraction of histones 1.0ml 0.1N NaOH was added to the de-histoned chromatin pellet. The pellet material was resuspended by repeatedly filling and ejecting from a 1.0ml syringe, and stirred overnight at room temperature. The mixture was spun at 28,000g for 20 minutes on an MSE "Superspeed 50" centrifuge and the supernatant transferred to a 15ml glass centrifuge tube. 5.0ml of cold 6% TCA was added to the supernatant, (making the solution 5% with respect to TCA), and the tube contents mixed thoroughly. The DNA and acidic proteins were allowed to precipitate overnight at 4°C, and were then spun down at 1,200g for 15 minutes in an MSE "Mistral 4L" centrifuge. The pellet was drained and dissolved in 2.0ml 1% SLS at room temperature and aliquots were taken for scintillation counting. The alkali treatment removes phosphate groups from proteins; therefore, $^{32}$P-counts in the final pellet material are in the DNA fraction and $^3$H-counts, (from $^3$H-lysine), are in the nuclear acidic proteins.

(vii) Extraction of explant DNA

DNA was extracted and estimated by the method of Burton, (1956);
as described in section 2.C.(i). There were, however, the following additional steps in the procedure:

1. The explants were washed thoroughly in cold distilled water prior to homogenisation in TCA.

2. After the 2 washes in TCA each pellet was suspended in 0.3N NaOH for 1 hour at 25°C. TCA was then added to a final concentration of 5%, and the samples left overnight at 4°C to precipitate completely. After a further spin the pellets were hydrolysed in perchloric acid and the DNA estimated by the diphenylamine reagent, as described previously.

   Step number 2 was only used when the explants were labelled with ³H-thymidine, and was an additional precaution to ensure that all the label present in the pellet was in fact incorporated label.

   When DNA was extracted and estimated from explants labelled with ³H-thymidine the various supernatants and the perchloric acid-hydrolysed DNA were retained and counted for thymidine uptake data and incorporation data respectively.

   *(The PCA was first neutralised by addition of 2N KOH. After centrifugation the supernatant was removed and aliquots were counted for ³H-thymidine.)*

(viii) **Extraction of explant total nucleic acid**

This was by the method described in section 2.B.(iii). 4 - 6g of explants were washed in distilled water and used for the extraction.
(ix) Hydrolysis of labelled histone fractions from explants, and paper electrophoresis for serine and threonine phosphates

Hydrolysis of labelled histone fractions with HCl

Total histone samples were electrophoresed on urea - acetic acid - 15% acrylamide gels. After rapidly staining and destaining a histone 'marker' gel, the positions of the histone fractions in the other, (unstained), gels were calculated. These gels were washed in changes of 7% acetic acid for 2 hours at room temperature and the various histone regions were cut into 0.5mm slices. Each set of slices, (equivalent to a single histone region), was added to 5.0ml 7% acetic acid and the protein eluted out by shaking overnight at room temperature. The resulting histone solutions were made to 6N HCl by the addition of concentrated HCl and were transferred to glass vials. Each vial was evacuated and sealed, and the sample hydrolysed by heating at 105°C for 5 hours. (According to Kabat, 1971), these conditions of acid hydrolysis also cause a breakdown of slightly less than 50% of the serine-phosphate linkages.) After hydrolysis, the samples were removed and dried down in a rotary film evaporator. Each was then dissolved in 1.0ml of 2.5% formic acid, 7.8% acetic acid, (pH4.8), and subjected to high voltage paper electrophoresis.

Enzymatic hydrolysis of labelled histone fractions

From Balhorn et al. (1971).

Total histone samples were electrophoresed on polymacrylamide
gels, as described in the previous section. The sample gels were then washed at room temperature in changes of 0.25M Tris-HCl, pH7.8, until the acetic acid had been removed. (This took 2 - 3 hours.) The various histone regions of these gels were cut into 0.5mm slices. Each set of slices, (equivalent to a single histone species), was added to 5.0ml 0.25M Tris-HCl, pH7.8, and the protein eluted out by shaking overnight at room temperature. Pronase was added to the histone solution to a final concentration of c. 25% of that of the histone present, and calcium chloride was added to a final concentration of $9 \times 10^{-4} M$. The mixture was incubated at 37°C for 24 hours. After heat denaturation of the pronase at 100°C for 15 minutes, and subsequent cooling to room temperature, the digestion was continued by adding 0.1mg of 'activated' leucine aminopeptidase.* The solution for this second digestion contained 0.3M Tris-HCl pH8.5, and 0.01M manganese chloride. The mixture was incubated at 37°C for a further 96 hours.

After hydrolysis, the samples were dried down in a rotary film evaporator. Each was then dissolved in 1.0ml 2.5% formic acid, 7.8% acetic acid, (pH 1.8), and subjected to high voltage paper electrophoresis.

* ('Activation' of the leucine aminopeptidase solution was by heating at 40°C for 3 hours.)

Paper electrophoresis for serine and threonine phosphates

Each hydrolysed histone sample was streaked on Whatman No 3 chromatography paper and electrophoresed for 2 hours at 1,200V,
(c. 30V/cm). Each end of the paper was in contact with an electrophoresis medium of 2.5% formic acid, 7.0% acetic acid, (pH 3.8), whilst the remainder of the paper was suspended in white spirit. Direction of electrophoresis was from cathode to anode. A stock solution containing 'markers' of inorganic phosphate, serine phosphate and threonine phosphate in electrophoresis medium was prepared and aliquots, containing 200µg of each 'marker', were electrophoresed at the same time as the samples. After electrophoresis the paper was dried completely and the phosphates were visualised by spraying with phosphomolybdate reagent, (4ml 70% perchloric acid, 10ml 1.0N HCl, 25ml 4% ammonium molybdate), followed by irradiation with ultra-violet light. The sample regions were cut into 5.0mm slices and the $^{32}$P-phosphate label in each was measured by scintillation counting.
D. General

(i) **Phosphatase incubation of histones**

This was by the method of Balhorn et al., (1971), with some modifications.

Histones were extracted in acid and precipitated with cold ethanol, as described previously. The precipitate was spun down at 28,000 g for 1 hour and washed twice in cold ethanol. The pellet was well drained, and dissolved in phosphatase solution containing 0.01M Tris-HCl, (at the requisite pH for optimal enzyme activity), and incubated at 37°C. At the same time a 'control' sample was incubated in 0.01M Tris-HCl alone. The incubations were terminated after 22 hours by the addition of acetic acid, 2-mercaptoethanol and sucrose to final concentrations of 7%, 1% and 10% respectively. The samples were stored at -20°C until required for electrophoresis on urea - acetic acid - 15% acrylamide gels.

*(The phosphatase concentration in the tubes was c. 5% of that of the histone samples.)*

(ii) **Electrophoresis of histones on urea - acetic acid - 15% acrylamide gels**

This was by the method of Panyim and Chalkley, (1969), with some modifications.

The following solutions were prepared:

1. A running buffer of 7% acetic acid, (pH 1.8).
2. A filtered 2.5M solution of urea. This was made to 0.2% ammonium persulphate, (AMPS), immediately prior to use.

3. An acrylamide stock solution of 60% acrylamide, 0.4% bis-acrylamide. This was filtered and stored in the dark.

4. A stock solution of 4% H,N,N,N - tetramethylethylene diamine, (TEMED), 43% acetic acid. This was stored at 4°C and re-prepared every 2 months.

5. An overlaying solution of 1.0M urea, 5% acetic acid. This was filtered.

6. A staining solution of 0.25% Coomassie Brilliant Blue R, 45:45 methanol, 9.2% acetic acid. The dye was dissolved by vigorous stirring and the resulting solution filtered before use.

7. A destaining solution of 5% methanol, 7% acetic acid.

8. A solution of 7% acetic acid, 1% 2-mercaptoethanol, 10% sucrose, containing the histone sample.

The urea - AMPS, acrylamide stock and TEMED stock solutions were mixed at room temperature in the ratio 5:2:1. After mixing the first two the solution was de-gassed. After addition of the TEMED the solution was gently stirred and pipetted into glass gel tubes sealed at the lower end with parafilm. Care was taken to ensure that there were no air bubbles in the tubes after addition of the gel solution. A small volume of overlaying liquid was carefully layered on top of the gel solutions, and these were left to
polymerize at room temperature away from direct sunlight. Polymerization took 30 - 60 minutes, depending on the temperature of the laboratory.

(The final gel composition was 15% acrylamide, 0.1% bis-acrylamide, 1.56M urea, 5.37% acetic acid, 0.5% TEMED and 0.125% AMPS.)

The gel tubes were 12.6cm long and 7mm wide, with a bore of 5mm. The gels prepared were 10cm in length.

Electrophoresis was from anode to cathode at a temperature of 4°C, with the gel-containing parts of the tubes immersed in the lower running buffer solution. This buffer was stirred continuously. (These precautions reduced ohmic heating in the gels to a minimum.)

The gels were first pre-run for at least 6 hours at 80V; they were then loaded and run at 130V, <1mA/gel. For a total histone gel electrophoresis was for 12 hours under these conditions, but when histone H1 was electrophoresed to near the lower end of the gel 24 hours was required. After electrophoresis the gels were carefully removed from the tubes and stained for 2 hours in the Coomassie Brilliant Blue solution. They were then destained at 45°C in changes of the methanol/acetic acid destaining solution. When completely destained, the gels were scanned in a Chromoscan, (Joyce-Loebl and Co. Ltd), using a 575mm filter.

*(Staining in Coomassie Blue was considered to be preferable to staining in Amido Black since the former is a much more 'sensitive' stain, and can therefore be used on smaller quantities of histone. If care is taken to ensure that the amount of protein
in each band is no more than about 6μg then the staining is also quantitative. (See section 2 of the Results, and Figure 16.)

(iii) Determination of histone molecular weights by electrophoresis on SLS - 15% acrylamide gels

Sample preparation

Total histone samples were dissolved in 10% glycerol, 5% 2-mercaptoethanol, 2.3% SLS, 0.0625M Tris-HCl, pH6.8.

For samples of single histones the individual histone species were eluted off the appropriate regions of urea - acetic acid - 15% acrylamide gels, after electrophoresis of total histones. This was by a method similar to the one described in section 3.C.(ix) for samples required for enzymatic hydrolysis. The histone fractions were eluted into a solution containing 10% glycerol, 5% 2-mercaptoethanol, 2.3% SLS, 0.0625M Tris-HCl, pH6.8.

The samples were stored at -20°C until required for use.

Gel electrophoresis

This was by the method of Laemmli, (1970), with some modifications.

The following solutions were prepared:

1. A running buffer of 0.1% SLS, 0.3% Tris, 1.44% glycine, pH8.3.
2. An acrylamide stock solution of 30% acrylamide, 0.8% bis-acrylamide. Both reagents had been recrystallised. The solution
was filtered and stored in the dark at 4°C.

3. A separating gel buffer of 0.4% SLS, 1.5M Tris-HCl, pH8.8. The solution was filtered and stored at 4°C.

4. A stacking gel buffer of 0.4% SLS, 0.5M Tris-HCl, pH6.8. The solution was filtered and stored at 4°C.

5. A 10% solution of ammonium persulphate, prepared fresh when required.


7. A stock solution of molecular weight 'markers' in 10% glycerol, 5% 2-mercaptoethanol, 2.3% SLS, 0.0625M Tris-HCl, pH6.8. This contained 100μg/ml of each 'marker'. The molecular weight 'markers' used were; Bovine Albumin, Egg Albumin, Myoglobin, Ribonuclease-A and Cytochrome C, with molecular weights of 65,000, 45,000, 17,000, 14,000 and 12,400 daltons respectively.

Separating gels were prepared by mixing the acrylamide stock, the separating gel buffer, and distilled water in the ratio 2 : 1 : 1. After de-gassing, polymerization was initiated by the addition of TEMED and 10% ammonium persulphate to the mixture in the ratio 0.1 : 1 : 100. The solution was gently stirred and pipetted into glass gel tubes. A small volume of distilled water was carefully layered on top of each gel solution. Polymerization took less than 30 minutes at room temperature.

The final separating gel composition was 15% acrylamide, 0.4% bis-acrylamide, 0.1% SLS, 0.375M Tris-HCl, pH8.8. These gels
could be prepared the day before use and stored at $4^\circ C$ until required.

Stacking gels were prepared by mixing the acrylamide stock, the stacking gel buffer and distilled water in the ratio 2 : 5 : 13. After de-gassing, polymerization was initiated by the addition of TEMED and 10% ammonium persulphate to the mixture in the ratio 0.1 : 1 : 100. The solution was gently stirred and pipetted onto the separating gels; (the overlaying liquid having been removed with tissue prior to this). A small volume of distilled water was carefully layered on top of the gel solutions. Polymerization took less than 30 minutes at room temperature.

The final stacking gel composition was 3% acrylamide, 0.08% bis-acrylamide, 0.1% SLS, 0.125M Tris-HCl, pH6.8. The gels were prepared immediately prior to use.

The gel tubes were 12.6cm long and 7mm wide, with a bore of 5mm. The separating gels and stacking gels were 9cm and 1cm in length respectively.

Separate gels were loaded with the 'marker' solution and sample solutions, and a few grains of the dye Bromophenol Blue were added to the upper running buffer solution. (An aliquot of the 'marker' solution containing 5μg of each protein was found to be a suitable loading.) Because of the presence of a stacking gel, samples of up to 500μl in volume could be electrophoresed by this method.

Electrophoresis, (from cathode to anode), was carried out at room temperature, (c. $20^\circ C$), 60V and less than 1mA/gel. The gel-
containing parts of the tubes were immersed in the lower running buffer solution, which was stirred continuously. Electrophoresis was terminated when the band of Bromophenol Blue reached the bottom of the separating gel. This took approximately 18 hours. (The Bromophenol Blue migrates just behind the solvent front.) After electrophoresis, the gels were stained for 2 hours in Coomassie Brilliant Blue and destained at 45°C in changes of the methanol-acetic acid destaining solution. When completely destained, the gels were scanned in a chromoscan, (Joyce-Loebl and Co. Ltd), using a 575nm filter. A calibration curve of log \( e \) molecular weight against percentage mobility was drawn for the molecular weight 'markers' and this was used to determine the molecular weights of the sample histones.

(iv) **Electrophoresis of nucleic acids on SLS - 2.5% acrylamide gels**

This was by the method of Loening, (1967).

The following solutions were prepared:

1. A double-strength "F" running buffer stock solution containing 80mM Tris, 66.3mM sodium acetate, 3.97mM disodium EDTA; pH to 7.8 with 6.3mL/L glacial acetic acid.

2. An acrylamide stock solution of 25% acrylamide, 1.25% bis-acrylamide. Both reagents had been recrystallised. The solution was filtered and stored in the dark at 4°C.
3. A 0.1% ammonium persulphate solution, prepared fresh when required.

4. A 4.1% solution of TEMED, prepared fresh when required.

The tubes used for these gels were perspex, 9cm long, 1cm in diameter, with a bore of 7mm. The gels were 8cm in length. The lower ends of the tubes were sealed with parafilm to hold the unpolymerized liquid, and a small hollow insert prevented the polymerized gels from falling out of the tubes.

Gels were prepared by mixing the acrylamide stock, the double-strength buffer solution and distilled water in the ratio 4 : 20 : 14.4. The mixture was de-gassed and 0.8ml of each of the ammonium persulphate and TEMED solutions was added. The solution was gently mixed and pipetted into the gel tubes, taking care that no air bubbles were trapped. A small volume of distilled water was carefully layered on top of the gel solutions. Polymerization took 10 - 15 minutes at room temperature.

The final gel composition was 2.5% acrylamide, 0.125% bis-acrylamide, 40mM Tris, 33.1mM sodium acetate, 1.98mM disodium EDTA.

Electrophoresis was from cathode to anode, at room temperature. The running buffer was double-strength "E" buffer diluted 1 : 1 with distilled water and made to 0.2% SLS. (The composition of the running buffer was therefore 40mM Tris, 33.1mM sodium acetate, 1.98mM disodium EDTA, 0.2% SLS, pH7.8.)

The gels were pre-run for 1 hour at 5mA/gel. They were then loaded and run for 2 - 3 hours at 5mA/gel, (c. 50V). After
electrophoresis, the gels were removed from the tubes and washed in distilled water for 1 hour. Each gel in turn was placed in a quartz cell and the nucleic acids visualised by scanning at 265nm in a Joyce-Loebl polyfrac U.V. scanner.

The nucleic acid samples electrophoresed on these gels were dissolved in running buffer containing 0.2% SLS and 12% sucrose.
THE DEVELOPMENT OF ADEQUATE CHROMATIN ISOLATION PROCEDURES

A. Introduction

In the initial investigations I extracted chromatin from pea shoot tips. Substantial work had already been published on chromatin from pea tissues, (Bonner et al., 1968; Fambrough et al., 1968; Panyim and Chalkley, 1969), so I felt that this was a good opportunity to become familiar with the methods of extraction and purification whilst at the same time comparing my results with those of others. Only then would I attempt to extract chromatin from Jerusalem artichoke tubers, a tissue not previously investigated in this manner.

Starting with the chromatin isolation method of Bonner et al., (1967), I attempted to determine the optimum conditions for the extraction of pea shoot chromatin, followed by chromatin from artichoke tubers and explants.

The aim was to maximise yield and purity of the chromatin concerned; however, since these two parameters normally show an inverse relationship, certain concessions had to be made. Where the amount of tissue was never a limiting factor, as in the case of the pea shoot tips and artichoke tubers, the chromatin could be purified to a high degree and still yield adequate amounts for experimental purposes. When only a small amount of tissue was available, as in the case of the artichoke explants, the yield had to be maximised at the expense of purity.

The chromatin extraction procedures described in Chapter 3 were
the final procedures adopted.

(i) Criteria for estimating yield and purity of chromatin

Chromatin yield was taken to be equivalent to the amount of DNA in the purified sample. This was estimated by the absorption at 260 nm of a solution of the chromatin. Chromatin purity was determined by the 260/280 nm and 320/260 nm absorption ratios on the U.V. scan. According to Bonner et al. (1967), these should be 0.20 and less than 0.1 respectively for a pure sample. An additional indication of purity is the Protein : DNA ratio in the chromatin. A ratio of 1.5 : 1 or less is generally considered to indicate a relatively pure sample.

If the chromatin sample is electrophoresed on 3% - 2.5% acrylamide gels the nucleic acids present can be separated and estimated by U.V. absorption. In this way not only can the amount of DNA be determined by its peak area, (using a calibration curve obtained with purified DNA), but the presence of DNA alone, or in large excess with respect to the other nucleic acids, would indicate a fairly pure sample.

(ii) Corrections for light scattering in a chromatin solution

The absorption of U.V. light by a turbid chromatin solution is the sum of the losses of transmittance caused by absorption and by scatter. Therefore, when the U.V. absorption spectrum of a chromatin solution is determined, correction must be made for the scattering. This correction is made by measuring apparent absorption in the region 320 - 360 nm, in which nucleoprotein does
not absorb, and extrapolating to lower wavelengths. An effective procedure for making the extrapolation is to plot log\(\text{absorbance, (optical density), against log\'wavelength, as suggested by Leach and Sheraga, (1960). The line connecting optical density points for wavelengths at which chromatin does not absorb is merely extrapolated linearly. The corrected baseline for the U.V. scan can thus be determined and the true chromatin absorption calculated. A typical correction curve is illustrated in Figure 3.}

This method of correction has been used for all chromatin scans; however uncorrected data are also presented.
FIGURE 3.

Typical correction curve for light scattering in a chromatin solution.

Log$^*$ absorbance is plotted against log$^*$ wavelength. The corrected baseline is a continuation of the line connecting absorbance values for wavelengths at which chromatin does not absorb (320 - 360 nm).
FIG. 3. CORRECTION CURVE FOR LIGHT SCATTERING IN A CHROMATIN SOLUTION.

\[ \log Q\Delta \]

\[ \log^1 \text{wavelength} \]

-1

260 nm

280 nm
B. Chromatin isolation and purification from pea shoot tissue.

(Plumular hook.)

(i) Initial preparations

The initial extractions of pea shoot chromatin were carried out as described in Chapter 3, with the following differences:

- Triton X-100 was not added to the homogenate.
- There was no wash in EDTA + NaCl.
- The 73,000g spin through sucrose was for only 100 minutes.

Figure 4(A) shows the U.V. absorption spectrum of the final extract in a typical experiment, (Experiment 3), and Table 1 shows the data obtained relating to yield and purity of the chromatin.

The yield was very low, and the absorption ratios showed that the final extract was not very pure. The low 260/280 nm absorption ratio indicated the presence of contaminating non-chromatin proteins, (since proteins absorb at 280 nm), and the high 320/260 nm ratio indicated probable contamination by cell wall fragments.

(ii) Final preparations

Three major improvements were made to the original extraction procedure:

- Triton X-100 was added to the homogenised tissue to dissociate membrane material. This would tend to reduce contamination by cell organelles, and also increase the yield of chromatin by detaching the outer nuclear membrane and any organelles associated with it.
- A chromatin wash with EDTA + NaCl was inserted after the
U.V. absorption spectra of pea shoot tip chromatin preparations.

(A) Initial extraction procedure. After spin through sucrose.

(B) Final extraction procedure. Before spin through sucrose.

(C) " " " After spin through sucrose.

(D) " " " After a spin through sucrose and suspension in 1% SLS.

For (A), (B) and (C) the samples were suspended in 0.01M Tris-HCl, pH7.3, and scanned using a 'blank' of Tris-HCl.

For (D) the 'blank' was 1% SLS.
Yield and purity of pea shoot tip chromatin.

Initial and final extraction procedures.

Experiments 3 and 6 were typical experiments carried out using the initial and final pea shoot chromatin extraction procedures respectively.

The spin through sucrose was the final stage in chromatin purification. Cell wall contaminants were removed by dissolving the chromatin in 1% SDS and spinning the insoluble material out of solution.

The 260/280nm absorption ratio is shown before and after correction for turbidity of the chromatin solution.

Determination of DNA was by absorption at 260nm.

(1.0 O.D. ≈ c. 55μg DNA.)

Determination of protein was by the method of Lowry et al., (1951).
| TABLE 1 |

<table>
<thead>
<tr>
<th></th>
<th>Initial procedure (Experiment 3.)</th>
<th>Final procedure (Experiment 6.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After sucrose spin</td>
<td>Before sucrose spin</td>
</tr>
<tr>
<td>Chromatin yield (μg DNA/g fresh weight)</td>
<td>13.3</td>
<td>-</td>
</tr>
<tr>
<td>Uncorrected 260/280 nm absorption ratio</td>
<td>1.37</td>
<td>1.44</td>
</tr>
<tr>
<td>Corrected 260/280nm absorption ratio</td>
<td>1.47</td>
<td>1.60</td>
</tr>
<tr>
<td>320/260nm absorption ratio</td>
<td>0.198</td>
<td>0.196</td>
</tr>
<tr>
<td>Protein : DNA ratio</td>
<td>No data</td>
<td>1.40 : 1</td>
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</table>
first two washes in Tris-HCl. It was hoped that the chelating properties of the EDTA would break down cell wall complexes and thus reduce contamination from cell wall material. It would probably also reduce contamination from ribosomes by destroying their aggregating properties. After this wash the pellet was spun down by greater centrifugation than in the Tris-HCl washes, since it had been reported that EDTA treatment causes chromatin to disperse to a less dense structure.

The final washed chromatin was given a longer spin through sucrose than before, in order to ensure that most of the chromatin was pelleted.

Figures 4(B)-(D) show the U.V. absorption spectra of a chromatin sample in a typical experiment, (Experiment 6), (B) before the sucrose spin, (C) after the sucrose spin, and (D) after a further spin in SLS to remove any cell wall debris still remaining. Table 1 shows the data obtained relating to yield and purity of the chromatin at the different stages in the preparation.

Yield of chromatin per unit fresh weight of tissue was increased nearly 5-fold. The absorption ratios indicated that the chromatin was purer than in the initial experiments even before it was spun through sucrose. There was further purification after the sucrose spin and the SLS spin. The final corrected 260/280 nm absorption ratio was 1.70, with a 320/260 nm ratio of well below 0.1.

Protein : DNA ratios in this preparation showed values of less than 1.5 : 1, as expected for purified chromatin, (Table 1); and
scans of 3LS - 2.5% acrylamide gels run for total nucleic acids also indicated the purity of the final chromatin sample, (Figure 5). In the latter case practically all of the nucleic acid present was DNA.

Using the improved extraction method described above pea shoot chromatin was regularly prepared with this yield and degree of purity; however it was found impossible to increase the 260/280 nm absorption ratio to a value closer to that suggested by Bonner et al, (1967), of 2.0.
Fig 5

DNA

Solvent front
C. Chromatin isolation and purification from artichoke tuber tissue

(i) Initial preparations

The extraction procedure first adopted was very similar to the final procedure used for pea shoot chromatin, with the exceptions that polyvinyl pyrrolidone, (PVP, 40,000) was added to the grinding medium, and sodium metabisulphite was included in both the grinding medium and the washing solutions. PVP binds polyphenols from the artichoke extract, (Core, 1973), and sodium metabisulphite is a general protease inhibitor.

The method used was as described in Chapter 3, but excluding the wash in EDTA + NaCl. It was felt that inclusion of this wash in later experiments would show whether or not it was really necessary following treatment with Triton X-100.

Table 2 presents the data from two experiments here, Experiments 12 and 13. Figure 6 shows the U.V. absorption spectrum of the final extract in Experiment 13.

Chromatin yield was fair, being 20 - 30% of that estimated to be present, and the U.V. absorption ratios indicated that the samples were very pure. However, there was contamination from protein and RNA. (This is probably ribosomal contamination.) The Protein : DNA ratios were about 2.2 : 1, whereas a value of significantly less than 2 : 1 would be expected. Also, gels run for total nucleic acids indicated the presence of ribosomal RNAs. (Figure 7B.) However, it must be pointed out that this contamination was very small and there had been considerable selective removal of RNA species in the extraction procedure. Indeed, the
Yield and purity of Jerusalem artichoke tuber chromatin. 

Initial and final extraction procedures.

Experiments 12, 13 and 14(A) were typical experiments carried out using the initial artichoke tuber chromatin extraction procedure. Experiments 14(B) and 17 were typical experiments carried out using the final extraction procedure, (i.e. the inclusion of a wash in EDTA + NaCl).

In each case the chromatin pellet was dissolved in 1% SDS after the spin through sucrose. Insoluble contaminants were removed by centrifugation and the supernatant collected for estimation of protein yield and for measurement of the U.V. absorption spectrum.

The 260/280 nm absorption ratio is shown before and after correction for turbidity of the chromatin solution.

Determination of chromatin DNA extracted was by absorption at 260 nm. (1. O.D. = c. 55 µg DNA).

Determination of total DNA in the tissue was by the method of Burton, (1956).

Determination of protein was by the method of Lowry et al, (1951).

The percentage DNA in chromatin nucleic acid was determined after polyacrylamide gel electrophoresis for total nucleic acids.
<table>
<thead>
<tr>
<th></th>
<th>Initial procedure</th>
<th>Final procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>% DNA in chromatin nucleic acid</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Chromatin yield.</td>
<td>26.3</td>
<td>30.0</td>
</tr>
<tr>
<td>DNA extracted as % DNA present.</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Uncorrected 260/280nm absorption ratio.</td>
<td>1.75</td>
<td>1.67</td>
</tr>
<tr>
<td>Corrected 260/280nm absorption ratio.</td>
<td>1.53</td>
<td>1.75</td>
</tr>
<tr>
<td>320/260nm absorption ratio.</td>
<td>0.046</td>
<td>0.063</td>
</tr>
<tr>
<td>Protein : DNA ratio.</td>
<td>2.19 : 1</td>
<td>2.20 : 1</td>
</tr>
</tbody>
</table>
FIGURE 6.

U.V. absorption spectrum of purified artichoke tuber chromatin.

Chromatin from Experiment 13, (using the initial extraction procedure), is shown. Chromatin from experiments using the final extraction procedure showed similar U.V. absorption scans.
(A) **Scan of total nucleic acids from artichoke tuber after electrophoresis.**

(B) **Initial extraction procedure for artichoke tuber chromatin:**
   - scan of chromatin nucleic acids after gel electrophoresis.

(C) **Final extraction procedure for artichoke tuber chromatin:**
   - scan of chromatin nucleic acids after gel electrophoresis.

Gels electrophoresed for different periods of time.

18s RNA = transfer RNA.
proportion of DNA had increased from c. 8% in tuber total nucleic acid to c. 90% in the final chromatin extract. (Figure 7(a), (b).)

(ii) Final preparations

The only change to the previous method of extraction was the inclusion of a chromatin wash in EDTA + NaCl. In Experiment 14 the 'crude' chromatin pellet was split two ways, one sample being given an EDTA + NaCl wash, the other being washed once more in Tris-HCl.

The results obtained are presented in Table 2. With no EDTA + NaCl treatment the results, as expected, were very similar to those for Experiments 12 and 13. However, when this wash was included certain changes were seen; and these were repeatable; (Experiment 17, Table 2). There was a general increase in chromatin purity as evidenced by a halving of the Protein : DNA ratio and by the almost total absence of RNA in the samples. (Figure 7(c).) Unfortunately, this was not without some decrease in final yield, and there was a fall by one-third to a new value of about 17%.

The slight fall in the 260/280 nm absorption ratio I do not believe to be very significant since it occurred in both samples in Experiment 14. In no experiment did this ratio reach a value of greater than 1.83, (after correction for light scattering).

In all subsequent extractions I included the wash in EDTA + NaCl. Since the amount of tissue available was not a limiting factor here I thought it wise to use the method producing the cleanest chromatin, at the same time suffering the relative decrease in yield.
I should point out that, unlike the pea shoot chromatin extractions, there was no 'selection' of pellet material here after each centrifugation. This was because no starch was present.
D. Chromatin isolation and purification from artichoke explant tissue

When artichoke tubers were used for chromatin extraction approximately 50 - 70g of tissue was used. However, in any extraction from explants less than 10g of tissue was available. Consequently the extraction procedure used for tubers was found to be inadequate for explants. The chromatin was as clean as before, but yield was down to 10% (in real terms this means 10μg DNA from 1g of freshly cut explants). In order to obtain adequate quantities of chromatin in any single extraction it was necessary to increase this yield by making the extraction procedure less rigorous. The method finally used is described in Chapter 3. In general, the number of chromatin washes was reduced and the amount of centrifugation at each stage was increased. (However the wash in EDTA + NaCl was retained.) In this way the yield of chromatin was doubled, so that 1g of freshly cut explants now produced 20μg DNA. Consequently, the purity of the samples declined, with 260/280 nm and 320/280 nm absorption ratios in the order of 1.50 and 0.090 respectively, and with Protein : DNA ratios of about 2.2 : 1. There was also 5% contamination by RNA species.

(Where necessary, the values above have been corrected for the RNA content of the chromatin and for the effects of light scattering on the U.V. absorption spectra.)
E. Summary and discussion

Fairly good extraction procedures have been developed for pea shoot chromatin and artichoke tuber and explant chromatin. The sparsity of information concerning the purity of the initial extractions from pea shoot tissue was an oversight on my part.

Various questions can now be raised concerning the validity of the methods used for determining the purity of the chromatin samples, and indeed for extracting and purifying the chromatin in the first place.

The value of 2.0 proposed by Bonner et al., (1967), for a 260/280 nm absorption ratio for purified chromatin seems to be unrealistic. This is because a sample of pure DNA gives a ratio of only c. 2.0; (see Figure 3). Therefore, since chromatin contains proteins, (which absorb at 280 nm), the ratio must have a theoretical maximum of significantly less than 2.0. Probably a value of 1.7 - 1.8 is more realistic.

Another case for controversy is the correction for light scattering in the chromatin U.V. absorption scan. Although I know of no discussion of this in the literature, I feel that the correction is only really valid when the chromatin sample is very pure, and therefore it may have been incorrect to use it for some of my samples.

Where possible I have attempted to purify chromatin to a high degree in order to reduce contamination from other substances. However, it could be argued that this 'purification' may remove some of the less tightly bound chromatin components and therefore I
FIGURE 8.

U.V. absorption spectrum of purified calf thymus DNA.

The commercially-obtained DNA was dissolved in 1/3 SLS and scanned using a 'blank' of 1/3 SLS.

260/280 nm absorption ratio = c. 2.0.
may be selectively extracting certain fractions of the chromatin. This would be made worse by the fact that the more rigorous the extraction procedure the lower is the yield and the less 'representative' the sample may be. This problem is insoluble since there is no real definition of chromatin; however, it does emphasise the absolute requirement for a standardised procedure within any series of experiments. All further extractions were performed with this aim in view.
2. ISOLATION AND CHARACTERIZATION OF HISTONES

A. Pea shoot histones

(i) Extraction

Acid-extraction of histones from purified pea shoot chromatin was as described in Chapter 3. Lowry protein assays were used to determine the amount of basic protein extracted. Basic Protein : DNA ratios in the range 0.8 : 1 to 1.1 : 1 were found. Although this ratio varies with the tissue used, values of about 1.3 : 1 are generally considered to be reasonable. For pea shoot chromatin Huang and Bonner, (1965), obtained a Basic Protein : DNA ratio of 1.33 : 1. My results indicated a slightly lower yield.

(ii) Gel electrophoresis

The histone samples were electrophoresed on urea - acetic acid - 15% acrylamide gels. Figure 9 shows a scan of a total histone gel after staining and destaining. The banding pattern was very similar to that obtained by Fambrough et al, (1968), for histones from the same tissue. Labelling of the histone peaks was as in Fambrough et al, (1968); but using the new nomenclature proposed at the CIBA Foundation Symposium, (1974). Figure 10 shows photographs of typical gels.

The slowest-migrating histone type was H.1, composed of 3 subspecies H.1a, H.1b and H.1c. The fastest-migrating histone was H.4. Between these two there was a double peak comprising histones H.2A, H.2B and H.3. Under the reducing conditions used histone H.3, the
FIGURE 2

Scan of total histones from pea shoot tissue after polyacrylamide gel electrophoresis.

(Proteins stained with Coomassie Brilliant Blue R)
Fig 9

- OD 575 nm
- Mobility

H2+3
H4
H1a
H1b
H1c
H1d
Photographs of total histones from pea shoot tissue after polyacrylamide gel electrophoresis.

(A) Two gels from separate experiments. High histone loadings. (c. 30 - 40μg).

(B) One gel from a third experiment. Low histone loading. (c. 15 - 20μg).

(Proteins stained with Coomassie Brilliant Blue R.)
only histone containing a cysteine residue, was present as the monomer, migrating at approximately the same speed as histone H.2B. If the samples had been prepared, and the gels run, under non-reducing conditions histone H.3 would have been present as a dimer, and would have migrated at about the same rate as histone H.1c, (Fambrough et al, 1968).

It was of interest to observe that the histone H.1 subspecies were stained blue-red with Coomassie Blue, whereas the other histone fractions were all stained blue. Perhaps this is related to the fact that of all the histones, H.1 has by far the highest basic : acidic amino acid ratio.
B. Artichoke tuber and explant histones

(i) Extraction

Acid-extraction of tuber and explant histones was as described in Chapter 3. The double-extraction in 0.2N and 0.4N H₂SO₄ was replaced by a single extraction in 0.4N H₂SO₄ when explant chromatin was used. The intention here was to minimise loss by reducing the number of steps in the procedure. It was subsequently found that exactly the same yield of basic protein was obtained in the one-stage extraction as in the two-stage extraction.

For both artichoke tuber and explant tissues the Basic Protein : DNA ratio was in the range 0.7 : 1 to 1.2 : 1.

(ii) Gel electrophoresis

The histone samples were electrophoresed on urea - acetic acid - 15% acrylamide gels. Figure 11 shows a scan of a tuber total histone gel after staining and destaining. Explant total histone gels were identical to this. The banding pattern is similar to the pea shoot histone pattern but with the absence of the two faster-migrating histone H₁ subspecies, H₁b and H₁c. There were, however, two very small peaks near to where these subspecies would be. These two proteins, arbitrarily called A and B, may represent subspecies of histone H₁, and in fact they did show the same blue-red coloration as the main peak when stained with Coomassie Blue.

Figure 12 shows a photograph of tuber total histone gels. All the fractions mentioned above can be distinguished. Further details, (absent from the scans), can be observed. On the higher-loaded gel
FIGURE 11.

Scan of total histones from Jerusalem artichoke tuber after polyacrylamide gel electrophoresis.

(Proteins stained with Coomassie Brilliant Blue R.)
FIGURE 12.

Photograph of total histones from Jerusalem artichoke tuber after polyacrylamide gel electrophoresis.

The gels show different histone loadings and different periods of electrophoresis.

(Proteins stained with Coomassie Brilliant Blue R.)
histone H$_{4}$ appears to have a minor slower-migrating component. (This is best observed 'by eye' than in a photograph.) It could be an acetylated fraction of histone H$_{4}$, since it has been reported that this histone is frequently acetylated, (Sung and Dixon, 1970; Louie and Dixon, 1972). The central histone region, comprising histones H$_{2}$A, H$_{2}$B and H$_{3}$, appears to consist of 4 peaks, two faint outer ones and two dense inner ones. This is only noticeable on the lower-loaded gels. A possible explanation of this is that the slowest migrating band is a phosphorylated or an acetylated form of the second band, histone H$_{2}$A. The remaining two bands would be histones H$_{2}$B and H$_{3}$, in either order. Under the electrophoresis conditions used here, phosphorylated or acetylated protein fractions would be more negatively charged than their unmodified counterparts. They would therefore migrate at a slower speed. According to Chalkley et al. (1973), a single phosphate group would decrease the speed of migration by approximately 1%.

I must point out that the naming of the histone bands in the H$_{2}$ - H$_{3}$ region is purely arbitrary and that I have no data to support it, nor have I any evidence for the histone modifications I propose.

Another observation from the photographs is the presence of two or more bands within the main histone H$_{1}$ peak area. When this histone was electrophoresed to the lower end of the gels the various subfractions present could be distinguished. Figure 13 shows the histone H$_{1}$ scan patterns from tubers and explants in 1974. Figure 14 shows these patterns from the 1975 crop. In all cases there was a main-band with 2 or 3 slower-migrating subfractions.
FIGURE 13.

Scanning patterns of histone H.1 from Jerusalem artichoke tuber and explant material, (1976), after polyacrylamide gel electrophoresis.

Horizontal scale expansion of 9 : 1.

(Proteins stained with Coomassie Brilliant Blue R.)
Fig 13

Tuber (45% main band) vs. Auxin cultured explants (30% main band)

OD 575 nm

Mobility
Scan patterns of histone H.1 from Jerusalem artichoke tuber and explant material, (1975), after polyacrylamide gel electrophoresis.

Horizontal scale expansion of 9 : 1.

(Proteins stained with Coomassie Brilliant Blue R.)
Fig 14.

Tuber.
(48% Main-band.)

Auxin-cultured explants.
(43% Main-band.)

Control explants.
(52% Main-band.)

Absorbance, 575 nm.

Mobility
The percentage of the total area under the main-band was carefully calculated in each case, and was within the range 30-60%. To correct for a non-horizontal base-line in some scans the peak areas were determined after adjustment to a new base-line, (see Figure 15). It had previously been determined that Coomassie Blue did give quantitative staining of protein within the range used on these gels, (see Figure 16). Therefore, the percentage of the total area which was under the main-band did actually represent the percentage of histone H\textsubscript{1} within this region.

The observed heterogeneity in histone H\textsubscript{1} appeared to change from one year to the next; and, since the differences were so small, no real conclusions could be drawn concerning the effects on this heterogeneity of culturing explants in the presence or absence of auxin. No scan was obtained for histone H\textsubscript{1} from 1974 explants cultured in the absence of auxin. (By the time I overcame the yield problems here the new tuber crop was in use.)

The slower-migrating subfractions of histone H\textsubscript{1} may be phosphorylated, methylated or acetylated derivatives, or any combination of these. In these cases the metabolic state, or age, of the tubers used may also affect the heterogeneity. On the other hand there may simply be a variety of unmodified histone H\textsubscript{1} molecules differing in molecular weight or primary structure.

I conclude that in artichoke tubers and explants, histone H\textsubscript{1} exists in a heterogeneous state. The possibility that this heterogeneity is in part caused by phosphorylation has been investigated. The results of these experiments are presented in sections 3 and 4 of the Results.
Correction of a histone gel scan to a horizontal base-line.

The new base-line is first drawn. Absorbance values at each point are then reduced by the difference in absorbance between the old and new base-lines. A 'corrected' scan is now drawn through the new absorbance values.
Curve showing the relationship between intensity of histone H.1 staining after gel electrophoresis and the amount of total histone loaded. (Staining with Coomassie Brilliant Blue R.)

The proportion of histone H.1 in the total histone sample remained unchanged. Thus, for total histone loading of < 30μg staining of single-protein peaks, (i.e., of histones H.1 and H.4), was quantitative.
Fig 16

Area under histone H1 peak (arbitrary units)

Total Histone Loaded (μg)
(iii) Histone molecular weights

The molecular weights of artichoke tuber histones were determined by electrophoresis on SLS - 15% acrylamide gels, as described in Chapter 3. Molecular weight 'marker' proteins were run at the same time as total histone samples. In addition, the individual histone fractions were eluted off urea - acetic acid - 15% acrylamide gels and electrophoresed on SLS gels, at the same time as 'markers'.

The photographs presented as Figures 17(A) and (B) show typical gels from one experiment after staining and destaining. Figures 18(A)-(D) show scans of these gels. The percentage mobility of the molecular weight 'markers' was plotted against log molecular weight and the standard curve drawn - (see Figure 19). This was used to calculate the molecular weights of the histone fractions from their percentage mobilities. The results are presented in Table 3, together with molecular weight data, from the literature, on animal histones. To my knowledge there has been no published work on the molecular weights of histones from plant material.

Histone H4 had a molecular weight of c. 11,300 daltons. This is in very close agreement with animal histone data and lends support to the opinion that its primary structure is very highly conserved, (DeLange et al, 1969). I did not attempt to separate out and identify histones H2A, H2B and H3, instead I simply determined the approximate molecular weights of the slower - and faster - migrating halves of this histone region. The values obtained of 16,500 and 15,600 daltons respectively were slightly
Photographs of Jerusalem artichoke tuber histone gels and molecular weight 'marker' gels after co-electrophoresis.

(A) Left-hand gel:  - Molecular weight 'marker' proteins.
      Right-hand gel:  - Tuber total histones.

(B) Left-hand gel:  - Molecular weight 'marker' proteins.
      Centre gel:      - Tuber histone H₁.
      Right-hand gel: - Slower-migrating half of tuber histone H₂ + 3 band region.

* The photograph does not show this up.

(Proteins stained with Coomassie Brilliant Blue R.)
FIG. 17. (A)
FIGURE 18.

Scans of Jerusalem artichoke tuber histones and molecular weight 'marker' proteins after co-electrophoresis on polyacrylamide gels.

(A) Molecular weight 'marker' proteins.

(B) Tuber total histones.

(C) Slower-migrating half of tuber histone H$_2$ + 3 band region.

(D) Tuber histone H$_1$.

(Proteins stained with Coomassie Brilliant Blue R$_2$)
Fig 18

(A) 
12,400
14,000
17,000
43,000
65,000

(B) 
H.2 + 3

(C) 
slow-migrating half of H.2 + 3

(D) 
H.1
H.4

Absorbance, 575 nm.
Mobility
Graph of $\% \text{ mobility}$, (on polyacrylamide gels), of molecular weight 'marker' proteins against log$_e$ molecular weight.
TABLE 3.

The molecular weights of Jerusalem artichoke tuber histones, (and animal histones).

Values presented for tuber histones are the mean values from 'total histone' and 'single histone' gels.
### Table 3

<table>
<thead>
<tr>
<th>(A) Artichoke tuber histone</th>
<th>Molecular Weight, (daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H.1.</td>
<td>42,700</td>
</tr>
<tr>
<td>A.</td>
<td>32,500</td>
</tr>
<tr>
<td>B.</td>
<td>26,900</td>
</tr>
<tr>
<td>Slower-migrating part of H.2 + 3 region.</td>
<td>16,500</td>
</tr>
<tr>
<td>Faster-migrating part of H.2 + 3 region.</td>
<td>15,600</td>
</tr>
<tr>
<td>H.4.</td>
<td>11,300</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(B) Animal histone</th>
<th>Molecular Weight, (daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H.1.</td>
<td>19,000-22,000</td>
</tr>
<tr>
<td>H.2a.</td>
<td>14,500-15,500</td>
</tr>
<tr>
<td>H.2b.</td>
<td>13,000-14,000</td>
</tr>
<tr>
<td>H.3.</td>
<td>14,000-15,000</td>
</tr>
<tr>
<td>H.4.</td>
<td>11,000-12,000</td>
</tr>
</tbody>
</table>
higher than expected on the basis of the animal histone data. Histone H4 had a molecular weight of c. 42,700 daltons. This is approximately twice the value for animal histone H4. Because of the reducing conditions in the sample media, (that is, the presence of 2-mercaptoethanol), there is no reason to believe that dimerisation, or any other form of aggregation, has taken place. Therefore, this molecular weight is probably the molecular weight of a single histone H4 molecule, and would represent about 420 amino acids.

The finding that it is almost twice the size of the corresponding histone from animal tissues may or may not be significant. It could have resulted from a 'doubling-up' of an original sequence; however it may simply represent a general increase in the size of the histone in response to some selection pressure for structural or other reasons. Investigations on the primary structure of this molecule may, in the future, distinguish between these two possibilities.

The two 'new' histones A and B had molecular weights of c. 32,500 and 26,900 daltons respectively. If these are in fact subspecies of histone H4, then their presence could be in favour of the theory for a general increase in the size of the histone, they themselves representing metabolically-stable intermediate forms.

Although the molecular weights of plant histones have not previously been determined a few researchers have presented photographs or scans of plant histone gels. In all cases where electrophoresis has been alongside animal histones plant H4 migrates much slower than animal H4, and indeed plant H2A, H2B and H3 all migrate slightly slower than their animal counterparts.
(See, for example, Spiker, 1975.) Despite the fact that electrophoresis in these cases is on urea – acetic acid – acrylamide gels, (which separate molecules by charge as well as by size), these findings point strongly to molecular weight differences similar to those I have presented in this section.
C. Summary and discussion

Pea shoot histones were extracted from purified chromatin and electrophoresed on acrylamide gels. The scans were identical to data produced by Fambrough et al., (1968), from the same tissue. This assured me that the histone extraction and electrophoresis procedures adopted were quite valid. No further work was done on pea shoot histones although, in retrospect, it would have been interesting to determine the molecular weights of the various fractions, particularly the H₁₁ subspecies.

Artichoke tuber and explant histones were extracted and the various components identified by acrylamide gel electrophoresis and comparison with pea shoot histone gels. The molecular weights of the tuber histones were determined. Not only was histone H₁₁ heterogeneous in nature but it had twice the molecular weight expected from data on animal histones. Possible causes of these two phenomena have been discussed.

The findings that histone H₁₁ differs in components between pea shoot and artichoke tuber, and that its molecular weight in plants is probably much higher than in animals, add to the widely expressed belief that this histone is species- and tissue-specific and is therefore the least conserved of all the histones.

The other histone fractions were fairly similar in molecular weight and mobility on gels to their counterparts from animal tissues, (particularly histone H₁₄). This apparent conservation of primary structure is as expected since these four histones are probably concerned with maintenance of the chromatin ultrastructure,
Because further work was to be concerned with phosphorylation of histone H1 I had to be reasonably certain that my identification of H1 was correct. The following characteristics were in agreement with the identification:

1. The protein is present in purified chromatin.
2. It is acid-extractable, and therefore has basic properties.
3. It migrates on acrylamide gels in a similar manner to pea shoot histone H1.
4. Its staining behaviour with Coomassie Brilliant Blue R is similar to that of pea shoot histone H1.
5. Its molecular weight is higher than that of the other acid-extractable chromatin proteins.
6. It is the only acid-extractable chromatin protein showing a degree of species- or tissue-specificity.
3. HISTONE H\textsubscript{1} - PHOSPHATE. ENZYMATIC DATA FROM NON-LABELLED ARTICHOKE TUBER AND EXPLANT TISSUES

A. Introduction

Histone H\textsubscript{1} from artichoke showed a number of slower-migrating fractions after gel electrophoresis. It was possible to use the enzyme phosphatase to study whether or not these fractions were phosphorylated derivatives of the main-band. If they were in fact phosphorylated derivatives, and if dephosphorylation was accomplished by incubation with enzyme, then it would be expected that the proportion of protein in the slower fractions would decrease relative to the main-band.

In these experiments samples of total histones were incubated in the presence or absence of phosphatase as described in Chapter 3, and then electrophoresed on urea - acetic acid - 15\% acrylamide gels to determine the amount of protein within each of the histone H\textsubscript{1} subfractions. The gels were stained with Coomassie Blue and, after destaining, the relative peak areas determined. With the amounts of histone used here the staining was quantitative and therefore the relative peak areas did represent relative amounts of protein in each fraction.
B. Enzymatic dephosphorylation of tuber histone H₁₁

Histone H₁₁ from the 1974 tuber crop consisted of 45% main-band protein, with the remainder being present as 2 slower-migrating fractions. After a 22-hour incubation of histone in alkaline phosphatase at 37°C there was a marked change in the relative amounts of protein in these 3 peaks. (Figures 20(A) & (B).) The slower-migrating fractions decreased from 55% to 38% of the total histone. This represents a 31% decrease in these 2 fractions. There was no effect on the histone subfractions in the control samples, incubated in Tris-HCl alone. Similar results were obtained whether incubation was in alkaline phosphatase pH optimum 8.0 or alkaline phosphatase pH optimum 10.4. This was, therefore, a preliminary indication of the presence of phosphorylated species within the tuber histone H₁₁.

After 40 hours of enzyme incubation there was no further decrease in the proportion of histone in the slower-migrating fractions. This remaining protein may or may not be phosphorylated. If the former state is true then we must assume that for structural reasons the enzymes used cannot bring about dephosphorylation. If the latter is true then the remaining heterogeneity in histone H₁₁ may be due to acetylation or methylation of protein molecules, or to the presence of unmodified H₁₁ molecules differing from the main-band in molecular weight.

When acid phosphatase, pH optimum 4.8, was used on histone from the 1974 crop there was no change in the histone H₁₁ gel pattern. This may be due to structural or conformational problems.
Scan patterns of alkaline phosphatase-incubated and Tris-incubated histone H1 from Jerusalem artichoke tuber, (1974), after polyacrylamide gel electrophoresis.

Horizontal scale expansion of 9 : 1.

(Proteins stained with Coomassie Brilliant Blue R.)
Fig 20(A)

Phosphatase-incubated
(32% Main-band)

Tris-incubated
(45% Main-band)

Absorbance, 575nm.
Photograph of alkaline phosphatase-incubated and Tris-incubated histone H1 from Jerusalem artichoke tuber, (1974), after polyacrylamide gel electrophoresis.

Left-hand gel: - Phosphatase-incubated. Top band region is the phosphatase. Bottom band region is histone H1.

Right-hand gel: - Tris-incubated. Lower band region is histone H1.

(Proteins stained with Coomassie Brilliant Blue R.)
Since these enzymes are all heterologous enzymes this would not be an unreasonable explanation.

When alkaline phosphatase incubation was carried out on histones from the 1975 and 1976 tuber crops there was no significant change in the histone H.1 heterogeneity. (Figure 21(A).) After repeating these experiments, and after using fresh enzyme stocks, there was still no apparent effect on the histone. In these tuber crops it seemed that, unlike the previous one, there were no phosphatase-labile subfractions of histone H.1. This reveals an apparent biochemical difference between successive crops of artichoke tubers. Differences of this nature may be due to factors such as degree of dormancy or age of the stored tubers.

Under the non-reducing incubation conditions used it may be expected that histone H.3 molecules would dimerise across their cysteine residues. If the subsequent reduction with 2-mercaptoethanol, prior to electrophoresis, is not completely effective then there may be a proportion of the dimers remaining. Figure 21(B) shows gels of 1976 tuber histone H.1 for incubated and non-incubated samples. It is seen that the incubated samples have a protein band migrating slightly slower than the histone H.1 band, and this is practically absent in the non-incubated sample. This band may be the histone H.3 dimer; although from my molecular weight data it would probably be expected to migrate just faster than histone H.1. If it is indeed the dimer then I must consider that the reducing action of 2-mercaptoethanol either cannot completely break the cystine linkage here, or that it takes a few
Scan patterns of alkaline phosphatase-incubated and Tris-incubated histone H.1 from Jerusalem artichoke tuber, (1975), after polyacrylamide gel electrophoresis.

Horizontal scale expansion of 9 : 1.

(Proteins stained with Coomassie Brilliant Blue R.)
Fig 2(A)

Tuber. (Phosphatase-incubated.)
(47% Main-band.)

Tuber. (Tris-incubated.)
(48% Main-band.)
**FIGURE 21(B)**.

Photograph of alkaline phosphatase-incubated, Tris-incubated and non-incubated histone H1 from Jerusalem artichoke tuber, (1976), after polyacrylamide gel electrophoresis.

Left-hand gel: - Tris-incubated.
Centre gel: - Phosphatase incubated. Top band region is the phosphatase.
Right-hand gel: - Non-incubated.

In each case the lowest band region is Histone H1.

(Proteins stained with Coomassie Brilliant Blue R.)
hours for total reduction to take place. In these experiments, after termination of incubation, (and addition of 2-mercaptoethanol), the samples were left for only 1 - 2 hours before gel electrophoresis.
C. Enzymatic dephosphorylation of explant histone H1

The explants used for these experiments were 3 - 4 days old, and therefore the cells in the auxin-cultured explants should have been in asynchronous division. Before proceeding it was first necessary to ensure that the auxin was having the expected effect on the cultured explants. Cell number and DNA determinations were carried out for freshly-cut and 3-day old explants cultured in the presence and absence of 2,4-D. In explants cultured without auxin there was no significant increase in cell number or DNA content over the time period used. However, the explants cultured in the presence of auxin showed a 3-fold increase in cell number, (16,700 - 53,000 cells per explant), and a similar increase in DNA, (0.6 - 1.6 µg per explant). These results confirmed the expected auxin effect of cell division.

I have no data on explant histone H1 phosphorylation from the 1974 tuber crop since the histones were almost completely broken down on incubation, (see part D of this section). However, using larger amounts of histone it was possible to study the effects of phosphatase incubation on histone from explants of the 1975 tuber crop. Figure 22 shows gel scans of histone H1 from explants cultured in the presence and absence of the auxin 2,4-D. In each case the samples were incubated in the presence and absence of alkaline phosphatase. In the control explants there was 52% main-band histone after Tris- HCl incubation, and there was virtually no additional change in the phosphatase-incubated samples. In the auxin-cultured explants phosphatase-incubated histone H1 did show
FIGURE 22.

Scan patterns of alkaline phosphatase-incubated and Tris-incubated histone H1 from Jerusalem artichoke tuber explants, (1975), after polyaerylamide gel electrophoresis.

Auxin-cultured and control explants.

Horizontal scale expansion of 9 : 1.

(Proteins stained with Coomassie Brilliant Blue R.)
Fig 22

Auxin-cultured explants.
(Phosphatase-incubated.)
(58% Main-band.)
(Tris-incubated.)
(48% Main-band.)

Control explants.
(Phosphatase-incubated.)
(53% Main-band.)
(Tris-incubated.)
(52% Main-band.)

Absorbance, 575 nm.
Mobility
a small decrease in the proportion of protein in the slower-
migrating fractions when compared to the Tris-HCl-incubated
sample. This decrease from 52% to 44% represented a loss in these
fractions of 15.4%. The same results were obtained with each of
the alkaline phosphatase enzymes. Acid phosphatase was not used in
these experiments.

Thus, it appears that in 2,4-D-cultured, (dividing), explants
there is a small proportion of phosphatase-labile histone H1, whereas in control, (non-dividing), explants there is none. The
differences involved, however, are very small and may not be
significant. If there is indeed a 'division-related' phosphoryla-
tion of this histone it would need to be confirmed by radioactive
labelling experiments. Using the artichoke explant culture system
it should also be possible to distinguish between phosphorylation
related to histone synthesis and phosphorylation not related to
synthesis. These labelling experiments are described in section 4
of the Results.
D Breakdown of histones on incubation

With explant histones it was found initially that there were large losses of protein in the phosphatase-incubation experiments. This apparent breakdown was investigated in time-course experiments using tuber histones, and then histones from auxin-cultured explants.

Samples of equal histone content were incubated for different lengths of time. They were then electrophoresed on urea - acetic acid - 15% acrylamide gels and the amounts of histone determined by staining with Coomassie Blue. The results were approximately the same irrespective of the tissue-source of the histone, the pH used, (8.0 or 10.4), and the presence or absence of alkaline phosphatase. Figure 23 shows graphs of the time-course of breakdown of histones H1 and H4 in a typical experiment. The mean of the phosphatase-incubated and Tris-HCl-incubated samples is plotted for each sample time. In this experiment each tube contained initially 25μg of total histone. After 6 hours of incubation approximately half of each of the histones had been broken down. This initially high rate of breakdown gradually declined with time. Here c. 30%, (1.5μg), and c. 43%, (2.0μg), of histones H1 and H4 respectively were left after 22 hours of incubation. Thus it is easily seen how an initially low yield of histone could be almost completely lost during a phosphatase-incubation experiment.

Time-course experiments carried out using auxin-cultured explants from the 1975 tuber crop indicated that a 6-hour incubation was not sufficient for the 'phosphatase effect' to be
FIGURE 23.

(A) **Time-course of histone H$_1$ breakdown during incubation at 37°C in alkaline pH.**

(B) **Time-course of histone H$_4$ breakdown during incubation at 37°C in alkaline pH.**
Incubation time (hours).

% of initial Histone H.1.

Histone H.1

Incubation time (hours).

% of initial Histone H.4.

Histone H.4
seen, although it was seen in the 22-hour samples. Therefore, it was impossible to improve final yield in these experiments by shortening the incubation time to less than 6 hours, and consequently in subsequent experiments it was necessary to allow for breakdown.

This histone breakdown on incubation was most likely by enzyme activity. I can only presume that it is caused by an acid-soluble protease which is active in alkaline pH. The source of the protease may be the chromatin itself, but could be contaminating cell wall material.
E. Summary and discussion

The results presented in this section show that in some tuber crops the slower-migrating histone H.1 subfractions may contain phosphorylated proteins. The presence of these phosphoproteins appears to vary between crops and may indicate in some way the metabolic state of the stored tubers. This electrophoretic heterogeneity within the tuber histone H.1 is probably not due solely to phosphorylation of some of the molecules, but may also reflect different histone modifications and/or the presence of different unmodified H.1 subfractions.

There is some evidence to suggest that histone H.1 from auxin-cultured explants contains phosphorylated subfractions, whereas the histones from non-dividing explants contain none. The differences here, however, are very small and may not be significant. $^{32}$P-phosphate labelling experiments must be carried out on explant material before any conclusions can be drawn.

The breakdown of histones is an unfortunate consequence of the incubation conditions used in these experiments, and very little could be done to prevent it. I had considered treating the initial chromatin samples with the specific protease inhibitor phenylmethylsulphonyl fluoride, (PMSF), as recommended by Chong et al, (1974), and Ballal et al, (1975); however I considered that the loss due to the extensive dialysis required would be no less than loss due to protease activity on incubation. The source of the protease may be the chromatin itself since it was present in tuber histone extracts, (which were prepared from highly purified...
chromatin), but cell wall contaminants cannot be ruled out as a possible source.

It is unlikely that activity of the protease has caused any of the apparent 'dephosphorylations' attributed to phosphatase activity since it would also be present in the control tubes. A more serious criticism of the results would be that the phosphatase itself has specific proteolytic activity, and that it has not dephosphorylated the histone H1 subfractions but selectively broken them down.
With the aid of radioisotopes the artichoke tuber explant culture system, (Yeoman et al, 1965), was used to study histone synthesis and phosphorylation in asynchronously-dividing cells, and in the first division cycle of synchronously-dividing cells. In the latter experiments emphasis was placed on histone H.1 in order to investigate the possibility of a phosphorylation, (not directly related to synthesis), during the G2/early M-phase of the cell-cycle, (Gurley et al, 1973; Bradbury et al, 1974a, 1974b). In each experiment the control was usually non-dividing tissue of the same age as the dividing tissue. The method of explant preparation is described in Chapter 3. The culture medium for these experiments contained no phosphate.
SUB-SECTION 1. EXPERIMENTS ON ASYNCHRONOUSLY-DIVIDING TISSUE

A. Histone synthesis and phosphorylation in asynchronously-dividing tissue

Artichoke tuber explants were cultured for 3 days in the presence or absence of the auxin 2,4-D. 750μCi 32P-phosphate and 25μCi 3H-lysine were added to each flask and the tissue cultured a further 4 hours. Histones were extracted and electrophoresed on urea - acetic acid - 15% acrylamide gels. The gels were stained, destained and scanned. They were then sliced into 1.0mm lengths and the incorporated labels determined by liquid scintillation counting.

Figure 24 shows a gel scan of total histones from auxin-cultured explants, together with counting profiles for the two radioisotopes. Figure 25 presents similar data from control explants. Histones from dividing tissue show significant incorporation of both phosphate and lysine, whereas histones from non-dividing tissue show only low incorporation. These results are as expected since cells in division need to synthesise histones to keep pace with DNA synthesis, and previous data of my own did indicate an increase in phosphorylation in at least 2 histone fractions during auxin-induced division. Turnover of histones in non-dividing tissue would probably account for the incorporation in control explants, (Byvoet, 1966).

The peaks of phosphate incorporation are slightly masked by the presence of a background of 32P counts in the gels, decreasing
Scan of total histones from 3-day old auxin-cultured explants after polyacrylamide gel electrophoresis, and the associated counting profiles for $^3$H-lysine and $^{32}$P-phosphate.

Labelling of explants was for 4 hours, with 1.56 and 47 μCi/ml of $^3$H-lysine and $^{32}$P-orthophosphate respectively.

(Background counts subtracted.)

(Proteins stained with Coomassie Brilliant Blue R.)
Scan of total histones from 3-day old control explants after polyacrylamide gel electrophoresis, and the associated counting profiles for $^3$H-lysine and $^{32}$P-phosphate.

Labelling of explants was for 4 hours, with 1.56 and 47.5 μCi/ml of $^3$H-lysine and $^{32}$P-orthophosphate respectively.

(Background counts subtracted.)

(Proteins stained with Coomassie Brilliant Blue R.)
Fig 25

- H₂ + 3
- H₂₁
- H₂₄

O.D. 575 nm

[^3H]-lysine incorporation (cpm)

[^32P]-phosphate incorporation (cpm)

Mobility
from the top. This could be caused by slow-migrating contaminants. The protein migrating slightly faster than histone H1 may be an example of this. (In the latter case, histone H1 could be identified by its colour after Coomassie Blue staining.) The effects of the \(^{32}\)P background were minimised in later experiments by electrophoresing the histones further down the gels.

In the dividing tissue the 2 incorporation peaks for the histone H2 + 3 peak area appear to show maxima at different places. This was investigated in a further experiment. Figure 26 shows the histone scan and label incorporation data. The lysine incorporation peak follows the shape of the scan, indicating a general synthesis of all the fractions. However, phosphate incorporation shows a peak on the slower-migrating side of the histone region. It appears, therefore, that a protein fraction in this area is being phosphorylated more, or at a faster rate, than the other fractions. This fraction may be either histone H2A or histone H2B. There are reports in the literature of each of the fractions being phosphorylated to some degree in dividing tissue, (Sung et al, 1971; Gutierrez-Cernosek and Hnilica, 1971).

It was not possible to adequately study the histone H1 incorporation peaks on total histone gels, (Figures 24 and 25), because of the high 'background' of counts from contaminating proteins. Therefore, histone H1 was electrophoresed to the lower end of the gels in all investigations on this protein. Gel scans and incorporation data for histone H1 from dividing and non-dividing tissue are presented in Figures 27 and 28 respectively.
Scan of the histone H2 + 3 band region from 3-day old auxin-cultured explants after polyacrylamide gel electrophoresis, and the associated counting profiles for $^3$H-lysine and $^{32}$P-phosphate.

Labelling of explants was for 4 hours, with 1.56 and 47 $\mu$Ci/ml of $^3$H-lysine and $^{32}$P-orthophosphate respectively.

(Background counts subtracted.)

(Proteins stained with Coomassie Brilliant Blue R.)
Scan of histone H1 from 3-day old auxin-cultured explants after polyacrylamide gel electrophoresis, and the associated counting profiles for $^3\text{H}$-lysine and $^{32}\text{P}$-phosphate.

Labelling of explants was for 4 hours, with 1.56 and 47 μCi/ml of $^3\text{H}$-lysine and $^{32}\text{P}$-orthophosphate respectively.

(Background counts subtracted.)

(Proteins stained with Coomassie Brilliant Blue R.)
Scan of histone H.1 from 3-day old control explants after polyacrylamide gel electrophoresis, and the associated counting profiles for $^3$H-lysine and $^{32}$P-phosphate.

Labelling of explants was for 4 hours, with 1.56 and 4.7 $\mu$Ci/ml of $^3$H-lysine and $^{32}$P-orthophosphate respectively.

(Background counts subtracted.)

(Proteins stained with Coomassie Brilliant Blue R.)
There is greater synthesis and phosphorylation in the dividing tissue, (as already indicated in the total histone gels). However, the non-dividing tissue still shows a significant incorporation of both labels. This would be in part due to turnover of the histones, but there may also be the effects of a very small amount of division.

The $^3$H-lysine and $^{32}$P-phosphate incorporation peaks are more or less coincident over the histone H$\alpha$ peak area. Since the rates of the incorporation processes in the different subfractions are unknown I cannot discuss the possibility, expressed in Section 3 of the Results, that the slower-migrating subfractions are more phosphorylated than the main-band.

It can be concluded that all fractions of histone H$\alpha$ were being synthesised and phosphorylated to some degree, and that this was predominantly in the dividing tissue.
B. Effect of alkaline phosphatase incubation on incorporated $^{32}$P-phosphate

Labelled histones from 3-day old auxin-cultured explants were subjected to alkaline phosphatase incubation, (pH 8.0). This was to ensure that the $^{32}$P-phosphate was incorporated and not just non-specifically attached to the proteins.

Figure 29 shows the label incorporation peaks for a non-incubated total histone sample. Figures 30 and 31 show similar data for phosphatase-incubated and Tris-HCl-incubated total histone samples respectively. Histone-associated $^{32}$P-lysine is not affected by the incubations. However, there is a marked decrease in histone-associated $^{32}$P-phosphate in the phosphatase-incubated sample. This decrease is in both the histone H4.2 + 3 peak area and in histone H4.1. In the latter case the differences are small and may have been affected by the $^{32}$P gel background mentioned before. It was therefore necessary to electrophoreze this histone further down the gels, and to use a larger amount of extract. This was done in a separate experiment. The histone scans and $^{32}$P-phosphate incorporation traces are presented in Figures 32(A)(B). $^{32}$P-phosphate associated with histone H4.1 is almost completely removed by the incubation in phosphatase.

These results indicate that the bulk of the histone-associated phosphate is indeed incorporated phosphate. In Figures 30 and 32(B) it can be seen that phosphatase-incubation does not completely remove all the $^{32}$P-phosphate in the histone regions. This may be because there is some non-specifically attached
Scan of total histones from 3-day old auxin-cultured explants after polyacrylamide gel electrophoresis, and the associated counting profiles for $^{3}H$-lysine and $^{32}P$-orthophosphate.

(This differs from Figure 24 in that it is the non-incubated control in a phosphatase-incubation experiment. See Figures 30 and 31).

Labelling of explants was for 4 hours, with 1.56 and 47 μCi/ml of $^{3}H$-lysine and $^{32}P$-orthophosphate respectively.

(Background counts subtracted.)

(Proteins stained with Coomassie Brilliant Blue R.)
Scan of alkaline phosphatase-incubated total histones from 3-day old auxin-cultured explants after polyacrylamide gel electrophoresis, and the associated counting profiles for $^3$H-lysine and $^{32}$P-phosphate.

Labelling of explants was for 4 hours, with 1.56 and 4.7 $\mu$Ci/ml of $^3$H-lysine and $^{32}$P-orthophosphate respectively.

(Background counts subtracted.)

(Proteins stained with Coomassie Brilliant Blue R.)
Scan of Tris-incubated total histones from 3-day old auxin-cultured explants after polyacrylamide gel electrophoresis, and the associated counting profiles for $^3$H-lysine and $^{32}$P-phosphate.

Labelling of explants was for 4 hours, with 1.56 and 47,000 Ci/ml of $^3$H-lysine and $^{32}$P-orthophosphate respectively.

(Background counts subtracted.)

(Proteins stained with Coomassie Brilliant Blue R.)
Scan of Tris-incubated histone H1 from 3-day old auxin-cultured explants after polyacrylamide gel electrophoresis, and the associated counting profile for $^{32}\text{P}$-phosphate.

Labelling of explants was for 4 hours, with 47 $\mu$Ci/ml of $^{32}\text{P}$-orthophosphate.

(Background counts subtracted.)

(Protein stained with Coomassie Brilliant Blue R.)
**FIGURE 32(b).**

Scan of alkaline phosphatase-incubated histone H₁ from 3-day old auxin-cultured explants after polyacrylamide gel electrophoresis, and the associated counting profile for $^{32}$P-phosphate.

Labelling of explants was for 4 hours, with 47 μCi/ml of $^{32}$P-orthophosphate.

(Background counts subtracted.)

(Protein stained with Coomassie Brilliant Blue R.)
phosphate, but is most probably caused by incorporation into sites unavailable to the enzyme. (This has also been suggested in Section 3 of the Results.)
C. Isolation and determination of serine phosphate and threonine phosphate residues from labelled histones

In these experiments only dividing, (auxin-cultured), explants were used. The explants were cultured for 3 days, then labelled for 4 hours with 750μCi 32P-phosphate per flask, prior to histone extraction. To confirm the presence of phosphorylated amino acid residues the histone H1 and H2 + 3 peak areas were eluted off urea-acetic acid - 15% acrylamide gels and hydrolyzed in 6N HCl. The hydrolysates were concentrated and subjected to paper electrophoresis with 'markers' of serine phosphate, threonine phosphate and inorganic phosphate. The paper was cut into strips and the 32P counts in each strip determined. All methods used are described fully in Chapter 3.

Figures 33 and 34 present the paper electrophoresis results for experiments on histone H1 and on the H2 + 3 histone region respectively. In the hydrolysed histone H1 sample there is a 3 : 1 ratio of serine 32P-phosphate to threonine 32P-phosphate.

Calf thymus histone H1 contains 6.7% serine and 5.4% threonine. If these proportions are approximately the same in artichoke tuber histone H1 then it would appear that the serine residues are being phosphorylated to a greater extent, or at a faster rate, than the threonine residues. Naturally, this interpretation would depend upon the acid lability of the 2 amino acid phosphates being approximately the same. On structural grounds there is no reason to believe that they differ very much. According to Kabat, (1971), the conditions used should represent just less than one half-life.
FIGURE 33.

$^{32}$P-phosphate counting profile after paper electrophoresis of acid-hydrolysed histone H1 from 3-day old auxin-cultured explants.

Positions of 'marker' phosphates shown. These were visualised by spraying with phosphomolybdate reagent followed by irradiation with ultra-violet light.

Labelling of explants was for 4 hours, with 47 μCi/ml of $^{32}$P-orthophosphate.

(Background counts subtracted.)
Fig 33

Mobility -

Origin

Threonine phosphate
Serine phosphate
Inorganic phosphate

32P counts/min

200
400
600
800
1000
\[^{32}\text{P}\]-phosphate counting profile after paper electrophoresis of the acid-hydrolysed histone H.2 + 3 region from 3-day old auxin-cultured explants.

Positions of 'marker' phosphates shown. These were visualised by spraying with phosphomolybdate reagent followed by irradiation with ultra-violet light.

Labelling of explants was for 4 hours, with 47 μCi/ml of \[^{32}\text{P}\]-orthophosphate.

(Background counts subtracted.)
Fig 34

Origin

Threonine phosphate  Serine phosphate  Inorganic phosphate

32P counts/min

Mobility
of serine phosphate, (and approximately the same for threonine phosphate). This means, therefore, that two-thirds of the 
$^{32}$P-labelled inorganic phosphate is unaccounted for, since this peak contains 3 times as much label as the sum of the other 2 peaks. The presence of acid-labile amino acid-phosphate linkages in the protein may explain this additional phosphate. (These linkages would have been broken down immediately on addition of the $6\text{M HCl}$.) Acid-labile lysine and histidine phosphate linkages have been reported by Smith et al, (1973), and Chen et al, (1975), in work on animal tissues. The only other explanation for the presence of this excess of labelled inorganic phosphate is that it may have been present in the protein as non-specifically attached phosphate.

In the hydrolysed sample of the 2 + 3 peak area there is a $15:1$ ratio of serine $^{32}$P-phosphate to threonine $^{32}$P-phosphate. Together calf thymus histones H.2A and H.2B contain approximately 8.0% serine and 5.5% threonine. If this is also the case in artichoke tuber then, as in histone H.1, it would appear that the serine residues are being phosphorylated to a greater extent, or at a faster rate, than the threonine residues. Again, two-thirds of the labelled inorganic phosphate is unaccounted for, (since this peak contains 3 times as much label as the sum of the other 2 peaks).

From these results I can conclude that at least part of the histone phosphorylation observed is due to the formation of specific amino acid-phosphate residues, in the form of serine phosphate and threonine phosphate.

In the following experiments I attempted to investigate the
possibility that there were acid-labile amino acid-phosphate linkages in the histones and to see if these accounted for the 'excess' labelled inorganic phosphate observed in the previous experiments. Enzymatic hydrolysis of the histone fractions was attempted to prevent breakage of the amino acid-phosphate linkages. If all the phosphate present was in fact bound to amino acids it would be expected that no labelled inorganic phosphate would be detected after paper electrophoresis of the hydrolysed samples.

Figures 35 and 36 present the paper electrophoresis results for these experiments on histone H4 and on the H2 + 3 histone region respectively. In both cases the enzymes failed to hydrolyse the proteins, since no labelled serine and threonine phosphate residues were detected, and the 32P-phosphate remained at the origin, on proteins or peptides. However, an interesting observation is the absence of any significant 32P-labelled inorganic phosphate. This indicates that there was probably no non-specifically attached phosphate in these proteins.

I can conclude that in each of the acid-hydrolysed samples one-third of the labelled inorganic phosphate came from breakdown of largely acid-stable serine and threonine phosphate linkages, and the remaining two-thirds probably came from the total breakdown of acid-labile amino acid-phosphate linkages. Therefore, in the unhydrolysed histones there must have been an approximately 1 : 1 ratio of acid-labile to acid-stable amino acid-phosphates.
Figure 25.

$^{32}$P-phosphate counting profile after paper electrophoresis of enzyme-hydrolysed histone H1 from 3-day old auxin-cultured explants.

Positions of 'marker' phosphates shown. These were visualised by spraying with phosphomolybdate reagent followed by irradiation with ultra-violet light.

Labelling of explants was for 4 hours, with 47 μCi/ml of $^{32}$P-orthophosphate.

(Background counts subtracted.)
Fig 35

Origin

Threonine phosphate  Serine phosphate  Inorganic phosphate

32P counts/min

Mobility
FIGURE 36.

$^{32}$P-phosphate counting profile after paper electrophoresis of the enzyme-hydrolysed histone H$_{2}$ + 3 region from 3-day old auxin-cultured explants.

Positions of 'marker' phosphates shown. These were visualised by spraying with phosphomolybdate reagent followed by irradiation with ultra-violet light.

Labelling of explants was for 4 hours, with 47 µCi/ml of $^{32}$P-orthophosphate.

(Background counts subtracted.)
D. Summary and discussion of labelling experiments on asynchronously-dividing tissue

These experiments showed that in dividing explants there was histone phosphorylation and general histone synthesis. The phosphorylation was mainly of histone H₁ and a protein within the histone H₂ + 3 region. In non-dividing explants there was much less histone synthesis and phosphorylation. This may be due to turnover, as well as perhaps some small amount of division.

The phosphorylation of histones in dividing tissue was shown to be largely phosphatase-labile and was therefore probably due to the formation of specific amino acid-phosphate linkages. This was confirmed by acid hydrolysis of the phosphorylated regions, followed by the identification of serine $^{32}$P-phosphate and threonine $^{32}$P-phosphate.

The absence of non-specifically bound inorganic phosphate was indicated by the results of enzymatic hydrolysis experiments, and it was inferred that approximately half of the incorporated $^{32}$P-phosphate present in these histones was in the form of acid-labile residues.

Unfortunately, the presence of acid-labile amino acid phosphates could not be confirmed by the enzymatic hydrolysis of the histones. The failure of this hydrolysis, however, is not too surprising since the methods had previously only been used on animal histone H₁.

If sufficient time had been available, it would have been interesting to study the amino acid phosphates in histones from...
non-dividing explants. Langan and Hohmann (1975), have shown for rat liver that in histone H1 threonine phosphate is only present in dividing tissues, whereas serine phosphate is present in both dividing and non-dividing tissues. This could have been followed by similar studies on the products of N-Bromosuccinimide cleavage of histone H1 to determine any possible difference in the amino acid phosphates from the two fragments.

A criticism of the methodology could be that very little is known about what is happening in the control explants. Therefore, although they contain cells which are essentially non-dividing, as true 'controls' should, they may be undergoing metabolic changes which are not occurring in the dividing explants. This would not affect interpretations of results from dividing tissues, but would detract from comparisons between dividing tissues and their non-dividing counterparts. A further problem is that only about half of the cells in auxin-cultured explants actually divide. Nothing is known about the metabolism of the non-dividing cells, or of its possible effects on the results.
SUBSECTION 2. EXPERIMENTS ON SYNCHRONOUSLY-DIVIDING TISSUE.

(CELL-CYCLE EXPERIMENTS)

A. Determination of phases in the first division cycle

The first synchronous division of explants cultured in the presence of 2,4-D was studied to determine the timing and approximate lengths of the various phases in the cell-cycle. Two parameters were used: $^3$H-thymidine incorporation by DNA, and cell number per explant. Labelling was with 10uCi $^3$H-thymidine per flask for 90 minutes prior to sampling. The explants were cultured for up to 40 or 50 hours. At intervals, two flasks were removed and 5 explants were taken from each for cell number determination. DNA was extracted from the remaining explants in each flask and measured by the diphenylamine reaction. The specific activity of each DNA sample was determined by liquid scintillation counting of an aliquot.

Experiment 1. (Using auxin-cultured explants only)

Figure 37 shows the specific activity of extracted DNA through a 40-hour culture period. There appears to be an S-phase peak at 20-24 hours from the beginning of the incubation. The second peak, at 36 hours, probably represents the following S-phase. It is known that after the first division cycle the cells become less synchronous, (Yeoman and Evans, 1967). This would, therefore, explain the lower second peak and the indication that after 40 hours the specific activity will level off at perhaps 50-60% of the first peak value.
Specific activity of $^3$H-thymidine-labelled DNA during growth of auxin-cultured explants. ('Experiment 1')

Duplicate samples of explants were taken at intervals of 4 hours. The two values for each time are plotted, together with the mean.

Labelling was for 1.5 hours at a concentration of 0.96 $\mu$Ci/ml of $^3$H-thymidine.

No correction is made for uptake of the label by the tissue.

(Background counts subtracted.)
Fig 37

3 H-thymidine incorporation (cpm/µg DNA)

Time in culture (hours)
Figure 38 presents the cell number data. There is a relatively large variation between duplicate samples. However, the increase between 24 and 28 hours is substantial, and probably represents the completion of the first division. A trend to asynchrony is again observed in that after 28 hours there is a steady increase in cell number per explant.

When these two figures are superimposed, Figure 39, the approximate timing of the phases in the first division cycle can be determined. From 0-16 hours after initiation of the cultures there is little or no incorporation of $^{3}H$-thymidine into DNA; this represents the G.1-phase, (or putative G.1-phase). Next comes an S-phase from 16-24 hours, followed by a G.2/M-phase from 24-28 or 30 hours. Because of the close proximity of the $^{3}H$-thymidine incorporation peak and the cell number increase distinction between S, G.2 and M is not very clear. All that can be said is that from 16-22 hours there is an 'S-rich' cell population, and from 24-28 hours there is a 'G.2/M-rich' cell population. Another inconvenience is the fact that about 50% of the cells in the explants do not divide, and therefore these could impose a 'masking effect' on any cell-cycle data.

This experiment was performed in early spring, when the explant cells were most synchronous in culture, (Yeoman et al, - Personal Communication). In addition, the timing of events in the first division cycle is highly predictable at this time of the year, (Yeoman et al - Personal Communication). Consequently, the experiments on the different phases in the first division cycle, (described in part B of this sub-section), were all carried out
FIGURE 38.

Changes in cell number during growth of auxin-cultured explants. (*Experiment 1*)

Duplicate samples of explants were taken at intervals of 4 hours. The two values for each time are plotted, together with the mean.
Figure 39.

Specific activity of $^3$H-thymidine-labelled DNA and changes in cell number during growth of auxin-cultured explants. ('Experiment 1')

(This figure combines the data from Figures 37 and 38.)

Duplicate samples of explants were taken at intervals of 4 hours. The mean value for each time is plotted.
within a few weeks of the experiment above, using the phase timings observed.

Experiment 2. (Using auxin-cultured explants only)

This was essentially a repeat of Experiment 1, but was carried out 15 weeks later. In the previous experiment I had not taken into account the uptake of $^{3}H$-thymidine by the tissue. The label 'available' for incorporation is that which is taken up by the tissue, and it is therefore believed by some researchers that incorporation data should be expressed as specific activity per unit uptake of label, not simply specific activity of the labelled molecule. There can, however, be arguments against this. For example, if the rates of incorporation and uptake of a particular label are linked in some way, then correction for uptake might lead to anomalous results.

The aim of this experiment was to see if correction for uptake of $^{3}H$-thymidine had any marked effect on the timing of the S-phase determined solely by the incorporation data. Uptake of label was taken to be equivalent to the counts in the supernatant after the first TCA-spin, when DNA was being extracted from the explants.

Figure 40 shows the uncorrected specific activity of the DNA through the experiment. Figures 41 and 42 show, respectively, the uptake of $^{3}H$-thymidine after a 90 minute labelling, and the specific activity of the DNA when corrected for this uptake. Uptake of the label is not constant throughout the time period, and shows a peak in the G1-phase. However, when S-phase is reached the uptake has
Specific activity of $^3$H-thymidine-labelled DNA during growth of auxin-cultured explants.

('Experiment 2').

Duplicate samples of explants were taken at intervals of 4 hours. The two values for each time are plotted, together with the mean.

Labelling was for 1.5 hours at a concentration of 0.96 μCi/ml of $^3$H-thymidine. No correction is made for uptake of the label by the tissue.

(Background counts subtracted.)
Uptake of $^3$H-thymidine during growth of auxin-cultured explants. ("Experiment 2")

Duplicate samples of explants were taken at intervals of 4 hours. The two values for each time are plotted, together with the mean.

Labelling was for 1.5 hours at a concentration of 0.96 µCi/ml of $^3$H-thymidine.

"Uptake" of label was taken to be equivalent to the counts in the supernatant after the first TCA-spin during DNA extraction. This is expressed as a percentage of the initial counts in the incubation medium shortly after label addition.

(Background counts subtracted.)
Specific activity of $^3$H-thymidine-labelled DNA during growth of auxin-cultured explants. ("Experiment 2")

Duplicate samples of explants were taken at intervals of 4 hours. The two values for each time are plotted, together with the mean.

Labelling was for 1.5 hours at a concentration of 0.96 μCi/ml of $^3$H-thymidine.

Correction for total uptake of the label, (expressed as cpm), is made here, (see Figure 44).

(Background counts subtracted.)
Fig 42

Specific Activity of DNA (% counts entering tissue) vs Hours in culture.
approximately levelled off, and subsequently changes very little. When correction is made for this uptake, (Figure 42), there is an S-phase peak in the 24-32 hour period. This peak is in the same position as that in the uncorrected data, (Figure 40). I do not consider the peak at 8 hours in the uncorrected specific activity data to be significant, since there was no indication of such a peak in Experiment 1. It seems that a 'more realistic' graph may be produced when specific activity is corrected for uptake, however the S-phase peak does remain in the same place. This indicates that the specific activity graph in Experiment 1, (Figure 37), does in fact give an accurate estimation of when S-phase occurs. There is, therefore, no reason to suppose that any cell-cycle timing errors were produced in Experiment 1 by not correcting the incorporation data for uptake of the $^3$H-thymidine label.

Figure 43 shows the corrected incorporation data and cell number counts for Experiment 2. These results are very similar to those for Experiment 1, (Figure 39), but there is a time difference of about 8 hours, the cells entering S-phase 8 hours later in Experiment 2. This apparent increase in the length of G1 with the age of the tubers used is frequently observed in cultured explants. It emphasises the need for experiments on particular phases of the cell-cycle to be performed as soon as possible after the phase-determination experiments.

**Experiment 3.** (Using both auxin-cultured and control explants)

This experiment was carried out to ensure that there was little or no indication of an S-phase or a cell number increase in
Specific activity of $^3$H-thymidine-labelled DNA and changes in cell number during growth of auxin-cultured explants. ('Experiment 2')

(This figure combines data from Figure 42 with cell number data.)

Duplicate samples of explants were taken at intervals of 4 hours. The mean value for each time is plotted.
Fig 43

- Mean cell no./explant
- Cell number
- \(^{3}\)H-Thymidine incorporation
- Specific Activity of DNA/\% Counts entering tissue (cpm/\mu g DNA)

Time in culture (hrs)
control explants. Explants were cultured in the presence or absence of auxin for up to 50 hours, and duplicate samples were taken at intervals.

Figure 44 shows the uptake of $^{3}$H-thymidine by auxin-cultured and control explants. There is little difference between the two graphs, indicating that, in this system, uptake of thymidine is not linked to any 'division-related' processes. Figure 45 shows the specific activity of the extracted DNA, adjusted for the uptake of label. Figure 46 presents the cell number data. The auxin-cultured explants show an S-phase $^{3}$H-thymidine incorporation peak and a cell number increase, both very similar to the results for Experiment 2. Control explants show no significant $^{3}$H-thymidine incorporation or any cell number increase. This confirms that the control explants apparently show no processes related to cell division.
Uptake of $^3$H-thymidine during growth of auxin-cultured and control explants. ('Experiment 3')

Duplicate samples were taken at intervals. The mean value for each time is plotted.

Labelling was for 1.5 hours at a concentration of 0.96 μCi/ml of $^3$H-thymidine.

'Uptake' of label was taken to be equivalent to the counts in the supernatant after the first TCA-spin during DNA extraction. This is expressed as a percentage of the initial counts in the incubation medium shortly after label addition.

(Background counts subtracted.)
Specific activity of $^3$H-thymidine-labelled DNA during growth of auxin-cultured and control explants. (Experiment 3)

Duplicate samples of explants were taken at intervals of 4 hours. The two values for each time are plotted, together with the mean.

Labelling was for 1.5 hours at a concentration of 0.96 μCi/ml of $^3$H-thymidine.

Correction for total uptake of the label, (expressed as cpm), is made here, (see Figure 44).

(Background counts subtracted.)
Changes in cell number during growth of auxin-cultured and control explants. (Experiment 3)

Duplicate samples of explants were taken at intervals of 4 hours. The two values for each time are plotted, together with the mean.
B. Histone synthesis and phosphorylation during the first division cycle

(i) Introduction

Using the timing data from Experiment 1, in Part A of this sub-section, I attempted to study histone synthesis and phosphorylation in the different phases of the first division cycle in auxin-cultured explants. Particular emphasis was placed on histone H4 to investigate the possibility of a 'division-related' phosphorylation in G2/Early M as reported for animal tissues, (Lake et al., 1972; Gurley et al., 1973), and Physarum, (Bradbury et al., 1974a, 1974b).

In each experiment, 10 flasks of auxin-cultured explants and 10 flasks of control explants were prepared. There was 10ml of culture medium in each flask. For the last 4 hours of the culture period 40μCi $^{32}$P-phosphate and 20μCi $^{3}$H-lysine were added to each flask. After termination of incubation the histones were extracted and electrophoresed on urea - acetic acid - 15% acrylamide gels. Gels were run for both total histones and for histone H4 alone. After staining and destaining, each gel was scanned. The radioactivity in the histone fractions was determined by scintillation counting. The $^{32}$P-phosphate : $^{3}$H-lysine incorporation ratio for histone H4 and for the phosphorylated protein in the H2 + 3 histone region was calculated for each phase of the cell-cycle. This gave an indication of the relative amounts of histone synthesis and phosphorylation occurring. The ratio of $^{32}$P-phosphate-labelling
of DNA: $^3$H-lysine-labelling of chromatin acidic proteins was also determined. All ratios were corrected for uptake of the 2 labels. Uptake data was obtained by scintillation counting of the first spin supernatants in the chromatin extraction procedure. All methods are described fully in Chapter 3.

(ii) Experiments performed

4 experiments were carried out. These investigated the following phases:

- Early G$_1$-phase. Explants were cultured for 8 hours.
- Late G$_1$-phase. Explants were cultured for 16 hours.
- S-phase. Explants were cultured for 22 hours.
- G$_2$/M-phase. Explants were cultured for 28 hours.

Labelling was for the last 4 hours of the culture period. The timing in these experiments was such that the label was 'available' to explants rich in cells at the required phase in the division cycle.

(iii) Uptake of $^{32}$P-phosphate and $^3$H-lysine by the explants

Table 4 presents the uptake data for these experiments. Because there was inevitably some variation in the amount of label added to flasks in different experiments, the figures of significance are those presenting uptake as a percentage of the label in the culture medium. Figure 47(A)(B) shows histograms of these percentages. Both isotopes show the same general trends. In the auxin-cultured tissue uptake is low in Early G$_1$ (3-4%).
TABLE I.

Uptake of $^{32}\text{P}$-phosphate and $^{3}\text{H}$-lysine during the cell-cycle of Jerusalem artichoke tuber explants.

The amount of radioisotope added was determined by counting the incubation medium immediately after isotope addition.

Uptake of radioisotopes was determined by counting the first spin supernatants in the chromatin extraction procedure.

(Machine background counts are subtracted from data.)
<table>
<thead>
<tr>
<th>Presence of 2,4-D.</th>
<th>Early G1</th>
<th>Late G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>1.536</td>
<td>1.665</td>
<td>2.790</td>
<td>5.182</td>
</tr>
<tr>
<td>Presence of 2,4-D.</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
(A) Uptake of $^{32}\text{P}$-phosphate and $^3\text{H}$-lysine during the first division cycle in auxin-cultured explants.

(B) Uptake of $^{32}\text{P}$-phosphate and $^3\text{H}$-lysine during growth of control explants for a period equivalent to the first division cycle in dividing explants.

Labelling of explants was for 4 hours, with 36.4 and 1.82 $\mu$Ci/ml of $^{32}\text{P}$-orthophosphate and $^3\text{H}$-lysine respectively.

$^{32}\text{P}$ - refers to $^{32}\text{P}$-phosphate uptake.

$^3\text{H}$ - refers to $^3\text{H}$-lysine uptake.

Uptake data was obtained by counting of the first spin supernatants in the chromatin extraction procedure. This is expressed as a percentage of the initial counts in the incubation medium shortly after label addition.

(Background counts subtracted.)
Fig 47

(A)

Phase in the cell-cycle of dividing explants

(B)

% Uptake of $^{32}$P-phosphate & $^3$H-lysine

- Early G1
- Late G1
- S
- G2/M
increasing slightly in late G.1, (5-7%). In S-phase the maximum uptake is reached, with the explants taking about 25% of each of the labels. This decreases after S-phase to values of 14-15%. In the control tissue there is no peak of uptake in the S-phase; instead, from early G.1 through to G.2/M there is a steady increase. In the latter, uptake is in the range 22-24%. In each phase, with the exception of S, uptake of the isotopes is slightly higher in the control explants than in the auxin-cultured explants.

It is of interest to note that the percentage uptake patterns for $^{32}$P-phosphate and $^3$H-lysine are virtually identical, and are completely different from the trends observed for $^3$H-thymidine uptake through the division cycle, (Sub-section 2.A - Experiments 2 and 3). The S-phase incorporation peaks in the auxin-cultured tissue may be related in some way to auxin or the cell division it stimulates.

(iv) **Synthesis of DNA and acidic proteins**

The data on $^{32}$P-phosphate incorporation into DNA and $^3$H-lysine incorporation into chromatin acidic proteins is presented in Table 5, and expressed as a histogram in Figure 43. The values are corrected for uptake of the 2 labels by the tissue.

DNA synthesis, (as measured by the phosphate incorporation), is high only in the S- and G.2/M-phases determined by $^3$H-thymidine incorporation. In the auxin-cultured explants there is a maximum in the S-phase, with a slightly lower level in G.2/M. The presence of the latter would be due to asynchrony between cells. In the control explants there is a smaller peak of phosphate incorporation.
Incorporation of $^{32}$P-phosphate into DNA and $^3$H-lysine into chromatin acidic proteins during the cell-cycle of Jerusalem artichoke tuber explants.

DNA and acidic proteins were extracted from de-histoned chromatin by stirring in 0.1N NaOH: (See Methods 3.C.(vi)).

Uptake of radioisotopes was determined by counting the first spin supernatants in the chromatin extraction procedure.

(Machine background counts are subtracted from data.)
<table>
<thead>
<tr>
<th>Phase in the cell-cycle of dividing e-plants</th>
<th>Early G1</th>
<th>Late G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of 2,4-D_6_</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>P</strong>-phosphate incorporation into DNA. (c.p.m./mlask x 10^-3).</td>
<td>2.509</td>
<td>3.557</td>
<td>4.374</td>
<td>4.924</td>
</tr>
<tr>
<td><strong>P</strong>-phosphate incorporation into DNA, per unit uptake of <strong>P</strong>-phosphate. (x 10^-4).</td>
<td>1.533</td>
<td>2.170</td>
<td>1.560</td>
<td>0.950</td>
</tr>
<tr>
<td><strong>H</strong>-lysine incorporation into acidic proteins. (c.p.m./mlask x 10^-5)</td>
<td>1.674</td>
<td>2.915</td>
<td>1.42</td>
<td>3.554</td>
</tr>
<tr>
<td><strong>H</strong>-lysine incorporation into acidic proteins, per unit uptake of <strong>H</strong>-lysine. (x 10^-3).</td>
<td>1.121</td>
<td>1.524</td>
<td>1.768</td>
<td>0.996</td>
</tr>
<tr>
<td><strong>P</strong>-phosphate-DNA/<strong>H</strong>-lysine-acidics incorporation ratio. (Corrected for uptake).</td>
<td>0.18</td>
<td>0.142</td>
<td>0.069</td>
<td>0.136</td>
</tr>
</tbody>
</table>
FIGURE 48.

(A) Incorporation of $^{32}$P-phosphate and $^3$H-lysine into DNA and acidic proteins respectively during the first division cycle in auxin-cultured explants.

(B) Incorporation of $^{32}$P-phosphate and $^3$H-lysine into DNA and acidic proteins respectively during growth of control explants for a period equivalent to the first division cycle in dividing explants.

(C) The ratio of DNA synthesis to acidic protein synthesis for auxin-cultured and control explants. (From histograms 48(A) and (B).)

Labelling of explants was for 4 hours, with 36.4 and 1.82 μCi/ml of $^{32}$P-orthophosphate and $^3$H-lysine respectively.

Correction for total uptake of each label, (expressed as cpm), is made here, (see Figure 47).

$^{32}$P - refers to $^{32}$P-phosphate incorporation into DNA.

$^3$H - refers to $^3$H-lysine incorporation into acidic proteins.

'2,4-D' - refers to auxin-cultured explants.

'C' - refers to control explants.

(Background counts subtracted.)
Incorporation of $^{32}\text{P}$-phosphate into DNA
and $^{3}\text{H}$-lysines into acidic proteins
\(\text{(cpm/flask} \times 10^4 (^{32}\text{P}) \text{or} 10^3 (^{3}\text{H}))\)

Phase in the cell-cycle of dividing explants

Incorporation of $^{32}\text{P}$-phosphate into DNA
\(\text{(cpm/flask} \times 10^4 (^{32}\text{P}) \text{or} 10^3 (^{3}\text{H}))\)

$^{3}\text{H}$ ratio from histograms (A) and (B)

**Fig 48**
This may be due to DNA synthesis, but appears to be much higher than expected on the basis of the $^3$H-thymidine incorporation data in Experiment 3, (Sub-section 2.A).

Acidic protein synthesis, (as measured by the lysine incorporation), shows the same trend as DNA synthesis, with an S-phase peak in both the auxin-cultured explants and the controls.

The indication that chromatin acidic protein synthesis is not constant through the division cycle, is in disagreement with results obtained by some researchers using other tissues, (Jeter and Cameron, 1974).

The ratio of DNA synthesis to acidic protein synthesis was determined, (Table 5), and is presented as Figure 48(c). As would be expected from the results, there is little difference in this ratio between auxin-cultured and control explants. There is a minimum in Late G1 in both cases, with a maximum in S for dividing explants, and a maximum in G2/M for non-dividing explants.

It could be suggested that the similarity between the incorporation patterns for auxin-cultured explants and control explants is due to the presence of a division cycle in the controls. Because $^3$H-thymidine labelling has indicated that this is probably not the case I can only suggest that the peaks of incorporation in the controls may represent an increase in the turnover rates of the DNA phosphates and the acidic proteins.

The DNA and acidic proteins were not specifically purified or fractionated in these experiments. Therefore, errors may have arisen because of the presence of labelled 'contaminants' in the
chromatin samples. In addition, bacterial contamination cannot be totally excluded as a possible source of labelled molecules. There may also be the problem of hydrolysis of some of the acidic proteins in the alkaline solution, (Gronow et al., 1976). Consequently, I do not place much significance on these results.

(v) Synthesis and phosphorylation of explant histone H1

Table 6 presents data on phosphorylation and synthesis of histone H1 through the division cycle. Figure 49 presents these results as a histogram. The histone had been electrophoresed to the lower end of the gels to obtain minimum interference by the 32P 'gel background' counts. The counts in the histone peak have been corrected for uptake of the labels, and are presented on a per unit histone basis, (Equivalent to per unit peak area).

Synthesis of histone H1 in auxin-cultured explants appears to have a maximum in late G1 and S. Phosphorylation of this histone, on the other hand, shows a maximum in S and G2/M. This difference is more clearly observed in the 32P-phosphate / 3H-lysine incorporation ratio for histone H1 through the cell-cycle, (Figure 49(C)). There is a maximum in G2/M, with a value of 3-times the corresponding value for S-phase.

Histone H1 from control explants appears to show an S-phase peak for both synthesis and phosphorylation. However, in each case the peak is much lower than that for histone from dividing explants.

Table 7 presents data on synthesis and phosphorylation of histone H1 from gels run for total histones. Only the S-phase and G2/M-phase data for dividing explants are included. These results
Incorporation of $^{32}$P-phosphate and $^{3}$H-lysine into histone H.1 during the cell-cycle of Jerusalem artichoke tuber explants.
(From a gel run for histone H.1 alone.)

The relative peak area of histone H.1 was determined by scanning the stained gel in a chromoscan under standardised conditions.

Uptake of radioisotopes was determined by counting the first spin supernatants in the chromatin extraction procedure.

(Machine background counts are subtracted from data.)
<table>
<thead>
<tr>
<th>Presence of 2,4-D</th>
<th>Early G1</th>
<th>Late G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative peak area of histone H1 (Arbitrary units).</td>
<td>73 43</td>
<td>6 13</td>
<td>41 42</td>
<td>33 54</td>
</tr>
<tr>
<td>$^{32}$P-phosphate incorporation into histone H1 (c.p. 4 min.)</td>
<td>189 80</td>
<td>23 253</td>
<td>1,455 460</td>
<td>1,673 755</td>
</tr>
<tr>
<td>$^{32}$P-phosphate incorporation, per unit histone H1, per unit uptake of $^{32}$P-phosphate (c.p. 4 min. x 10$^5$)</td>
<td>1,665 1,104</td>
<td>1,373 3,831</td>
<td>2,315 5,365</td>
<td>7,267 1,310</td>
</tr>
<tr>
<td>$^3$H-lysine incorporation into histone H1 (c.p. 4 min.)</td>
<td>2,324 1,000</td>
<td>1,170 205</td>
<td>11,635 2,820</td>
<td>9,806 3,126</td>
</tr>
<tr>
<td>$^3$H-lysine incorporation, per unit histone H1, per unit uptake of $^3$H-lysine (c.p. 4 min. x 10$^5$)</td>
<td>2,132 1,216</td>
<td>6,313 0,309</td>
<td>7,533 3,130</td>
<td>2,103 0,256</td>
</tr>
<tr>
<td>$^{32}$P-phosphate/$^3$H-lysine incorporation ratio in histone H1 (Corrected for uptake)</td>
<td>0.0079 0.0091</td>
<td>0.0022 0.1240</td>
<td>0.0118 0.0171</td>
<td>0.0345 0.0493</td>
</tr>
</tbody>
</table>
(A) Incorporation of $^{32}\text{P}$-phosphate and $^3\text{H}$-lysine into histone H$_{1}$ during the first division cycle in auxin-cultured explants.

(B) Incorporation of $^{32}\text{P}$-phosphate and $^3\text{H}$-lysine into histone H$_{1}$ during growth of control explants for a period equivalent to the first division cycle in dividing explants.

(C) The ratio of histone H$_{1}$ phosphorylation to synthesis during the cell-cycle in auxin-cultured explants.

(From histogram 49(A).)

Labelling of explants was for 4 hours, with 35.4 and 1.82 μCi/ml of $^{32}\text{P}$-orthophosphate and $^3\text{H}$-lysine respectively.

Correction for total uptake of each label, (expressed as cpm), is made here, (see Figure 47).

$^{32}\text{P}$ refers to $^{32}\text{P}$-phosphate incorporation into histone H$_{1}$.

$^3\text{H}$ refers to $^3\text{H}$-lysine incorporation into histone H$_{1}$.

(Background counts subtracted.)
Incorporation of $^{32}$P-phosphate & $^{3}H$-lysine into histone H.I.

(A) FIG 49

(B)

(C)

Ratio in histones H.I from histogram (A)

Phase in the cell-cycle of dividing explants
Incorporation of $^{32}$P-phosphate and $^3$H-lysine into histone H.1 during the cell-cycle of Jerusalem artichoke tuber explants.
(From a gel run for total histones.)

Only S- and G.2/M-phase data from dividing explants is included. Loadings of other samples on gels were too low to give significant counts in the histone H.1 region.

The relative peak area of histone H.1 was determined by scanning the stained gel in a chromoscan under standardised conditions.

Uptake of radioisotopes was determined by counting the first spin supernatants in the chromatin extraction procedure.

(Machine background counts are subtracted from data.)
<table>
<thead>
<tr>
<th>Phase in the cell-cycle of dividing explants.</th>
<th>S.</th>
<th>G.2/M.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Presence of 2,4-D.</strong></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Relative peak area of histone H.1. (Arbitrary units).</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>( ^{32} )P-phosphate incorporation into histone H.1. (c.p. 4 min.)</td>
<td>1,078</td>
<td>-</td>
</tr>
<tr>
<td>( ^{32} )P-phosphate incorporation, per unit histone H.1, per unit uptake of ( ^{32} )P-phosphate. (c.p. 4 min. x 10(^7))</td>
<td>35,207</td>
<td>-</td>
</tr>
<tr>
<td>( ^{3} )H-lysine incorporation into histone H.1. (c.p. 4 min.)</td>
<td>15,389</td>
<td>-</td>
</tr>
<tr>
<td>( ^{3} )H-lysine incorporation, per unit histone H.1, per unit uptake of ( ^{3} )H-lysine. (c.p. 4 min. x 10(^6)).</td>
<td>305,336</td>
<td>-</td>
</tr>
<tr>
<td>( ^{32} )P-phosphate/( ^{3} )H-lysine incorporation ratio in histone H.1. (Corrected for uptake).</td>
<td>0.0115</td>
<td>-</td>
</tr>
</tbody>
</table>
are in agreement with those presented above.

Synthesis and phosphorylation of histone H1 in dividing tissue appear to be separate events, with the phosphorylation occurring perhaps 4-6 hours later than the synthesis. This sequence of events is very similar to that reported by workers using Physarum, (Bradbury et al., 1974a, 1974b), and synchronous animal cell cultures, (Gurley et al., 1973). These authors interpret their results by suggesting that there is a 'bulk' histone H1 phosphorylation during Late G2/Early M, and that this phosphorylation causes structural changes in the chromatin, resulting in chromosome condensation. I would like to propose that my results present the first indication of this phenomenon in plant tissues, and that they lend support to the theory that it is a fundamental event in mitotic cell division.

The results presented in this section indicate that histone synthesis begins in Late G1, and therefore probably occurs out of phase with DNA synthesis. Figure 50 presents histograms of DNA and histone synthesis for dividing explants in different phases of the cell-cycle. The trends are as predicted, with histone synthesis being initiated first. The partial independence of these 2 events has been reported by Holoubek and Ruckert, (1964), and by Sadgopal and Bonner, (1963). In addition, there is some evidence in the literature to suggest that histone synthesis is initiated in Late G1, (Irvin et al., 1963; Block et al., 1967).
The relative rates of DNA and histone H.1 synthesis during the cell-cycle in auxin-cultured explants. (From histograms 48(A) and 49(A).)

'DNA' - refers to synthesis of DNA.

'H.1' - refers to synthesis of histone H.1.
DNA synthesis - Incorporation of $^{32}$P-phosphate (cpm/flask $\times 10^{-4}$) & Histone H1 synthesis - Incorporation of $^3$H-lysine (c.p. 4min/unit histone $\times 10^5$)
(vi) Synthesis and phosphorylation of a protein within the explant histone H$_2 + 3$ region

In sub-section 1 of this section the labelling data indicated that a histone in the H$_2 + 3$ region was phosphorylated in dividing tissue. Table 8 presents data on the synthesis and phosphorylation of this histone in S and in the G$_{2}$/M-phase for dividing explants. (The phosphorylated protein was taken arbitrarily to be that area in the H$_2 + 3$ region showing a $^{32}$P-phosphate incorporation peak.) As in histone H$_1$, it appears that the ratio of $^{32}$P-phosphate incorporation : $^3$H-lysine incorporation in this histone increases from S to G$_{2}$/M. Synthesis and phosphorylation may, however, be closely related here, since both show higher values in the G$_{2}$/M-phase.
Incorporation of \(^{32}\)P-phosphate and \(^{3}\)H-lysine into the phosphorylated region of the histone H\(_{2}\)A + 3 peak area during the cell-cycle of Jerusalem artichoke tuber explants.

Only S- and G\(_{2}/M\)-phase data from dividing explants is included. Loadings of other samples on gels were too low to give significant counts in this histone region.

This histone was never selectively extracted so the relative peak areas could not be determined. In addition, the protein was taken arbitrarily to be that area in the histone H\(_{2}\)A + 3 region showing a \(^{32}\)P-phosphate incorporation peak.

Uptake of radioisotopes was determined by counting the first spin supernatants in the chromatin extraction procedure.

(Machine background counts are subtracted from data.)
### TABLE 8

<table>
<thead>
<tr>
<th>Presence of 2,4-D.</th>
<th>Phosphate in the cell-cycle of dividing explants.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S.</td>
</tr>
<tr>
<td>32P-phosphate counts in phosphorylated region of H.2 + 3 peak area, (c.p. 4 min.)</td>
<td>2,392</td>
</tr>
<tr>
<td>32P-phosphate counts in phosphorylated region of H.2 + 3 peak area, per unit uptake of 32P-phosphate, (c.p. 4 min. x 10^6).</td>
<td>627.6</td>
</tr>
<tr>
<td>3H-lysine counts in phosphorylated region of H.2 + 3 peak area, (c.p. 4 min.)</td>
<td>15,119</td>
</tr>
<tr>
<td>3H-lysine counts in phosphorylated region of H.2 + 3 peak area, per unit uptake of 3H-lysine, (c.p. 4 min. x 10^6).</td>
<td>4,193.7</td>
</tr>
<tr>
<td>32P-phosphate/3H-lysine incorporation ratio in phosphorylated region of H.2 + 3 peak area, (Corrected for uptake).</td>
<td>0.0149</td>
</tr>
</tbody>
</table>
C. Summary and discussion of labelling experiments on synchronously-dividing tissue

The timing of phases in the first division cycle of auxin-cultured explants was determined by $^3$H-thymidine incorporation into DNA and cell number counting. Uptake of $^3$H-thymidine was high initially, but decreased towards the beginning of S-phase. This occurred in both dividing explants and controls. It was probably some expression of a 'wound response' in the tissue after explant preparation. Control explants did not show the DNA synthesis (as measured by $^3$H-thymidine incorporation), or cell number increase exhibited by the dividing explants. Individual phases in the division cycle were studied in later experiments, using the timing determined. Because of the close timing of S-phase and mitosis it was only possible to study 'S-rich' and 'G$$_2$/M-rich' tissues here. In addition, tissues in early and late G$$_1$ were used.

Uptake of $^3$H-lysine and $^{32}$P-phosphate in the cell-cycle experiments were generally low in G$$_1$, increasing to maxima in S and G$$_2$/M for dividing and non-dividing tissues respectively. DNA synthesis (as measured by $^{32}$P-phosphate incorporation), and acidic protein synthesis in dividing explants showed maxima in the S-phase period determined by $^3$H-thymidine incorporation. Similar peaks were observed in the controls, but these were probably not related to cell division. Synthesis of histone H$$_1$$ in dividing explants occurred in late G$$_1$$ and in S-phase. There was reason to believe that it commenced before DNA synthesis. Phosphorylation of histone H$$_1$$ in dividing explants occurred 'out of step' with synthesis.
The ratio of phosphorylation: synthesis of this histone showed a maximum in the G.2/M-phase, of 3-times the corresponding S-phase value. This was interpreted as indicating that most of the phosphorylation of histone H.1 occurred later in time than, and independently from, synthesis. It is similar to the 'bulk' phosphorylation of histone H.1 observed during Late G.2/Early M in Physarum, (Bradbury et al, 1974a, 1974b), and in synchronous animal cell cultures, (Saxley et al, 1973). I concur with these authors in suggesting that this phosphorylation is related to some pre-mitotic event in the division cycle, (probably chromosome condensation). A protein in the histone H.2 + 3 region also showed an increase in the phosphorylation: synthesis ratio from 3 to G.2/M. Since both parameters were higher in the G.2/M-phase this may reflect, in part at least, a later synthesis of this histone than histone H.1.

It is unfortunate that the S-, G.2-, and M-phases of the cell-cycle in dividing explants were separated by only a few hours, rendering it impossible to obtain tissue containing cells in only one phase. In addition, since only about half of the cells were dividing, there was the problem of 'masking' by the non-dividing tissue. (It has been shown by Yeoman et al, (1965), that it is the central core of tissue which does not divide.) A greater temporal separation of S, G.2 and M would only have been achieved by using a different tissue, but would have given greater significance to the distinction between the processes of synthesis and phosphorylation in histone H.1. The Acer cell suspension culture system could have been useful here since not only do the cells divide synchronously,
but the division cycle is much longer than that in artichoke explants, (King et al., 1974). The non-dividing core of artichoke explant tissue is most likely a direct or indirect consequence of the absolute thickness of the explants, and may reflect a decrease in effective auxin activity as the hormone penetrates the tissue. The culturing of thin slices of artichoke tuber tissue could, in the future, prove to be successful in obtaining a 90-100% division of cells, (Unconfirmed data of my own).

As stated previously, care must be taken when comparing dividing and non-dividing tissues, since the controls may be undergoing metabolic processes which are absent from the dividing tissues. For this reason I believe that the only valid comparisons that can be made are those between the different phases of the division cycle.

If sufficient time had been available it would have been of interest to study the nature of the amino acid phosphates formed in histone H4 in the different phases of the division cycle. A distinction between phosphorylation occurring in Late G1 and S, and phosphorylation occurring in G2/M might have given greater credibility to the suggestion that the latter was not directly related to histone synthesis.

The phosphorylated protein within the histone H2+3 region was never selectively extracted and identified. The results for this histone must, therefore, be regarded as tentative. If time had been available, I would have isolated this histone fraction and investigated its synthesis and phosphorylation through the cell-cycle, in the same manner as for histone H4.
CHAPTER 5

DISCUSSION
For each section in the previous chapter the results were summarised and discussed. In this final chapter I shall present a brief interpretation of the significant results and suggest further work on the artichoke explant system. A critique of the methods used and the results obtained will also be included.

Other plant tissues, or culture systems, would have to be studied to give greater credibility to the interpretation of results from this system. Suggestions for research here will be presented.

Finally, I shall discuss the proposed role of histone H₁₁ phosphorylation in chromatin condensation, and the hypothesis that this may represent a control point in the cell-cycle.
A. Interpretation of results and suggestions for further experimentation

Histones were isolated by acid-extraction from purified chromatin. Identification of the various fractions was by relative mobility on polyacrylamide gels. This allowed histones H$_{1}$ and H$_{4}$ to be distinguished, with histones H$_{2A}$, H$_{2B}$ and H$_{3}$ migrating so closely together that specific identification was impossible.

Further work on artichoke histones should include selective extraction procedures for the isolation of histones from the H$_{2}+3$ region. Amido Black has different staining properties with 'lysine-rich' and 'arginine-rich' histones. It could, therefore, be used to identify the 'arginine-rich' histone H$_{3}$ within the H$_{2}+3$ region, since the other two proteins are 'lysine-rich'. A further method for specifically identifying histone H$_{3}$ would be to allow the molecules to dimerise under oxidising conditions, and to follow the changes in band position on polyacrylamide gels. Hopefully, this would result in a loss of part of the H$_{2}+3$ region with the simultaneous appearance of a band migrating at a much slower rate, (the dimer).

The molecular weight of histone H$_{1}$ was c. 43,000 daltons, approximately twice the reported value for the corresponding histone in animal tissues. To determine whether this represents a simple 'doubling-up' of an original sequence it would be necessary to carry out amino acid sequencing studies on a highly purified sample. The molecular weights of the other histone species appeared to be
similar to those for the corresponding fractions from animal tissues. These 4 histones are intimately concerned with the maintenance of chromatin ultrastructure, and it may be that this function necessitates a high degree of conservation of primary structure.

Histone H.1 from tuber and explant chromatin showed heterogeneity after polyacrylamide gel electrophoresis, with a main-band and 2 or more slower-moving subfractions. The results of alkaline phosphatase incubations indicated that these subfractions were, at least in part, phosphorylated derivatives of the main-band. However, they were not completely removed by enzyme incubation and the remaining portions may have been phosphorylated regions inaccessible to the phosphatase, acetylated or methylated derivatives of the main-band, or simply unmodified molecules of slightly different composition from the main-band. Studies on the effects of acetylase or methylase incubations on the 'dephosphorylated' histone H.1 may assist the interpretation here. In addition, if the various subfractions of histone H.1 can be adequately separated and isolated, (perhaps by column chromatography), then amino acid composition or sequencing studies may determine whether or not the subfractions remaining after phosphatase incubation do indeed have different primary structures.

The proportion of phosphatase-labile fractions of histone H.1 varied with the tuber crop used, and may indicate, in some way, the 'metabolic state' of the tissue. Since dormancy of the tubers decreases with storage it would be of interest to study the phosphatase-lability of the histone H.1 subfractions at different
times of the year. Perhaps some correlation between degree of dormancy and phosphatase-lability of histone H$_{1,1}$ would be found for the stored tubers.

Alkaline phosphatase incubations indicated that dividing explants may contain slightly more phosphorylated histone H$_{1,1}$ than control explants. However, the difference was small, and to be certain that the gel scans were correct these experiments would have to be performed many more times.

Phosphatase incubations were only carried out on histone H$_{1,1}$ from dividing explants when the divisions had become asynchronous. If methods could be devised for increasing the scale of this tissue-culture system it would be useful to study the gel banding pattern and phosphatase-lability of histone H$_{1,1}$ from cells in different phases of the first (semi-synchronous), division cycle. In this way, any heterogeneity resulting from phosphorylation of histone H$_{1,1}$ could perhaps be linked to a particular phase of the cell-cycle.

The phosphatase incubation conditions employed resulted in a large breakdown of histone, presumably by the activity of an acid-soluble protease in the samples. If the explant experiments could be performed on a larger scale, this would make PMSF-treatment of the extracted chromatin a viable proposition. (PMSF-treatment has been adopted by other researchers to inhibit the activity of a chromatin-bound acid-soluble protease, (Chong et al., 1974; Ballal et al., 1975).) Alternatively, it may be possible to separate the histones and protease by polyacrylamide gel electrophoresis and,
after removal of histones by diffusion, carry out phosphatase incubations without loss of protein by breakdown.

Radioactive labelling of histones with $^{32}$P-phosphate was used to study histone phosphorylation in explants more accurately. $^3$H-lysine incorporation gave an estimation of histone synthesis here. In asynchronously-dividing explant tissue there was general histone synthesis, as well as phosphorylation of histone H$_{\text{1}}$ and a histone within the H$_{\text{2+3}}$ region. Control, (non-dividing), explants showed very little histone synthesis or phosphorylation.

The 'phosphorylations' of histones from dividing explants were shown to be phosphatase-labile and were therefore most likely due to true incorporation of phosphate molecules. Acid-hydrolysis of the phosphorylated proteins indicated that approximately half of the incorporated phosphate was in the form of serine and threonine phosphate residues, whilst the remainder was probably in acid-labile linkages. Enzymatic hydrolysis experiments were unsuccessful. However, further attempts should be made here to confirm the presence of acid-labile phosphates. 'Markers' of lysine phosphate and histidine phosphate would be required for these investigations.

Phosphatase incubation and acid-hydrolysis experiments were only performed on 'labelled' histones from dividing explants. These experiments could usefully be extended to control explants so that the suggestion that threonine phosphate is present only in dividing tissues, (Langan and Hohmann, 1975), might be investigated. A further series of experiments could study the products of
N-Bromosuccinimide cleavage of 'labelled' histone H\(_{1}\). If the cleavage products were subsequently hydrolysed and subjected to paper electrophoresis then the nature of the phosphorylated residues could be determined for each half of the histone H\(_{1}\) molecule. The type and approximate positions of the phosphorylation sites in histone H\(_{1}\) from dividing and non-dividing tissues may thus be compared.

The approximate timing of the G\(_{1}\), S and G\(_{2}/M\)-phases in the cell-cycle was determined for dividing explants using 2 parameters, cell number per explant and specific activity of DNA when pulse-labelled with \(^{3}\)H-thymidine. Synthesis and phosphorylation of histone H\(_{1}\) were studied in these different phases by incorporation of \(^{3}\)H-lysine and \(^{32}\)P-phosphate respectively. It was found that synthesis occurred mainly in Late G\(_{1}\) and S, whereas phosphorylation occurred in S and G\(_{2}/M\). The ratio of histone H\(_{1}\) phosphorylation : synthesis increased 3-fold as the cells passed from S to G\(_{2}/M\). I interpret these findings as indicating a large G\(_{2}\)-related phosphorylation of histone H\(_{1}\). It is my belief that this phosphorylation is similar in nature to that found in animal tissues, (Lake et al., 1972; Gurley et al., 1973), and Physarum, (Bradbury et al., 1974a, 1974b), when also in the G\(_{2}\)-phase. My results provide further support for the hypothesis that a G\(_{2}\)-related phosphorylation of histone H\(_{1}\) is responsible for chromatin condensation prior to mitosis, and may therefore represent a 'mitotic trigger' of some form. To be more certain that these results are significant, and do actually

* Assuming that phosphorylation of histone H\(_{1}\) from control explants can be detected.
represent division-related changes, (and not changes occurring in the non-dividing core of cells), it is necessary to repeat these experiments a few more times, preferably over a number of years, with explant material showing a range of cell-cycle times. This and other problems posed by the presence of a non-dividing core of cells in auxin-cultured explants may be overcome by culturing thin slices of tuber tissue. There is reason to believe that if 1.0mm slices are used all the cells divide, (Stratton and Mungall, Unpublished data). However, it remains to be seen whether or not the initial division is synchronous.

Acid hydrolysis and enzymatic hydrolysis experiments could be performed on histone H1 from dividing explants in different phases of the cell-cycle. Similar experiments on the N-Bromosuccinimide cleavage products of this protein could also be carried out, in the manner suggested previously. In this way, the G2-related phosphorylation may be shown to differ in nature or site(s) from the phosphorylation occurring during synthesis.

In cell-cycle investigations inhibitors could be used to 'concentrate' cells in particular phases. 5-fluorodeoxyuridine, (FuDR), is an inhibitor of DNA synthesis and could be used to accumulate cells at the G1-S boundary if applied early in the culture period. 'Metaphase-arrest' of cells could be induced by application of colchicine at certain concentrations. A novel method of studying cells in the G2/M-phase could be by the simultaneous application of these two inhibitors immediately after the S-phase
peak. The colchicine would prevent exit of cells from metaphase, whilst the FuDR would prevent 'masking' of the G₂-associated metabolism by S-associated events. One experiment of this nature was performed, and the histone H₁ synthesis and phosphorylation results were in agreement with those for the G₂/M-phase investigation carried out without inhibitors. However, this approach was discontinued since the effects of these inhibitors on the cells were not known and interpretation would be highly speculative. In addition, without considerable experimentation I could not be certain that FuDR in fact inhibited S-associated histone metabolism, (even if it prevented DNA synthesis), or if colchicine truly 'arrested' histone metabolism in metaphase.
The artichoke explant culture system could be used in more widespread histone investigations. Possible topics of investigation would be:

1. The timing of synthesis of specific histones in the cell-cycle. This investigation would involve studies of the incorporation of labelled amino acids by the various histone fractions.

2. The timing of uptake by the nucleus of newly synthesised histones. This investigation would require methods for the isolation of histones from the cytoplasm. After a 'pulse' of a labelled amino acid the disappearance of label from cytoplasmic histones would be studied.

3. The specificity of uptake by the nucleus of newly synthesised histones. The properties of histones which enable them to be rapidly and specifically taken up by the nucleus would be investigated here. Using the method described in (2) above, the uptake by the nucleus of proteins of known primary sequence and/or basicity would be studied.

4. The phosphorylation of histones other than histone H1 in dividing and non-dividing tissues, and through the cell-cycle. Methods similar to those employed for studies of histone H1 phosphorylation would be used.

5. Other histone modifications, (e.g. acetylation, methylation), in dividing and non-dividing tissues, and through the cell-cycle. Incorporation of labelled acetyl and methyl groups

* Most of these investigations would require methods for the selective extraction of individual histone species.
would be studied.

6. The existence of extrachromatin pools of histones within the nucleus. The identification of histones in the nucleoplasmic, (soluble), phase of the nucleus would be attempted here.
B. A critique of the methods and results

Harvesting of tuber crops occurs in the winter, but no account is taken of the degree of frost treatment the tubers have received. Consequently, each crop is unique in its 'degree of dormancy' and 'metabolic state' when harvested. (The latter is indicated by the differences in phosphatase-lability of histone H1 between different tuber crops.) Comparisons between results from different crops can therefore be subject to errors. Dormancy of stored tubers decreases with time, and by the summer many show signs of growth. In addition, the response of explants to auxin varies according to the age of the stored tubers; the length of the first G1, (or 'lag'), phase increases with age. All investigations performed are, therefore, subjected to the influence of this 'background variation' in the tissue used.

When tuber tissue is cultured in the form of explants further problems arise. Only half of the cells in auxin-cultured explants actually divide in the first, (semi-synchronous), division. Not only do the remaining cells probably contribute 'masking' effects to the division-related changes, but they themselves may be undergoing specific metabolic changes. I have no conclusive proof that the 'division-related' or 'cell-cycle' events I have described are indeed occurring in the dividing cells. The fact that these events do not occur in control explants, (the cells of which do not divide), may not be sufficient to substantiate this, since there is no evidence to suggest a similarity between the metabolism of control explants and that of the non-dividing cells in auxin-cultured
A criticism of the cell-cycle experiments would be that phase-determination and specific phase investigations were not performed simultaneously. For technical reasons this was impossible, and I had to assume that the timing of the cell-cycle events changed very little in the early part of the year. Other researchers have reported that this is the case.

Possible proteolytic activity of the alkaline phosphatases used was never studied, nor were attempts made to inhibit the proteolytic breakdown of incubated histone samples by pre-treatment with PMSF. If, in the future, phosphatase incubation experiments are to be carried out routinely then these two factors will have to be considered.

Although most of this thesis presents investigations on histone H1, this protein was never selectively extracted. Attempts at selective extraction were in fact made, but were unsuccessful. Perseverance may have resulted in success here, but since I was reasonably certain of the histone H1 identification, (see Chapter 4, section 2), the selective extraction experiments were discontinued. Perhaps the higher than expected molecular weight for this protein may have hindered its separation from the other histones.

*(The selective extraction procedure adopted was by the reported
solubility of histone H.1 in 5% TCA, the other histone fractions being precipitated. However, it was found that 5% TCA precipitated, all the histone fractions from acid-extracted tuber chromatin. The insolubility of histone H.1 here may have been a result of its unexpectedly high molecular weight.)

Finally, 2 'correction' techniques employed in the investigations can be subject to criticism. These are; the 'correction' of the U.V. absorbance scan of chromatin for light-scattering effects, and the 'correction' of radioisotope incorporation data for uptake of the particular labels. The possible criticisms of these techniques have been mentioned previously, and although their use has probably resulted in no significant errors in interpretation of results, no attempts were made to confirm the validity of the 'corrections' used.
There are many examples in the literature of hormonal or other treatments stimulating plant cell division. If the apical region of the pea epicotyl is removed and replaced by lanolin paste containing the auxin indoleacetic acid, (IAA), then cambial cells in the sub-apical region are stimulated to divide rapidly to produce root primordia, (Fan and Maclachlan, 1966, 1967). Cut epicotyls treated with lanolin paste alone show no such division. Blakely et al., (1972), have shown that primary roots, or root segments, from newly germinated seedlings of Haplopappus, tomato and radish show rapid and abundant branch-root formation when cultured in the presence of auxin. Again, in the absence of auxin there is no significant root formation. It should be possible to extract histones from these 2 systems and to compare their synthesis and phosphorylation for dividing and non-dividing tissues. Each system would, however, have problems akin to those arising from the use of artichoke explants, there would be 'masking' effects by non-dividing tissues in the auxin-treated samples, and the metabolism of the controls would be uncertain. Nevertheless, general investigations using radioisotopes may provide useful data. I made various unsuccessful attempts to use these systems in order to provide support for my results on artichoke explants. Histones were in fact successfully labelled with \( ^3H \)-lysine, extracted and purified from pea epicotyls and radish roots, but I was unable to obtain any incorporation of \( ^{32}P \)-phosphate. I concluded that the applied concentrations of this label were too
low for adequate incorporation into proteins to take place. Increasing these concentrations was impossible for technical reasons.

A more useful system for studying dividing and non-dividing cells would be one such as the Acer cell suspension system, (Stuart and Street, 1969). These cells, when cultured in a nutrient medium, divide asynchronously until nitrate becomes limiting. Division then ceases. Not only can dividing and non-dividing cells be studied here, but in rapidly-dividing cultures 90 - 100% of the cells present are undergoing division. There is therefore no major problem of 'masking' by the effects of a non-dividing residue of cells. The potential of this system in the study of the cell-cycle increased markedly following the discovery that divisions could be synchronized by nitrate-starvation followed by low-density re-inoculation into fresh medium, (King et al., 1973, 1974). Histone synthesis and phosphorylation could, therefore, be studied through the cell-cycle. The 2 - 4 day cycle time and the fact that most of the cells divide would enable accurate and significant studies to be made on any parameter through the different phases of the cell-cycle. The only technical problems with this system appear to be the unpredictable length of the initial lag phase after re-inoculation, (3 - 10 days), and the variation in cell-cycle times in the synchronous divisions. These could be overcome by carrying out 'phase-determination' investigations in parallel with the removal of samples for histone or other work.

In a most interesting paper Usciati et al., (1972), have shown
that cells in the axillary buds of Chickpea, (*Cicer arietinum*), are 'arrested' in the G.2-phase of the cell-cycle. Following application of the cytokinin 6-Benzylaminopurine, (6-BAP), to the buds these cells rapidly enter mitosis. The cytokinin appears to act as a 'trigger' for the release of G.2-'arrest'. If histone H.1 phosphorylation initiates chromatin condensation prior to mitosis then it would not be unreasonable to propose that the cytokinin treatment results in activation of the 'growth-associated' histone H.1 phosphokinase. This, in turn, would initiate phosphorylation of histone H.1 and result in chromatin condensation and the onset of mitotic cell division. The proposed large-scale phosphorylation of histone H.1 could be investigated in this system by the simultaneous application of 6-BAP and $^{32}$P-phosphate to the axillary buds. $^{3}H$-lysine treatment could also be included, to show that histone synthesis was not related to the phosphorylation. The controls would simply be axillary buds treated with the radioisotope but with no cytokinin. I attempted an investigation of this nature, and extracted and purified the chickpea histones. Again, the amount of $^{32}$P-phosphate added was insufficient to allow incorporation into histones during the labelling period.

If phosphorylation of histone H.1 is concerned with chromatin condensation, (and perhaps the formation of chromosomes), then it may occur in meiotic, as well as mitotic cells. In developing anthers the pollen mother cells synchronously pass through meiotic division, (Stern and Hotta, 1969). Preparation for this division is
also synchronous, and therefore offers a unique opportunity to any researcher on the meiotic cycle. With the aid of radioisotopes it should be possible to study synthesis and phosphorylation of histone H1 in this system.

On a more general note, I would conclude by suggesting investigations on the heterogeneity and molecular weights of plant histones. The observation that artichoke histone H1 has approximately twice the molecular weight of its animal counterpart may be of evolutionary significance. However, data from many more plant species would be required to substantiate this view. Histone H1 sub-species have been shown for pea tissues, and at least indicated for Jerusalem artichoke tuber. Investigations across the plant kingdom may give some indication of the distribution and heterogeneity of these sub-species.
Chromatin condensation takes 2 forms. The first is shown by the localised occurrences of heterochromatin in interphase nuclei, and by the sperm head nucleoprotein during spermatogenesis. Condensation in these cases is undoubtedly regulated, but shows no signs of a structural organisation. The second type of chromatin condensation occurs during the G2-phase of the cell-cycle. Here, the nucleoprotein complex becomes organised into discrete bodies known as chromosomes. It is this latter case which is generally referred to when 'chromatin condensation' is discussed, although perhaps it should more correctly be termed 'chromosome condensation'.

In recent years a series of investigations have shown that the tendency to consider all 'condensations' of nucleoprotein as specific examples of a general phenomenon is, at the very least, misleading. Paradoxically, it has been shown that conditions required for the heterochromatin type of condensation would lead to decondensation of chromosomes, and vice versa. Therefore, although the 2 types of condensation have structural similarities they are markedly different in nature.

Heterochromatin organisation requires a high level of free Ca$^{2+}$ ions. If cations are removed then decondensation occurs, resulting in euchromatin, (Leake et al., 1972). Jacobs et al., (1976), have shown that presence of cations neutralises the repulsion forces between the free negatively charged DNA phosphates, thus allowing the nucleoprotein complex to become more densely packed.
Regions of uncondensed chromatin would be maintained by the presence of localised unneutralised DNA phosphates. As discussed in Chapter 1, specific phosphorylations of the nuclear acidic proteins and of histone H1 have been strongly implicated in specific gene-activation. It is not unreasonable to propose that these phosphorylations cause local decondensation of chromatin, resulting in transcription of the 'exposed' DNA sequences.

The condensation process in sperm head chromatin is less well understood, but there is evidence to suggest that it may be similar in nature to that involved in the maintenance of heterochromatin. A gradual dephosphorylation of the protamines occurs during condensation here, (Louie et al, 1973).

Chromosome condensation differs from the cases above in that it has a requirement for a low free Ca$^{2+}$ ion concentration, (Weisenberg, 1972). In addition, there is evidence for the involvement of a large-scale phosphorylation of histone H1 in this condensation. It is intriguing to discover that the factors one would suppose to be instrumental in the maintenance of diffuse chromatin may in fact be required for the condensation of chromosomes.

There is no simple explanation of these findings. Nevertheless, two suggestions can be made to account for this particular type of phosphorylation during the period of chromosome condensation. Forces of attraction may be set up between the nucleosomal histones and histone H1 when the latter is heavily phosphorylated. Assuming some specificity in the positioning, or the phosphorylation, of histone H1 molecules, it may be that the histones, (and their
associated DNA sequences), caused to attract one another are those from the same presumptive chromosome. In other regions of the chromatin, nucleofilaments from different chromosomes may repel one another by means of unneutralised DNA phosphates. Therefore, the situation would be one involving chromosome condensation and separation, (by histone-histone attraction forces and DNA-DNA repulsion forces respectively). The high degree of specificity of histone H1 function could result from a non-random distribution of the phosphorylated molecules; however it is possible that specificity could be conferred by the simultaneous modification of other histone molecules, or of acidic proteins.

The second proposal for the role of histone H1 phosphorylation in chromosome condensation differs from the first in that it does not assume that condensation is caused by the phosphorylation. Instead, I would suggest that the bulk phosphorylation of histone H1 causes chromatin decondensation by repulsion between histone-histone and histone-DNA phosphates. Once this is achieved a secondary process occurs which enables nucleoproteins from different presumptive chromosomes to condense separately. This condensation could result from the specific dephosphorylation of DNA-binding acidic proteins. Repulsion between nucleoproteins from different chromosomes could be by the histone H1-phosphates, (assuming the phosphorylated molecules to have some specificity of position), or alternatively by phosphorylation of specific acidic protein fractions. In this theory, histone H1 phosphorylation can be regarded as a mechanism to bring about the total decondensation of
Only when this is achieved, perhaps, can organised chromosomal condensation occur.

No direct 'cause and effect' relationship has been established for histone H1 phosphorylation and chromosome condensation, although they do appear to be simultaneous dependent events. The second proposal is, therefore, the more favourable of the two since it invokes only a temporal, not a causal, relationship between the two events. In addition, there are numerous reports of specific phosphorylations and dephosphorylations of the nuclear acidic proteins through the cell-cycle; perhaps chromosome condensation results from certain of these changes.

Whatever the mechanism of chromosome condensation, evidence suggests a requirement for the bulk phosphorylation of histone H1. There is, therefore, the possibility of a 'control point' within the cell-cycle after DNA synthesis, but prior to mitosis. Inhibition of the activity of 'growth-associated' histone H1 phosphokinase would prevent phosphorylation of histone H1, and thereby inhibit chromosome condensation and entry into mitosis. Cell 'arrest' in G2 would be the result. This will be discussed further in the following section.
The division cycle represents a progression of well-organised and contiguous metabolic events within a cell. Not only must these events be controlled with respect to one another but they must also be 'sensitive' to the internal and external environments if the cell is to survive. That the cell-cycle is under some form of direct or indirect genetic control would be undisputed, but it is only in recent years that definitive investigations have illustrated this control in eukaryotic cells.

Temperature-sensitive mutants have been isolated from many mammalian cell lines. Kane et al. (1976), have reported a mutation, 'arresting' cells in the G1-phase of the cell-cycle. Decrease in RNA-synthesising ability and ethidium bromide binding capacity of chromatin are correlated with the presence of this mutation. In hybrids between human cells and temperature-sensitive hamster cells, Ling et al. (1976), have shown that human chromosome 3 is responsible for conferring to the hybrids the ability to grow at the non-permissive temperature for the hamster cells; (i.e. the ability to overcome a G1 'block'). In a most interesting paper Hartwell et al. (1974), presented details of experiments on the Yeast cell-cycle. Mutants, each defective in one of the cell-cycle events, were isolated from haploid cells and complementation studies attempted. It was found that complementation, in the true genetic sense, was possible. These findings, together with other reports in the literature, suggest the genetic nature of the control of the cell-cycle.
In more natural, and less experimental, systems there appear to be 2 major points of 'arrest', (or 'blocks'), in the cell-cycle. Van't Hof has called these the Principal Control Points, (Van't Hof and Kovacs, 1972). One is in G.1 and the other, (found mainly in plant tissues), is in G.2. They can be regarded as points at which 'mitotic commitment' takes place in the pre- and post-synthetic periods respectively. The reason for cell 'arrest' is considered to be the lack of proteins for the G.1 → S or the G.2 → M transitions. In general, though, we can suppose that the cells are 'arrested' because the metabolic conditions required for the cell-cycle to proceed have not yet been achieved. Now, we may ask, how are these conditions achieved, and what is the nature and form of the 'triggering' event in each case?

Metabolic processes occurring during G.1 and G.2 are poorly understood, therefore the most fruitful way of studying the two 'commitment' events is to follow, respectively, the ensuing processes of DNA replication and chromosome condensation. Presumably, apparent commitment to DNA replication in some way reflects the passage of cells through the control point in G.1, and similarly, commitment to chromosome condensation reflects passage through the control point in G.2.

Rao and Johnson, (1970), have shown that a nucleus in G.1 begins DNA replication if the cell is fused with a cell that is in S-phase. In addition, work on hybrids made between cell-types having different cell-cycle schedules has shown that replication of DNA from the different strains in the hybrid begins simultaneously,
These findings argue for the involvement of a 'switch' mechanism in the initiation of DNA synthesis, rather than a gradual build-up during G1 of the ingredients for DNA replication. Steinhardt and Mazia, (1973), found that the polarization of the fertilized sea urchin egg following its first interaction with the sperm could be induced in an unfertilized egg by exposure to NH₄OH. (The egg remains unfertilized by the usual criteria and can be subsequently fertilized.) Chromosome replication normally begins soon after fertilization, and it was found that a true DNA replication did in fact occur in these cells after NH₄OH treatment, (Mazia and Ruby, 1974). This experiment demonstrates that, given the ingredients for DNA synthesis, the 'switch' mechanism initiating the process can be a simple change in the intracellular environment, (a pervasive 'change of state' of the cell).

The second Principal Control Point in the cell-cycle, governing passage of cells from G2 to mitosis, appears, at least superficially, to act in the same manner as the first Principal Control Point. Again, cell fusion studies have forced us to consider a 'switch' or 'signal'. Johnson and Rao, (1970), have shown that chromosomes of a cell in any stage of interphase will be forced into condensation when the cell is fused with one in which the chromosomes are condensed. Likewise, the presence of a pervasive 'switch' has been indicated by Mazia, (1974).

The triggering mechanisms for passage of cells through these major control points thus appear to be relatively simple in nature and non-cell-specific. They could, therefore, represent responses
to intracellular and extracellular molecular, (or ionic), environments. This suggestion of a direct monitoring and response by a cell to its environment has certain aesthetic qualities, since it is unreasonable to propose that the metabolism of a cell is not governed in any way by external influences.

Once a triggering process has occurred this would presumably initiate further, more complex, metabolic events, (such as DNA replication). I would propose that the increase in 'growth-associated' histone H,1 phosphokinase activity during G,2 is one such event occurring after passage through the second Principal Control Point. The resulting phosphorylation of histone H,1 may then, in some way, stimulate chromosome condensation.

To summarize; two Principal Control Points have been invoked to explain cell-'arrest' in the G,1 and G,2 phases of the division cycle. Both controls are suggested to operate via a simple 'switch' mechanism, triggered by the ionic or molecular environment of the cell. Once these control points have been passed the cells enter parts of the G,1 or G,2 phases leading to, respectively, DNA replication, or chromosome condensation and mitosis. (Cells in the pre-synthetic phase which are not 'committed' to progression through the division cycle are frequently said to be in a G,0-phase. Those which pass the first Principal Control Point and have become 'committed' to cell division are in the true G,1-phase.) It is conceivable that the 'switch' mechanism involved in passage of a cell through the second Principal Control Point is responsible for activation or synthesis of a specific histone H,1 phosphokinase.
I therefore present the notion that bulk phosphorylation of histone H4 is not only a prerequisite for chromosome condensation and mitotic cell division, but also a reflection of the operation of a 'mitotic trigger' mechanism in G2.
This thesis has presented details of investigations into phosphorylation of histone H1 in dividing and non-dividing tissues, and through the mitotic cell-cycle. The importance of this phosphorylation in the regulation of the cell-cycle has been discussed; however it is evident that work of this nature is in its infancy, and much more remains to be done.

The tendency for a purely descriptive approach by many researchers into histone phosphorylation has perhaps inhibited the development of other lines of investigation. A more functional approach should now be instigated in order to direct investigations into the purpose of histone phosphorylation. In this way, research may lead to a much better understanding of the cell-cycle and its regulation.
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