RNA METABOLISM IN
CULTURED PLANT CELLS

by

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A thesis submitted for the degree of
Doctor of Philosophy of the University
of Leicester

December, 1971
This thesis is the result of studies carried out between October, 1968 and October, 1971. All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

B. J. Cox

I certify that the above statement is correct.

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Date 18/11/72
ACKNOWLEDGEMENTS

I express my sincere thanks to my supervisor, Professor H. E. Street, for his advice, criticism and guidance during the course of this work, and to Dr. G. Turnock for his advice and assistance with the techniques used in this work.

I also wish to extend my thanks to Mr. E. Singer and the technical staff, especially Miss S. Pearcey and Miss P. Sharman for their help in the preparation of the figures and to Mrs. Barbara Birch for typing this thesis.

I also acknowledge the receipt of an S.R.C. studentship.
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ABBREVIATIONS

DNA       Deoxyribonucleic acid
RNA       Ribonucleic acid
TCA       Trichloroacetic acid
TCA cycle Tricarboxylic acid cycle
BSA       Bovine serum albumen
CMP       Cytidine monophosphate
UMP       Uridine monophosphate
UDP       Uridine diphosphate
UTP       Uridine triphosphate
UDP-glucose Uridine diphosphate glucose
dTTP       Thymidine triphosphate
IAA       Indole-3-acetic acid
Growth and development in higher plants arises from an orderly sequence of cell division, cell expansion and differentiation. It is characteristic of such development that a cell or group of cells, once set upon a particular route or pathway of development, continues inexorably along this pathway unless it is diverted by a fresh set of signals. Some of the agents which can cause alteration of developmental pathways have been identified but much less is known of the factors which control the progress of a cell, or cells, along and to the completion of a given pathway.

A striking feature of development is that a complex sequence of events can flow from a single act of induction. An example in higher plants is the process of flowering in which, as flower development proceeds, the meristematic apex swells, sequential flower primordia are initiated, the individual organs of the flower, sepals, petals and ovary appear, meiosis taking place and so on, all from an initial act of induction. It is as though the plant contained a series of preprogrammed plans of development which are selected and called into action by the presence of the appropriate signals or inducer substances. Such a concept immediately raises a number of questions: Of what does a developmental programme consist? How is it initiated and terminated? How do the individual cells achieve and maintain the correct morphology, physiology and biochemical characteristics for tissue and organ development? Are such developmental programmes reversible or, once determined, does a line of cells proceed inevitably upon the induced pathway of development?

The physiological and biochemical processes that characterise
a particular cell type are ultimately conducted under the auspices of enzymes. Protein synthesis must therefore be considered to be at the centre of control of growth and development, and factors which bring about marked changes in cell growth and development must be considered to have some effects, either directly or indirectly, upon protein synthesis.

The "central dogma" of molecular biology, founded upon the work of Crick and Watson (1953), considers that the information for protein synthesis is contained in the molecular structure of DNA and that there is a unidirectional flow of information from DNA to RNA, which then codes for the proteins that will determine the structure and function of the cell.

The observations that DNA is conserved throughout cell division and differentiation in living plant cells (Swift, 1950; Rasch and Woodward, 1959) and that cell cultures contain cells that are totipotent (possess the ability of forming embryo-like structures and in some cases the adult plant, see review by Steward, 1969) has led to the general view that differentiation does not involve loss of genetic material. This therefore provides a chemical basis for the concept of the developmental programme. It is possible to envisage the living cell as containing all the necessary information for development of the whole plant as a number of discrete programmes contained in the genome which can be switched on or repressed by specific inducing or repressing influences. An indication of how a genetic programme may be sequentially translated into the various events of cell division and differentiation comprising a developmental pathway comes from the work of Wetmore and Hie (1963).
They demonstrated that the introduction of sucrose and an auxin into callus cultures resulted in the formation of distinct rings of vascular tissue; the initiation not simply of randomly located centres of differentiation but a pattern of differentiation. Only cells occupying a particular position in the callus relative to the source of sucrose and auxin therefore show vascular differentiation, clearly implying that the cells can sense developmental stimuli from the environment about them. Gradients of nutrients and growth regulators in a tissue, the presence or absence of other cells and physical factors such as light and pressure together reflect the position of the individual cells and are the factors which control their development. The relationship between these factors and the way in which they interact with the genetic control of protein synthesis is therefore the central problem of growth and development in higher plants.

This is, however, a difficult subject to study experimentally. The interactions of an array of plant growth regulators with protein synthesis, over and above nutrient supplies, have recently received a lot of attention and out of this work has arisen a growing literature concerned with the relationship of nucleic acid metabolism to regulation of growth and development. Summaries of this recent work can be found in reviews by Key, 1970; Trewavas, 1968; Loening, 1967a; van Overbeek, 1965; Paleg, 1965; and Osborne, 1965. Although it has been shown that in many tissues responding to growth regulators there is a stimulation or repression of nucleic acid synthesis, it has yet to be demonstrated that these growth regulators exert their primary effect on some part of the protein synthesising machinery. The observations of Anstine, Jacobsen, Scandalios and Warner (1970)
that gibberellic acid initiates \textit{de novo} synthesis of specific peroxidases in germinating barley embryos and the discovery of cell division factors or cytokinins in transfer RNA (Letham and Ralph, 1967; Hall, Robbins, Stasiak and Thedford, 1966; review by Hall, 1970) are examples which suggest that some or possibly all of the growth regulators may eventually prove to have some direct interaction with nucleic acid metabolism at the molecular level.

The generally accepted scheme for the genetic regulation of protein synthesis in bacteria has arisen from the work of Jacob and Monod (1961) and most of the available evidence still supports the overall view that information contained in DNA passes to the ribosomes, the site of protein synthesis, in the form of a rapidly turned over intermediate species of RNA, the "messenger" RNA. A large amount of information is now available of the control of transcription of DNA and the complex system of translation of messenger RNA at the ribosome.

Protein synthesis in eukaryotes appears to be basically very similar to that of bacteria but the structural complexity of higher plant and animal cells and their ability to differentiate imposes a requirement for a much more complex and probably different control of the expression of genetic information. There are many observations, for example, the ability of the unicellular alga \textit{Acetabularia cliftonii} to differentiate several weeks after removal of the nucleus (Hamel, 1963) and the ability of frog oocytes to continue synthesising protein for many hours after enucleation (Smith and Ecker, 1965) which challenge the accepted view of a messenger RNA which is rapidly turned over and which have led some workers (Harris, 1967) to question whether such a species of RNA exists. Very recently,
however, a species of RNA, extracted from the polysomes of mammalian erythrocyte cells in the process of making haemoglobin, has been shown to be capable of stimulating synthesis of this protein \textit{in vivo} in amphibian oocytes into which it had been injected (Gurdon, Lane and Woodland, 1971). This very elegantly demonstrates the messenger function of certain species of RNA and provides a very good system for studying the rate of turnover and translation of eukaryote messenger RNA.

The recent demonstration by Temin and Mizutani (1970) that RNA tumour viruses can replicate DNA in the host cell and insert it into the host genome has raised some very fundamental questions concerning the once supposed unidirectional flow of information from DNA to RNA. The reverse transcriptase enzymes responsible for this phenomenon were found in a number of RNA viruses (Spiegelman, Burny, Das, Keydar, Schlam, Travnicek and Watson, 1970), but this was followed by the discovery (Scolnik, Stuart, Aaronson, Todaro and Parks, 1971) of the enzyme in normal cells from mice and from human skin, and almost every other tissue investigated. The transfer of information from RNA to DNA was classed in the central dogma as a special transfer unlikely to have generality and "it might indeed have profound implications for molecular biology if any of these special transfers could be shown to be general, or if not at least widely distributed" (Crick, 1970). It is not yet clear how widespread this phenomenon may be or whether there is reverse transcriptase function in normal cells as opposed simply to activity (Baltimore, 1970) but on this basis it would be possible to provide theoretical answers to many biological problems, particularly some aspects of
differentiation. It is possible to envisage information transfer between cells in the form of an RNA molecule - perhaps wrapped in a protein coat, travelling between cells and leaving a permanent imprint of itself in the form of DNA integrated into the genome of the cell it visits.

It is clearly necessary to learn more about the basic system of protein synthesis and information transfer in higher plant and animal systems in relationship to differentiation. In addition to the search for improved biochemical techniques for analysing the various aspects of protein synthesis there is also an obvious requirement for control of growth and development of the tissues in which these studies are to be carried out. The development of techniques of plant tissue culture (Gautheret, 1959; Street, 1966; Street and Henshaw, 1966; Street, 1969) provides a new approach to the study of the factors which control the growth and development of higher plants. Such techniques provide an opportunity to study the nutritional requirements of plant cells and their reaction to regulatory factors so that it may eventually be possible to reproduce in vitro the developmental programmes found in the whole plant.

One of the most useful systems for studying the biochemistry of cell division and development is the free cell or suspension culture. An ideal culture for such studies would contain a uniform population of random or synchronously dividing cells, which could be maintained in continuous steady states of growth or "which could be induced en masse to yield specialised cells or organ primordia of one type" (Halperin, 1969).

Suspension cultures are usually initiated by shaking callus
(derived by inducing cell division in explants from whole plants on solidified media) in liquid media. Callus from different sources shows a wide range of variability in its friability or capacity to break up into free cells and small aggregates in liquid medium. The callus formed from the stem cambium of *Acer pseudoplatanus* L. (Lamport, 1960) is particularly friable and forms a fine suspension of free cells and small aggregates when shaken in liquid medium. This tissue has therefore been adopted for extensive studies of the growth of plant cells in liquid culture and a considerable amount of information has been obtained of its cultural requirements and growth characteristics (Henshaw, Jha, Nehta, Shakeshaft and Street, 1966; Stuart and Street, 1969).

It is, however, a feature of plant tissue cultures that the individual cells are not uniform in behaviour or morphology. There is considerable evidence of morphological and cytological instability in callus cultures (Street, 1969), which even single cell cloning cannot exclude (Muir, Hildebrandt and Riker, 1958; Bergman, 1960; Sievert and Hildebrandt, 1965). The causes of such variation are not at present understood or controllable, nor is it clear whether chromosomal aberrations and endopolyploidy arise more frequently in culture than in ageing and differentiated tissues or whether the multiplication of such aberrant nuclei is promoted in culture (Street, 1969). It is therefore necessary to find conditions of culture in which cells of a particular morphological and genetic type can be stabilized.

Suspension cultures of *Acer pseudoplatanus* L. have been propagated by serial batch culture for a number of years. These cultures
have a well defined growth cycle of which the overall form is very reproducible and a number of biochemical and physiological processes have been investigated in relation to the growth cycle (Givan and Collin, 1967; Short, Brown and Street, 1969; Simpkins and Street, 1969; Brown and Short, 1969; Mackenzie and Street, 1970; Fowler, 1971; Wilson, 1971). There are, however, variations in the amount and rate of growth between individual cultures and there are marked changes in the morphology (Sutton-Jones and Street, 1968), physiology and biochemistry of the cells during the various phases of the growth cycle. The exponential phase of cell division, where the culture would be expected to be most uniform and synthetic processes would continue at a steady rate, is very short in duration and restricted to the early part of the division phase. In order to attempt to overcome the variability in growth of the cultures and to establish more uniform populations of cells, culture methods have recently been developed in this laboratory which enable cells to be grown continuously in a number of steady states of random growth or to be maintained in synchronous cell division for a number of full cell cycles (Wilson, King and Street, 1971). These developments provide a much improved system in which to study the physiological and biochemical processes underlying cell growth and development, and, together with techniques of reducing cell aggregation and single cell cloning may eventually lead to the ideal culture conditions specified by Halperin, 1969.

Most of the studies which will be reported in this thesis have been carried out using the batch culture technique. Previous work by K. C. Short has established the characteristic changes in the
levels of total RNA and DNA during the growth of the cultures and has shown that there are large changes in the cellular content of RNA during the various phases of cell growth. The aim of this work is therefore to study RNA metabolism in greater detail which involves, first, the development of techniques of labelling cellular RNA with radioactive precursors in an interpretable manner, and second, the development of techniques of extraction and fractionation of undegraded RNA.

It has not yet been possible to carry out any of these studies with the continuous steady state cultures but some preliminary experiments have already been carried out with synchronous cultures to attempt to define the overall pattern of synthesis of DNA, RNA and protein during the cell cycle. Eventually, it may be possible, using these cultural techniques, to establish the pattern of synthesis and degradation of individual species of RNA during the cell cycle and during a number of steady states of growth, and to relate this to the rate of cell division and to the particular type of cell produced.
GENERAL METHODS OF CELL CULTURE

Glassware and Chemicals

All glassware used in experimental work was Pyrex. New glassware was cleaned with chromic acid as described by Street and Henshaw (1966). Glassware used in radioactive experiments was decontaminated with Beckman decontamination compound and was then cleaned with chromic acid, otherwise all glassware was cleaned in Pyroneg detergent.

All chemicals used in experimental work were of analytical grade unless otherwise stated.

Serial sub-culture of suspension cultures of Acer pseudoplatanus L.

The suspension cultures were derived from callus cultures initiated from the stem cambium of Acer pseudoplatanus L. by Lamport and Northcote (1960) and obtained from Dr. D.H. Northcote (Dept. of Biochemistry, Cambridge). These were serially sub-cultured in a chemically defined medium as described by Street, Collin, Short and Simpkins (1969) but with kinetin omitted. The composition of the culture medium is shown in Table I. All stock solutions were made up fresh every three or four weeks and were checked for microbial contamination before use.

The culture medium was dispensed into the culture flasks and autoclaved at 15lb/sq.in (121°C) for 15 minutes. Urea, which is thermolabile, was obtained in sterile ampoules (Oxoid Ltd.) and was added to the culture medium just before inoculation of the cultures.

70ml batch cultures were grown in 250ml Erlenmeyer flasks and sealed with aluminium foil. The cultures were agitated by mechanical shaking on a horizontal orbital shaker at approximately 120 cycles
### TABLE I

**STANDARD SYNTHETIC CULTURE MEDIUM FOR GROWTH OF SUSPENSION CULTURES OF ACER PSEUODOPLATANUS L.**

<table>
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<th>Constituent</th>
<th>Concentration (mg/mL)</th>
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<tr>
<td><strong>Hellers Inorganic Salt Solution</strong></td>
<td></td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>KCl 75.0</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>NaNO₃ 60.0</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>CaCl₂·6H₂O 11.2</td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate</td>
<td>NaH₂PO₄·2H₂O 13.3</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>MgSO₄·7H₂O 25.0</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>ZnSO₄·7H₂O 0.1</td>
</tr>
<tr>
<td>Boric acid</td>
<td>H₃BO₃ 0.1</td>
</tr>
<tr>
<td>Manganese sulphate</td>
<td>MnSO₄·4H₂O 0.01</td>
</tr>
<tr>
<td>Copper sulphate</td>
<td>CuSO₄·5H₂O 0.003</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>KI 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>1.0</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>10.0</td>
</tr>
<tr>
<td>Cystine hydrochloride</td>
<td>10.0</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.5</td>
</tr>
<tr>
<td>meso-inosital</td>
<td>100.0</td>
</tr>
<tr>
<td>2,4-dichlorophenoxyacetic acid</td>
<td>1.0</td>
</tr>
<tr>
<td>Ferric chloride (Spectroscopically pure)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea (from 40% sterile ampoules)</td>
<td>200.0</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>20g/l.</td>
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pH adjusted to 5.2 with hydrochloric acid
per minute. The shakers were situated in a constant temperature room at 25°C under continuous illumination of approximately 500 lux intensity from tungsten filament lamps. The suspensions were sub-cultured every 21 days by transferring 10ml of stationary phase cells to 60ml of fresh culture medium. All transfers were carried out using sterilized automatic pipettes in a sterile cabinet. The cell density at inoculation was approximately $4 - 6 \times 10^5$ cells per ml and at this high density the cultures were maintained continuously in the synthetic culture medium shown in Table I. The standard synthetic culture medium described by Street, Collin, Short and Simpkins (1969) contains kinetin, but this can be omitted where cultures are initiated at high density without markedly altering the growth of the cultures.

Measurement of growth of the cultures

The three growth parameters of cell number, dry weight and packed cell volume were routinely measured as described by Henshaw et al (1966) with some minor technical modifications.

Packed cell volume was measured by taking samples of cell suspension with an automatic pipette and placing a known volume in a 15ml graduated conical centrifuge tube. The cells were allowed to settle so that a flat surface of the cell mass was obtained and the tubes were then centrifuged at 1000g for 5 minutes in a bench centrifuge. The volume of packed cells was read off the scale on the centrifuge tube and was expressed as a percentage of the total volume of the culture sample.

Dry weight was measured by resuspending the cellular material from the packed cell volume determination and collecting it on a
2.4cm glass fibre filter (Whatman GF/A) under vacuum. The cell material was washed three times with distilled water and was then dried in an oven at 55°C for 24 hours. The dried filters were then placed in a desiccator over silica gel and were dried to constant weight. The dry weight of cell material was then calculated and expressed as mg per ml of culture.

Cell number was measured by counting a dispersed sample of cells in a modified haemocytometer. Samples of cell suspension were added to an equal volume of 12% w/v chromium trioxide in water and the solution was heated at 55°C for 1 hour. It was then allowed to cool for a further 2 or 3 hours after which it was vigorously shaken on a mechanical shaker for approximately 20 minutes. This procedure breaks up the cell clumps and causes the protoplast to shrink thus enabling accurate counting to be carried out. The dispersed suspension was then diluted to a known volume and an aliquot was transferred to a pre-calibrated counting slide with a volume of 0.8μl per viewing field under 100x magnification. At least 30 fields were counted per sample and the standard error between fields was found to be, in general, less than 5%.

Growth of suspension cultures

The pattern of growth of suspension cultures of Acer pseudoplatanus L. in batch culture is well established (Henshaw et al., 1966; Short, Brown and Street, 1969). Fig.1(a) shows a typical growth curve of cell number per unit time for a culture grown in standard synthetic medium, with kinetin omitted, and inoculated at a cell density of 6.0 x 10^5 cells/ml.

Most of the work described in this thesis has been carried out
FIG 1(a)  
TYPICAL GROWTH CURVE OF SUSPENSION CULTURE OF ACER PSEUDOPLATANUS L IN STANDARD SYNTHETIC MEDIUM KINETIN OMMITTED

FIG 1(b)  
DIAGRAMATIC REPRESENTATION OF THE GROWTH CYCLE INDICATING THE VARIOUS GROWTH PHASES

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[Graphs showing growth curves with labels: Cell No. x 10^6 (ml culture), Time (days)]

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[Graphs showing growth phases: Exponential, Linear, Stationary, Decrease, Lag]
with batch cultures grown under these conditions and since reference will constantly be made to various phases of the cell division curve a brief preliminary description of this and some of its implications is necessary.

The growth curve can be divided into three distinct regions (Fig.1b) a lag phase, a phase of cell division and a stationary phase where cell division has ceased. The lag phase has a duration of approximately three days after which cell division commences. There is some evidence of a high degree of synchrony in the first division which then rapidly disappears during subsequent divisions. The rate of cell division during the early part of the division phase (4 – 6 days) is greater than at later times and the cell number increase during this period is very near to that of a culture exhibiting exponential growth. After this period, however, it can be seen that the increase of cell number with time is linear, not logarithmic, which means that either the rate of cell division is falling or only a proportion of the cells are dividing. Towards the end of the division phase (12 – 16 days) the rate of cell division rapidly decreases and finally ceases after approximately 18 days. The cells then enter a stationary phase when there is often an apparent small fall in cell number. It is not clear, however, if there is actual lysis of cells or simply an increased sensitivity to the fixative used for cell counting which causes some cells to break.
SECTION I

STUDIES OF THE INCORPORATION AND METABOLISM
OF RADIOACTIVE URIDINE IN SUSPENSION CULTURES
OF ACER PSEUDOPLATANUS L.
Introduction

In studies of the biosynthesis and turnover of macromolecules, exogenous radioactive precursor molecules are used as a means of introducing a label into the large molecules to trace the sequence in time of the various steps in their synthesis and degradation. An extensive literature exists describing investigations in which both specific and non-specific radioactive nucleic acid precursors have been supplied to higher organisms as well as to bacteria and other microorganisms.

The introduction of labelled precursor molecules into the cells, and thence into their macromolecules, relies upon the fact that although living cells maintain high concentrations of internally synthesised molecules, they are in many cases capable of concentrating small molecules from the external environment. Many of these small molecules are intermediates in macromolecular biosynthesis and are collectively termed the pool of metabolic intermediate or simply the "pool". The characteristics of incorporation of externally applied radioactive precursors into macromolecules will therefore depend upon a number of important factors: (i) the characteristics of entry of externally applied substances into the pool; (ii) the possible compartmentalization of compounds in the pool; and (iii) the relationship of compounds in the pool to synthesis and degradation of macromolecules.

Molecules obtained from the external environment may not necessarily exist in the same chemical state or occupy the same sites in the cell as internally synthesised molecules. Equally they may not necessarily share the same chemical fate as the endogenous
molecules. Since the spatial organisation of the cellular precursor pools and the mechanisms by which they are maintained is not at present understood, the term "pool" will, for the purposes of this investigation, be defined as "the total quantity of low molecular weight compounds that can be extracted from the cell under conditions such that the macromolecules are not degraded into low molecular weight sub-units" (Britten and McClure, 1962).

A number of studies have been made of the behaviour of exogenously applied substances in pool formation and macromolecular synthesis in microorganisms. The kinetics of incorporation of labelled bases into the pool and into the RNA of *E. coli* and *B. subtilis* (Gros, Gilbert, Hiatt, Attardi, Spahr and Watson, 1961; Salser, Janin and Levinthal, 1968; Buchwald and Britten, 1963; Nierlich, 1967; Nierlich and Vielmetter, 1968) have been studied in sufficient detail to allow the formulation of theoretical models of the formation of RNA precursor pools and their relationship to RNA synthesis. In higher organisms, however, although radioactive nucleic acid precursors have been extensively used to follow the synthesis and turnover of the various species of RNA, very little information is available regarding the formation of precursor pools and their relationship to macromolecular biosynthesis.

Suspension cultures of plant cells, free from contamination by microorganisms and which can be sampled quickly and easily, are a suitable experimental tool for investigating the short term kinetics of incorporation of radioactive nucleic acid precursors. Suspension cultures of *Acer pseudoplatanus L.* in the early part of the division phase of the growth cycle are sufficiently homogeneous and near to
exponential growth to attempt a critical examination of pool formation and its relationship to RNA synthesis by feeding a specific labelled precursor of RNA. $^{3}H$-uridine and $^{3}H$-cytidine have been shown to be incorporated into the RNA and DNA of wheat leaves (Bhattacharyya and Shaw, 1967) and Wasilewska and Reifer (1967) have shown that 2-$^{14}C$-uridine and 2-$^{14}C$-uracil are actively metabolised in wheat, green peas and spinach plants. However uridine was several times more active as a precursor of pyrimidine nucleotides than uracil and the plant homogenates retained a high uridine kinase activity.

A series of experiments has therefore been performed in which the kinetics of incorporation of radioactive uridine into the pool and into the RNA of plant cells in suspension cultures have been investigated. In addition, the effects of uridine upon growth of the cultures, and upon internal synthesis of uracil compounds and the composition of the pool has been investigated. The information gained from these experiments has then been used to study the turnover of RNA during the various phases of the growth cycle.
Materials and Methods

The incorporation of radioactive precursors of RNA into the pool and into the macromolecules of suspension cultures of *Acer pseudoplatanus* L.

Radiochemicals

2-\textsuperscript{14}C-uridine and 5-H\textsuperscript{3}-uridine were obtained in sterilized aqueous solution from the Radiochemical Centre Ltd., Amersham at specific activities of 62mCi/mM and 15000mCi/mM respectively. Labelled compounds were fed in small volumes to cultures under sterile conditions using a Hamilton syringe or sterilized pipette. Where appropriate the specific activity was reduced by the addition of unlabelled uridine, sterilized by autoclaving.

Cell Culture

Suspension cultures of *Acer pseudoplatanus* L. were grown as previously described in standard synthetic medium at 25°C and at an inoculation density of between 4 and 6 x 10\textsuperscript{5} cells per ml.

Measurement of the incorporation of RNA precursors into the pool and the macromolecules of plant cells in suspension culture.

The incorporation of labelled precursors of RNA into the cellular pool and into macromolecules was measured by a modification of the rapid filtering technique of Britten, Roberts and French (1955).

Samples of cell suspension were taken with an automatic pipette or, for small samples of less than 1.0ml volume, a Marburg pipette.

Total incorporation was measured by passing samples of cell suspension very rapidly through glass fibre filters (Whatman GF/C 2.4 cm diameter) under vacuum. The cellular material collected was
then washed 5 times with medium containing unlabelled precursor at 100 times the concentration of the radioactive precursor fed to the cells. The filters were then dried at 55°C for at least 6 hours, usually overnight, and were stored for scintillation counting.

Incorporation into the macromolecular fraction of the cell was measured by adding samples of cell suspension to an equal volume of 10% w/v TCA containing a 100 times excess concentration of unlabelled precursor. These were allowed to stand for 2 hours at 0°C with occasional shaking. The TCA-insoluble precipitate was then collected on glass fibre filters and was washed 5 times with cold 5% w/v TCA. The filters were dried as before and stored for scintillation counting.

The incorporation of radioactive precursor into the pool or acid-soluble fraction of the cell was estimated as the difference between the total and TCA-insoluble incorporation.

To minimise binding of radioactive precursors the filters were equilibrated with medium or 10% w/v TCA, containing a 100 times excess concentration of unlabelled precursor, for several hours before use. In addition a zero time sample was always taken to distinguish between non-specific binding of radioactive precursor to the filters or cellular material and actual incorporation in subsequent samples. The zero time control is shown on all graphs in the results section unless otherwise stated.

Estimation of the distribution of radioactivity in the TCA-insoluble fraction.

The distribution of radioactivity in the TCA-insoluble fraction of the cell between RNA, DNA, protein and non-nucleic acid components was estimated by selective degradation of the TCA-insoluble precipitates.
Total nucleic acids were removed by treatment of the TCA-insoluble precipitate with hot TCA to obtain an estimate of the amount of radioactivity present as protein and non-nucleic acid material. RNA was removed from another sample of the TCA-insoluble precipitate by treatment with warm dilute alkalis (Schmidt and Thannhauser, 1945) under conditions which do not degrade DNA or protein. The relative proportions of radioactivity in RNA and DNA can then be calculated, the RNA from the difference between the total TCA-insoluble radioactivity and the radioactivity remaining after treatment with dilute alkalis (DNA + protein and non-nucleic acid components), and the DNA from the difference between the radioactivity remaining after treatment with dilute alkalis (DNA + protein and non-nucleic acid components) and the radioactivity remaining after treatment with hot TCA (protein and non-nucleic acid components).

The Schmidt-Thannhauser procedure has been shown (Short, Brown and Street, 1969) to be the most suitable available method for the quantitative extraction and separation of nucleic acids from this tissue. TCA insoluble extracts of cell suspension were washed once with cold 5% w/v TCA, resuspended in 0.3 N KOH and were incubated at 37°C for 18 hours. The resulting solution was cooled to 0°C and made 10^{-3}M with MgCl₂. The pH was adjusted to 1.6 with 50% w/v TCA and 2 volumes of absolute ethanol at -18°C were added to facilitate the precipitation of DNA and protein (Smillie and Krotkov, 1960). The solution was then stored for several hours at -18°C. The precipitated material was collected on glass fibre filters, washed 5 times with cold 5% w/v TCA, dried and stored for scintillation counting.

A hot acid treatment was employed to remove total nucleic acids
and leave an insoluble fraction consisting predominantly of protein. TCA-insoluble extracts of cell suspension were washed once with cold 5% W/v TCA, were resuspended in hot 5% W/v TCA containing 100μg/ml carrier BSA and were incubated at 90°C for 20 minutes. The solution was allowed to cool to 4°C and the remaining insoluble material was collected on glass fibre filters, washed 5 times with cold 5% W/v TCA, dried and stored for scintillation counting.

Osborne (1962) has reported that the Schmidt-Thannhauser degradation of cold TCA precipitates of plant material removed not only RNA but also some protein. This could lead to possible errors in the determination of the proportion of radioactivity contained in RNA if there is a high level of labelling in protein. Hot acid digests of cells labelled with uridine have, however, shown that only a very small amount of incorporation of radioactivity into protein and non-nucleic acid components occurs, especially in short term experiments of only a few hours duration (see results section). It is therefore probable that the estimation of the relative proportions of radioactivity in RNA and DNA is not seriously affected by possible solubilization of protein in the dilute alkaline treatment.

Measurement of removal of radioactive compounds from the medium

Samples of culture were rapidly chilled to 0°C in ice and were then centrifuged at 3000g for 5 minutes to sediment cellular material. The medium was then removed with a pipette and stored for scintillation counting.

Scintillation counting

Samples dried onto glass fibre filters and samples of medium were counted in a Beckman LS-100 scintillation spectrometer using Beckman "Fluoralloy" DXA universal scintillation cocktail of the following composition:-
8.0 gm/l Butyl-PBD
0.5 gm/l PBBO
1.0 gm/l Cab-O-Sil™
100 gm/l Naphthalene

Scintillation grade dioxane

Butyl-PBD (2-(4'-t-Butylphenyl)-5- (4''- biphenyl)-1,3,4-oxadiazole.
PBBO 2-(4-Biphenyl)-5-phenyl-1,3,4-oxadiazole.
Cab-O-Sil Registered trade mark of Cabot Corp., Boston.

This scintillation cocktail can be used in the Beckman room temperature system for counting aqueous samples or samples dried onto glass fibre filters.

Samples of medium were counted with a ratio of medium to scintillation cocktail of 1:15 which gave a counting efficiency of approximately 70% for carbon-14. Samples dried onto glass fibre filters were counted in the minimum amount of scintillation fluid required to cover the filter with an efficiency slightly greater than 70% for carbon-14 which was slightly higher than that obtained in a toluene-based scintillator. Both the aqueous and glass fibre filter samples were counted with a maximum variation of less than 5% in counting efficiency, as indicated by external standard channels ratio measurements, and the results are therefore expressed directly as counts per minute.

Counting of samples dried onto filters has the advantages that the samples are easily prepared and are counted under identical conditions at high efficiency. Glass fibre filters (Whatman grade GF/C) have been preferred to millipore or collodion membranes as they are relatively inexpensive and retain plant materials easily without
clogging. In addition they have been shown (Davies and Cocking, 1966) to cause less quenching than do other kinds of filter. The disadvantages of this method are that variation in counting efficiency may result from changes in the orientation of the filters within the counting vial or by self absorption of radioactivity by the sample. The filters were therefore folded against the inside surface of \(1\frac{1}{2}''\) x \(\frac{3}{8}''\) disposable glass vials. This ensured that all filters were counted with the same counting geometry and in addition enabled a reduced volume of scintillation fluid to be used. The relationship between the volume of cell material on the filters and the amount of radioactivity counted was investigated by adding increasing amounts of TCA-insoluble precipitate or whole cells from a six day-old culture (cell density \(1.0 \times 10^6\) cells/ml, dry weight 4.28mg/ml), labelled with \(^{14}\text{C}-\text{uridine}\) or \(^{3}\text{H}-\text{uridine}\), to glass fibre filters. A linear relationship was obtained for \(^{14}\text{C}-\text{labelled}\) samples up to a maximum volume of 1.5ml for the TCA-insoluble material and 1.6ml for whole cells on 2.4cm diameter filters. The size of samples was always kept well within these limits and self absorption was therefore not an important source of error. With \(^{3}\text{H}-\text{labelled}\) samples, however, self absorption became an important factor at much smaller volumes of cell material and therefore \(^{14}\text{C}-\text{labelled}\) compounds have been preferred for most of the experiments in this investigation.
Measurement of $^{14}\text{CO}_2$ evolution from plant cell suspension cultures

1) The kinetics of $^{14}\text{CO}_2$ evolution

The kinetics of evolution of $^{14}\text{CO}_2$ from actively dividing cultures fed 2-$^{14}$C-uridine has been investigated using the specially designed culture vessel shown in Fig. 2a. Cultures were grown in the usual way for 6 days with aluminium foil covering the tops of the flasks. Two hours before addition of isotope the foil was replaced with a ground glass stopper, 10% w/v KOH was placed in the side arm of the flask and the sampling device was fitted. The culture was then replaced on the shaker to equilibrate for two hours. The isotope solution was added and small samples of the KOH (0.25ml) were taken at zero time and then at regular intervals for several hours. The large volume of KOH contained in the side arm of the flask, kept in continual motion by the shaking of the culture vessel, enabled a large number of samples to be taken. These were then stored frozen for scintillation counting. The total $^{14}\text{CO}_2$ released by the cultures with time could therefore be calculated, taking into account the gradual reduction in the volume of KOH by continued sampling.

Evolution of $^{14}\text{CO}_2$ through the growth cycle

The kinetics of $^{14}\text{CO}_2$ evolution throughout the growth cycle of cultures fed 2-$^{14}$C-uridine at inoculation were investigated using a standard culture vessel fitted with a centre well (Fig. 2b). The cultures were inoculated in the usual way and 2-$^{14}$C-uridine was added to the medium under sterile conditions. 3.0mls of 10% w/v KOH, sterilized by autoclaving at 151°lb/sq.inch for 15 minutes, were added to the centre well with an automatic pipette. KOH was removed from
FIG. 2 (a)

SIDE-ARM FLASK FOR MEASURING THE SHORT TERM KINETICS OF $^{14}$CO$_2$ EVOLUTION

- Ground glass stopper
- 1ml syringe
- Luer lock needle
- Rubber stopper
- Flexible sampling tube
- 10ml KOH
- Culture

FIG. 2 (b)

CENTRE-WELL FLASK FOR MEASURING $^{14}$CO$_2$ EVOLUTION THROUGH THE GROWTH CYCLE

- Flask sealed with aluminium foil
- Centre well containing 3ml KOH
- Culture
the centre well at regular intervals (carefully washing the centre well out with sterile distilled water which was then added to the KOH) and replaced with fresh absorbant. The total $^{14}$CO$_2$ evolved by the culture over a period of time could thus be determined and used to calculate the total amount of $^{14}$CO$_2$ released with time throughout the growth cycle.

Aluminium foil was used to cover the flasks throughout the culture period since complete sealing of the flasks would have markedly affected the growth characteristics of the culture.

The effectiveness with which both types of modified culture vessel could trap the CO$_2$ evolved by the cultures was estimated by comparing the efficiency with which a standard amount of $^{14}$CO$_2$ was trapped in comparison with that trapped by a Warburg manometric flask. Identical amounts of $^{14}$C-sodium bicarbonate were added to the flasks in 0.01 M Tris buffer, pH 7.8, and $^{14}$CO$_2$ was rapidly released by the addition of 3N H$_2$SO$_4$. The total amount of $^{14}$CO$_2$ trapped by each flask in 30 minutes was then measured by scintillation counting. The stoppered culture vessel with the side arm was found to be 88% as efficient as a Warburg manometric flask in trapping $^{14}$CO$_2$. The aluminium foil covered culture vessel was slightly more efficient and trapped 90% of the $^{14}$CO$_2$ trapped by the Warburg flask.

Measurement of the proportion of incorporated 2-$^{14}$C-uridine degraded to $^{14}$CO$_2$ by the sycamore suspension cultures.

In order to estimate the proportion of incorporated 2-$^{14}$C-uridine which is degraded by plant cell suspension cultures with release of the carbon-14 atom as $^{14}$CO$_2$, a balance sheet was compiled of the total
loss of carbon-14 radioactivity from the medium, the total amount incorporated by the cells and the total amount released as $^{14}\text{CO}_2$. An effective balance sheet can only be obtained by converting all the radioactive samples to the same chemical composition for scintillation counting. The total $^{14}\text{CO}_2$ evolved by cultures was therefore collected and samples of medium and cell material were degraded to CO$_2$ using the persulphate oxidation described by Osburn and Werkman (1952) and adapted by Weinhouse (1949) and Anthony and Long (1952) for carbon-14 assay.

Small volumes of cell suspension (approximately 5.0mls) were transferred, under sterile conditions to small flasks containing 2.0mls of CO$_2$-free 10% w/v KOH in a centre well. Two flasks were set up at each concentration, one containing the cell culture, the other containing an equivalent amount of culture medium filtered free of cells. Both flasks were then fed an identical amount of 2-$^{14}$C-uridine. The flask containing the cell culture was sealed with a Suba-seal stopper and incubated on a rotary shaker (120 rpm) at 25°C for a period of time dependant upon the concentration of uridine supplied and determined from the kinetic experiments of $^{14}\text{CO}_2$ evolution. The cultures were then cooled to 0°C, the stoppers were removed and the KOH was carefully removed from the centre well and stored frozen. The cells were collected on glass fibre filters (Whatman GF/C), washed five times with medium containing an excess of unlabelled uridine and the washings were added to the "stale" medium. The fresh medium from the replicate flask, the stale medium plus the cell washings and the cell material were carefully transferred to 100ml centre well flasks. About 500 to 600 mg of solid potassium persulphate were
placed in each flask so that none of the salt was introduced into the centre well. Water was then added to give a volume of from 5 - 15 mls and the mixture was acidified with a few drops of dilute sulphuric acid to liberate any carbonate present. 1.0ml of 4% w/v silver nitrate solution was added to the mixture and 2.0mls of CO\textsubscript{2}-free 10% w/v KO\textsubscript{H} were placed in the centre well. The flasks were closed with Suba-seal stoppers and were evacuated by inserting a 20-22 gauge hypodermic needle connected to a vacuum line in order to remove any dissolved CO\textsubscript{2} and provide a tight seal for the flask during combustion. The flasks were then placed in an oven at 70\degree C. The contents of the main well darkened and gas evolution commenced until at the end of the combustion period the dark colour disappeared and the solution became water clear. The flasks were then allowed to cool on the bench for several hours and the KO\textsubscript{H} of the centre well was carefully removed and stored frozen for scintillation counting.

The amount of carbon-14 radioactivity removed from the medium could therefore be calculated and compared directly with the amount of radioactivity present in the cells and the total amount evolved as \textsuperscript{14}CO\textsubscript{2}.

Scintillation counting of \textsuperscript{14}CO\textsubscript{2} trapped in KO\textsubscript{H}.

The investigation of the kinetics of \textsuperscript{14}CO\textsubscript{2} evolution by suspension cultures of \textit{Acer pseudoplatanus} L. required the development of an efficient and rapid means of trapping \textsuperscript{14}CO\textsubscript{2} and counting the radioactivity of a large number of samples quickly and reproducibly.

The measurement of \textsuperscript{14}CO\textsubscript{2} derived from biological systems has received considerable attention. Many workers have measured \textsuperscript{14}CO\textsubscript{2}
by precipitation as $\text{Ba}^{14}\text{CO}_3$ which is either counted on planchets or in suspension in liquid scintillator. In order to obtain reliable results with this method great care has to be exercised with sample preparation since trace elements and moisture can cause serious interference with the count and the maximum amount of $\text{Ba}^{14}\text{CO}_3$ which can be measured at any one time is severely limited. This method was found to be unsuitable for measuring $^{14}\text{CO}_2$ evolved from the plant cell suspension cultures as the amounts of $\text{Ba}^{14}\text{CO}_3$ obtained were very large and varied quite markedly between some samples thus complicating sample preparation.

Hyamine has been extensively used as a trapping agent for $^{14}\text{CO}_2$ and has the advantage that it can be counted in toluene or xylene-based scintillators. Hyamine, however, is supplied dissolved in methanol and cannot therefore be used to trap $^{14}\text{CO}_2$ directly from plant cell suspension cultures as growth of the cultures is adversely affected by methanol vapour. The method originally described by Passman et al (1956) which involved trapping $^{14}\text{CO}_2$ in KOH or Ba(OH)$_2$ then releasing it with acid and retrapping in hyamine was therefore investigated. It was found however, that a gaseous product of the cells carried over into the hyamine slowly produced a red colouration in the counting vials with accompanying fall in the counting efficiency due to colour quenching.

It was therefore decided to investigate the possibility of trapping $^{14}\text{CO}_2$ in KOH and counting the radioactivity directly. Harlan (1961) has described trapping of $^{14}\text{CO}_2$ in sodium hydroxide followed by counting of the resultant Na$_2$CO$_3$ or NaHCO$_3$ in a water miscible scintillation cocktail to which has been added a thixotropic gelling
agent such as Cab-O-Sil (Registered trade mark of Godfrey L. Cabot, Inc.). Sauermann (1966), Bhagavan, Coursin and Dakshinamurti (1965) and Passeron, Savageau and Harary (1968) have also counted $^{14}\text{CO}_2$ trapped in NaOH or KOH directly in water miscible scintillators with or without a thixotropic gelling agent.

A number of water-miscible scintillators have been examined in the Beckman LS-100 room temperature spectrometer and the toluene/Triton X-100 scintillant of Patterson and Greene (1965) has been found to be the most useful of those investigated for direct counting of KOH in this system. KOH solution must be added to the scintillator to at least 12% v/v to obtain reliable counting. The counting efficiency was approximately 30% and was unaffected by the molarity of $K_2\text{CO}_3$ and only slightly affected by large changes in the alkalinity. The addition of finely divided silica powder was found to increase the counting efficiency to approximately 45% and the scintillator was therefore used with the following composition:

\[ 5.5 \text{ gml/l PPO (2,5-Diphenyloxazole)} \]
\[ 2:1 \text{ v/v toluene:Triton X-100} \]
\[ 5 \text{ gml/l fumed silica powder (Beckman Tube-O-Gel}^{\text{TM}}) \]

The results are expressed directly as counts per minute since in any one experiment all the samples were treated in an identical way, the amount of $^{14}\text{CO}_2$ trapped as $K_2^{14}\text{CO}_3$ being the only variable, and the maximum variation in counting efficiency was less than 5% as indicated by external standard channels ratio measurements.
Measurement of the efficiency of incorporation of $^{14}$C-uridine into the RNA of suspension cultures of *Acer pseudoplatanus* L.

The efficiency of incorporation of $^{14}$C-uridine into the RNA of sycamore cells in suspension cultures was investigated by comparing the specific activity of carbon-14 in each of the four constituent bases of RNA with that of the labelled uridine fed to the cells.

Plant cell suspension cultures were fed 2-$^{14}$C-uridine at high external concentration, approximately $10^{-3}$ M (the specific activity diluted with unlabelled uridine), at inoculation, and were grown under standard cultural conditions for 6 days. An aliquot of medium was removed immediately after addition of the isotope and was stored for determination of the specific activity of the labelled uridine. After 6 days the cells were harvested, washed once in cold distilled water and then homogenized in 5% w/v TCA at 0°C for 1 minute with a Polytron homogenizer. The extract was allowed to stand for 1 hour at 0°C and was then centrifuged at 10,000g for 20 minutes at 0°C. The pellet was resuspended twice in cold 5% w/v TCA and after the final centrifugation was resuspended in 0.3 N KOH. This suspension was then incubated for 18 hours at 37°C to hydrolyse RNA to its constituent nucleotides. At the end of the incubation period the solution was quickly cooled to 0°C and centrifuged at 10,000g for 5 minutes. The supernatant was carefully removed with a pasteur pipette and adjusted to pH 7 by adsorption of KOH on pyridinium Dowex ion exchange resin (Dowex 50x 2-200, dry mesh 100-200, exhaustively washed to remove U.V. absorbing impurities and converted to the pyridinium form by washing in 10% v/v aqueous pyridine). The resin was added directly to the RNA hydrolysate and was removed by low speed centrifugation.
The solution was then reduced to small volume in vacuo on a rotary film evaporator and was loaded directly onto chromatograms.

The four major nucleotides of RNA were separated by two dimension-
al thin layer chromatography on plastic sheets precoated with cell-
ulose, MN - Polygram CEL 300 (Camlab Ltd.) in the following solvents:-
Dimension I isobutyric acid/ ammonia/ water (100:4:2:55:8)
Dimension II isopropanol/ conc. hydrochloric acid/ water (130:33:37)
The precoated plastic sheets were first washed in methanol and given a preliminary development in the first dimension solvent. Careful washing of the plates and thorough drying between solvents was found to be essential for good separations. The nucleotide spots were located by viewing the chromatograms under a Hanovia U.V. lamp and were then cut out and separately eluted with 0.01 N HCl. The optical density of each eluate was measured at the wavelength of maximum absorption of the nucleotide at pH 2.0 in a Unicam SP 500 spectrophotometer against a blank consisting of a 0.01N HCl eluate of a portion of the chromatogram as near to the spot as possible. The radioactivity of each nucleotide was then determined by scintillation counting in solution in the dioxane-based "Fluoralloy" scintillator. It was therefore possible to calculate, knowing the extinction coefficient of each nucleotide at its absorption maximum at pH 2.0 and after correction of background counts from the blank, the specific activity of each nucleotide in counts per minute per millimole (cpm/mM).

The labelled uridine originally present in the medium was separated by chromatography from the aliquot of medium withdrawn immediately after addition of the isotope by the technique used to separate the nucleotides. The specific activity was then determined as previously
described and compared with that of each nucleotide from the extracted RNA. The efficiency of incorporation was expressed as the percentage of cpm/mli of the labelled uridine fed to the cells incorporated into each of the four constituent nucleotides of total RNA.

**Determination of the major labelled constituents of the pool when 2-\(^{14}\)C-uridine is fed to suspension cultures of *Acer pseudoplatanus* L.**

The major constituents of the pool or acid-soluble fraction of the cell that become labelled when 2-\(^{14}\)C-uridine is supplied to the cells have been investigated in cultures which were incorporating uridine at a constant rate and in which the size of the pool was constant (steady state labelling conditions).

2-\(^{14}\)C-uridine was fed to actively dividing cells at 5 \(\times\) \(10^{-4}\)M (the specific activity was diluted with unlabelled uridine) and the cultures were incubated for 12 hours to establish steady state conditions of labelling. The cells were then harvested by filtration and were carefully washed by resuspending five times in a medium containing an excess concentration of unlabelled uridine. The cell mass from 70ml. of cell suspension was homogenized for 1 minute in 25ml of ice-cold 0.75N perchloric acid using a Polytron ultrasonic homogenizer. After standing at 0°C for 15 minutes the homogenate was centrifuged at 10000g for 20 minutes at the same temperature. The supernatant was decanted, its pH was adjusted to 7.2 with KOH and the resulting precipitate of KClO\(_4\) was removed by centrifuging at 2500g for 15 minutes. The pH of the extract was then adjusted to 3.5 with acetic acid and further purification was carried out with the charcoal adsorption technique described by Brown (1962). A column 1.2cm diameter with a scintillated glass disc was packed with a mixture of 500mg NoritOL
charcoal and 500 mg of Celite Hyflo Supercel on a 2 mm bed of Hyflo Supercel. The extract was applied to the column and was then washed with 50 ml of 0.01 M EDTA at pH 7.0 followed by 50 ml of deionized water. This procedure ensures subsequent good elution of the nucleotide glycosyl compounds (Portis, Cabib and Leclor, 1957). The washings from the column were collected, bulked and checked for the presence of radioactive compounds by reducing them to small volume and counting in solution in "Fluoralloy" scintillator. The total radioactivity of the washings was only marginally above background and the column was therefore eluted with 50 ml of 25% v/v ethanol containing 0.5% v/v 880 ammonia. The eluate was reduced to dryness in a rotary film evaporator.

The radioactive constituents of the pool contained exclusively in the ethanolic ammonia eluate of the column were separated by thin layer chromatography on plastic sheets precoated with PEI (polyethylene-imine) cellulose (MN-Polygram CEL 300, PEI Camlab Ltd.) with a two dimensional techniques designed to separate a complex mixture of nucleotides (Randerath and Randerath, 1964) and with a one dimensional technique designed to perform a detailed separation of the nucleoside monophosphates (Randerath and Randerath, 1965).

The precoated plastic sheets were given a preliminary development in distilled water and were then dried in air overnight. Two dimensional chromatography was then carried out with a stepwise development, in the first dimension, with 0.2 M LiCl (2 minutes), 1.0 M LiCl (6 minutes), 1.6 M LiCl to 13 cm above the starting line. The plates were then immersed in absolute anhydrous methanol for approximately 15 minutes to remove lithium chloride. The second dimension development was carried out with 0.5 M sodium formate buffer pH 3.4 (30 seconds),
2.0M sodium formate buffer pH 3.4 (2 minutes), 4.0M sodium formate buffer pH 3.4 (to 15cm above the starting line). The plates were then carefully dried in air and the marker spots were located with a Hanovia U.V. lamp. Radioactive spots were located by autoradiography using Ilford Ilfex 25 EP X-ray film exposed to the labelled chromatogram for three weeks.

One dimension chromatography of the nucleoside monophosphates was performed by stepwise development with 1.0N acetic acid (to 4cm above the starting line), 0.3M LiCl (to 12cm above the starting line). The plates were then thoroughly air dried and the marker spots were located by viewing under a U.V. lamp. Radioactive spots were located by scanning the chromatogram with a Panax thin layer radioactivity scanner (RTLS - 1A) with which it is possible to obtain a quantitative estimation of the amount of radioactivity in each of the separated compounds.

**Extraction and fractionation of undegraded RNA**

RNA was extracted from cells harvested at various points during the growth cycle by the modified phenol/m-cresol/TNS method of Parish and Kirby (1966) described in detail in Section 2 of this thesis. Fractionation of the RNA was carried out by sucrose density gradient centrifugation which is also described in detail in Section 2.
Chapter I

The incorporation of $^{14}$C-uridine into the metabolic pool and into the RNA of Acer pseudoplatanus L. cells grown in suspension culture

The incorporation of $^{14}$C-uridine into the metabolic pool and the RNA of plant cells has been investigated experimentally using six day-old actively dividing cultures of Acer pseudoplatanus L. Cultures of this age approximate most nearly to exponential growth and are therefore the most suitable for a kinetic study of the utilization of externally applied nucleic acid precursors.

Major features of $^{14}$C-uridine incorporation

$^{14}$C-uridine was fed to six day-old cultures at varying external concentrations and total incorporation and incorporation into the TCA-insoluble fraction of the cell were followed with time. The amount of radioactivity in the pool was estimated as the difference between these two measurements. At low concentrations ($5 \times 10^{-7}$M and $10^{-6}$M) $^{14}$C-uridine was supplied undiluted at a specific activity of 62mCi/mM but at higher concentrations it was diluted 100 times with unlabelled uridine to 0.62mCi/mM.

Fig. 3 shows the results of feeding $^{14}$C-uridine at low concentration. At $5 \times 10^{-7}$M labelled uridine was rapidly taken up into the cells for approximately 140 minutes. During this period radioactivity was incorporated into the TCA-insoluble fraction of the cells without any detectable initial delay, at a rate corresponding to about one-eighth of the total rate of uptake by the cells. After 140 minutes when the uptake of $^{14}$C-uridine into the cells had ceased, the rate of incorporation into the TCA-insoluble fraction quickly fell by a factor of about 4. During this second phase the rate of incorporation of
FIG. 3

Incorporation of Low Concentrations of $2^{-14}$C-Uridine in Suspension Cultures of Acer pseudoplatanus L. Growing exponentially at 25°C. Cell density $1 \times 10^6$ cells/ml. Dry wt. 4.28 mg/ml.

![Graphs showing incorporation of 2-$^{14}$C-Uridine into Total cell and TCA insoluble fractions at concentrations of 5 x $10^{-7}$ M and 10$^{-6}$ M.](image-url)
uridine label fell very slowly (this was much clearer at higher concentrations).

At $10^{-6}$M very similar kinetics of incorporation were obtained. The period of uptake of labelled uridine into the cells was extended to 170 minutes and during this period incorporation of radioactivity into the TCA-insoluble fraction of the cell proceeded at a rate corresponding to one-quarter the total rate of uptake. A four-fold reduction in the rate of TCA-insoluble incorporation was again observed after uptake of labelled uridine into the cells had ceased but the transition to this rate was less sharp than at the lower concentration. A semi-log plot of the total acid-soluble radioactivity or pool radioactivity (calculated from the difference between the curves of total and TCA-insoluble incorporation) as a function of time (Fig. 4), after uptake of labelled uridine had ceased, indicated that the pool decayed exponentially.

Measurements of the removal of radioactivity from the medium (Table 2) showed that the same small amount of radioactivity remained in the medium after uptake of labelled uridine into the cells had ceased whether $10^{-6}$ or $5 \times 10^{-7}$M $2^{-14}$C-uridine had been fed to the culture. The amount of radioactivity removed from the medium at $5 \times 10^{-7}$M was very approximately balanced by the total amount of radioactivity taken up into the cells (total uptake of labelled uridine and removal of radioactivity from the medium were measured under different conditions of scintillation counting but with only slightly different counting efficiencies). At $10^{-6}$M, however, there was a large difference between the amount of radioactivity removed from the medium and the amount of total uptake into the cells recorded.
FIG 4

INCORPORATION OF 2-$^{14}$C-URIDINE INTO THE POOL

Data obtained from the difference between total and TCA-insoluble incorporation at $10^{-6}$ M.

Semi-log plot of decay of radioactivity of the $^{14}$C-uridine-labelled pool.
TABLE 2

REMOVAL OF 2-$^{14}$C-URIDINE FROM THE CULTURE MEDIUM

2-$^{14}$C-uridine was fed to cells at $5 \times 10^{-7}$M and $10^{-6}$M (specific activity 62mCi/mM) and the level of radioactivity per ml of medium and the total uptake of radioactivity activity into the cells per ml of culture were measured with time.

<table>
<thead>
<tr>
<th>Time of sampling</th>
<th>Total radio-activity present in the cells (cpm/ml culture)</th>
<th>Total radio-activity removed from medium (cpm/ml culture)</th>
<th>Percentage of radioactivity removed from medium present in the cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>$5 \times 10^{-7}$M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>435.00</td>
<td>22244.40</td>
<td>0</td>
</tr>
<tr>
<td>1 hr.</td>
<td>14034.70</td>
<td>10859.45</td>
<td>approx. 100%</td>
</tr>
<tr>
<td>3 hr.</td>
<td>21691.30</td>
<td>2853.75</td>
<td>approx. 100%</td>
</tr>
<tr>
<td>$10^{-6}$M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1056.25</td>
<td>36694.00</td>
<td>0</td>
</tr>
<tr>
<td>1 hr.</td>
<td>18066.50</td>
<td>10093.00</td>
<td>approx. 64%</td>
</tr>
<tr>
<td>3 hr.</td>
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<td>2800.00</td>
<td>approx. 77%</td>
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</tbody>
</table>
When labelled uridine was applied at a higher concentration (10^{-4}M) and the incorporation of radioactivity into the TCA-insoluble fraction of the cells was followed with time a two phase curve was obtained as shown in Fig. 5(a). An initial rate of incorporation was established without any detectable delay, as at lower concentrations, but an increase occurred after approximately 35 minutes, to a rate of incorporation at later times which was just 1.7 times the initial rate.

When labelled uridine was fed at still higher concentration (5 \times 10^{-4}M) and the rate of uptake into the cells and incorporation of radioactivity into the TCA-insoluble fraction were followed for a much longer period of time, the amount of radioactivity entering the pool continued to increase for approximately 8 hours (Fig. 5(b)). During this time the rate of incorporation of radioactivity into TCA-insoluble material continued to increase until it reached a final value which was then maintained throughout the period of sampling. At this point the pool became saturated with radioactive compounds and "steady state" conditions of labelling were obtained in which radioactive compounds entered the pool only at the same rate as the radioactive compounds were incorporated into the TCA-insoluble fraction of the cells.

Measurements of the distribution of radioactivity between the major constituents of the TCA-insoluble fraction of cells, RNA, DNA and protein, after 1.0 and 6.0 hours incubation of the cultures in low and high concentrations of 2-^{14}C-uridine showed (Table 3) that almost all the TCA-insoluble radioactivity was present in RNA.

These data illustrate some interesting features of the incorporation of 2-^{14}C-uridine into the metabolic pool and into the RNA of
FIG. 5

Incorporation of $2^{-14}$C-Uridine at High Concentration (Specific Activity Diluted 100-Fold.)

(a) TCA Insoluble Incorporation at $10^{-4}$M

(b) Incorporated at $5 \times 10^{-4}$M Followed for an Extended Period of Time
TABLE 3

THE DISTRIBUTION OF RADIOACTIVITY IN THE TCA-INSOLUBLE FRACTION OF
THE CELLS OF ACER PSEUDOPLATANUS L. FED 2-$^{14}$C-URIDINE

<table>
<thead>
<tr>
<th>Time of Sampling</th>
<th>Cold TCA-insoluble radioactivity cpm/ml culture</th>
<th>Hot TCA-insoluble radioactivity cpm/ml culture</th>
<th>Warm dilute KOH-resistant radioactivity cpm/ml culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^{-6} M URIDINE Specific activity 62mCi/mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>207.50</td>
<td>12.80</td>
<td>18.65</td>
</tr>
<tr>
<td>1 hr.</td>
<td>1455.17</td>
<td>25.73</td>
<td>40.86</td>
</tr>
<tr>
<td>6 hr.</td>
<td>6029.19</td>
<td>62.46</td>
<td>265.16</td>
</tr>
<tr>
<td>10^{-4} M URIDINE Specific activity 0.62mCi/mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>26.00</td>
<td>14.80</td>
<td>15.80</td>
</tr>
<tr>
<td>1 hr.</td>
<td>267.50</td>
<td>26.45</td>
<td>25.50</td>
</tr>
<tr>
<td>6 hr.</td>
<td>2140.80</td>
<td>132.80</td>
<td>163.80</td>
</tr>
<tr>
<td>With background (0 time sample cpm) subtracted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^{-6} M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 hr.</td>
<td>1247.67</td>
<td>12.93</td>
<td>22.21</td>
</tr>
<tr>
<td>6 hr.</td>
<td>5821.69</td>
<td>49.66</td>
<td>246.51</td>
</tr>
<tr>
<td>10^{-4} M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 hr.</td>
<td>241.50</td>
<td>11.65</td>
<td>9.70</td>
</tr>
<tr>
<td>6 hr.</td>
<td>2114.80</td>
<td>118.00</td>
<td>148.00</td>
</tr>
<tr>
<td></td>
<td>Percentage RNA cold TCA-dil KOH x 100</td>
<td>Percentage DNA dil KOH-Hot TCA x100</td>
<td>Percentage protein Hot TCA x 100</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------</td>
<td>--------------------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td><strong>10^-6 M</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 hr.</td>
<td>96.70</td>
<td>1.2</td>
<td>2.1</td>
</tr>
<tr>
<td>6 hr.</td>
<td>95.40</td>
<td>3.5</td>
<td>1.1</td>
</tr>
<tr>
<td><strong>10^-4 M</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 hr.</td>
<td>95.20</td>
<td>0</td>
<td>4.8</td>
</tr>
<tr>
<td>6 hr.</td>
<td>93.00</td>
<td>1.4</td>
<td>5.6</td>
</tr>
</tbody>
</table>
cultured plant cells. $2^{-14}$C-uridine is very rapidly taken up by sycamore cell suspensions and is incorporated specifically into RNA in experiments of short duration (6 hours or less). A small proportion of the radioactive compound passes without any detectable delay into RNA whilst the rest enters a cellular pool which is very large, as indicated by the amount of radioactivity in the acid-soluble fraction of the cell compared with the amount entering RNA. Since the size of the pool is very large it would be expected that a time lag should exist between application of the labelled compound to the cells and the appearance of radioactivity in RNA. The experimental results, however, show that there is an undelayed component of incorporation into RNA. The initial rate of incorporation is increased after approximately 35 minutes by a delayed component of incorporation which is most obvious where labelled uridine is fed at high concentration and is only just discernable at low concentrations where the rate of uptake into the pool begins to fall soon after the addition of the labelled compound to the culture. In addition, as soon as the uptake of labelled uridine into the cells ceases there is an immediate and substantial fall in the rate of incorporation of radioactivity into RNA although the radioactivity of the pool is at its highest value.

These data may be explained by considering the theoretical model suggested by McCarthy and Britten (1962) to explain similar phenomena in bacteria and which is outlined in the schematic diagram below:—

\[
\text{exogenous uridine} \quad \xrightarrow{S} \quad P \xrightarrow{RNA} \quad \text{endogenous synthesis}
\]
A proportion of uridine entering the cell passes very rapidly into RNA through a series of intermediates $P$ which have very small pool sizes and are rapidly turned over with the result that their specific activity rises very quickly. The remainder of the $^{14}C$-uridine entering the cell equilibrates with a very large pool of uracil compounds $S$ which exchange with some compound in $P$. The rate of exchange between $S$ and $P$ is not fast and a component of incorporation is delayed by passage through the large pool $S$. After uptake of $^{14}C$-uridine from the medium ceases the by-pass flow through $P$ ceases to function with an accompanying fall in the rate of incorporation of radioactivity into RNA. Further incorporation into RNA then continues at a reduced rate by the flow of radioactive compounds from the large pool $S$ which consequently decays slowly.

An alternative explanation of these observations has been suggested by Gros, Gilbert, Hiatt, Attardi, Spahr and Watson (1961) from work on the incorporation of labelled guanine in mutants of $E. coli$ in which the conversion of guanine compounds into adenine compounds was blocked. Gros et al (1961) consider the possibility that the passage of bases through the large cellular pool is obligatory for their subsequent entry into RNA, and, that the kinetics of incorporation of such precursors into RNA are determined by different rates of synthesis and degradation of different species of RNA as represented by the following schematic diagram:

```
  exogenous precursor ——> S ———> T
```

$T$ represents a rapidly turned over fraction of the total RNA, $R$ represents a stable fraction of RNA and $S$ represents the pool.
If the rate of exchange $\beta$ between the pool and the unstable fraction of RNA $T$ is very rapid they will both have the same specific activity and a certain fraction of radioactivity entering the cell, determined by the relative sizes of $S$ and $T$, will appear without delay in the TCA-precipitable RNA. Initially the breakdown products of the unstable RNA are unlabelled but at later times as they become radioactive the specific activity of the nucleotide pool increases such that the stable RNA becomes labelled at a higher rate. Only at the beginning of labelling is the difference in the rates of synthesis and degradation of the stable and unstable RNA apparent from the shape of the incorporation curve of radioactivity into total RNA as at later times the specific activity of the pool will be unaffected by radioactive molecules chased from the unstable fraction of RNA.

When uridine is fed at high concentration ($5 \times 10^{-4} M$) the radioactivity of the pool eventually reaches a saturation level and a steady state of labelling is established. The rate of entry of labelled uridine into the observable acid-soluble pool continues at the same rate at which nucleotides are removed by the net synthesis of RNA. Consequently, if there is exchange of nucleotides between unstable fractions of RNA and the pool, incorporation of uridine during this period does not necessarily sum the synthesis of all forms of RNA, unstable and stable, as is often supposed.

It is clear from measurements of the radioactivity in the medium that incorporation ceases whilst there is still some radioactive compound remaining. This radioactive compound has not been identified but since the level of radioactivity remaining in the medium is the same in cultures fed uridine at different initial concentrations it
suggests that there may be a concentration, between 0.64 and 0.76x10^{-8}, below which uridine is unavailable to the cells. The discrepancy between the amount of radioactivity removed from the medium and the amount taken up into the cells in the culture fed 2^{-14}C-uridine at a concentration of 10^{-6}M (approximately 2.5% after 3 hours) can only be explained by loss of carbon-14 from the system as a gas. This possibility has been further investigated and will be described in chapter 3.

The relationship between the concentration of uridine supplied to the cells and its rate of incorporation into RNA.

The relationship between the concentration of 2^{-14}C-uridine fed to sycamore suspension cultures and its rate of incorporation into RNA was investigated in an experiment in which 2^{-14}C-uridine was fed to cultures at varying concentrations between 10^{-6} and 5 \times 10^{-4}M but at the same specific activity of 0.5mCi/mL. The total uptake of radioactivity into the cells and incorporation into the TCA-insoluble fraction were followed over a time period of 40 hours. The average rate of incorporation of radioactivity into the TCA-precipitable material and into the whole cell (the TCA-insoluble fraction + the acid-soluble pool) over the first hour of incubation was calculated from the incorporation curves and expressed as counts per minute per hour.

The curves of incorporation of 2^{-14}C-uridine into the total cell and into the TCA-insoluble fraction of the cell are shown in Fig. 6. These curves show the time for which continuous incorporation into the total cell was sustained at each initial concentration of uridine supplied to the cultures. At 10^{-6}M uridine incorporation into the total
FIG. 6
INTEGRATION CURVES OF 2-14C-URIDINE FED TO CULTURES OF ACET PSEUDOMONAS L

2-14C-URIDINE fed at varying concentrations but at the same specific activity.

- **10^{-5}M URIDINE**
- **10^{-4}M URIDINE**
- **5 \times 10^{-4}M URIDINE**
FIG. 7

THE RELATIONSHIP BETWEEN THE CONCENTRATION OF 2-$^{14}$C-URIDINE FED TO SYCAMORE CELL SUSPENSION CULTURE AND ITS INITIAL RATE OF INCORPORATION INTO THE TCA INSOLUBLE FRACTION OF CELLS.
cell continued for approximately 2 hours, at $10^{-5}$M for approximately 4 hours, at $10^{-4}$M for approximately 12 hours, and at $5 \times 10^{-4}$M incorporation continued throughout the period of sampling.

A log-log plot of the initial rates of incorporation of radioactivity into the TCA-insoluble fraction of the cell (measured as an average value over the first hour of incorporation) as a function of the external concentration of uridine is shown in Fig. 7. The rate of incorporation increased rapidly as the uridine concentration increased from $10^{-6}$ to $10^{-4}$M but beyond $10^{-4}$M very little further increase in the rate of incorporation was obtained.

These results show that the rate of incorporation of 2-$^{14}$C-uridine into RNA is dependent upon the external concentration of uridine supplied to the cells below a concentration of $10^{-4}$M. At this concentration the mechanism controlling the rate of incorporation into RNA saturates and further increases in the external concentration of uridine merely prolong the period of time for which uridine is incorporated.

**Pool expansion**

The question can be raised as to whether the amounts of compounds in the pool are dependent on the concentration of exogenous uridine. That is are the pools expandable? This question was investigated in an experiment in which $10^{-4}$ unlabelled $^{12}$C-uridine was supplied to the cells for 6 hours before adding $10^{-6}$M 2-$^{14}$C-uridine. The kinetics of incorporation of radioactivity into the TCA-insoluble fraction of the cells in this culture were compared with those of a control culture where the $^{12}$C-uridine was added with the $^{14}$C-uridine at zero time. The concentration of uridine remaining in the medium after 6 hours
incubation was estimated very approximately from the incorporation curves in the previous experiment as \(0.45 \times 10^{-5}\) M. A quantity of unlabelled uridine was therefore added to the control culture at zero time to adjust the concentration of the \(^{14}\text{C}-\text{uridine}\) to \(0.45 \times 10^{-5}\) M so that the concentration and specific activity of the \(^{14}\text{C}-\text{uridine}\) was roughly comparable in both cultures.

Fig. 8 shows the results of this experiment. The kinetic curves are drawn with the zero time background subtracted. The control culture showed the usual undelayed incorporation of radioactivity into the TCA-insoluble fraction of the cell but in the culture fed unlabelled uridine beforehand there was evidence of a short initial delay of approximately 4 minutes. There was also a reduced initial rate of incorporation and a more gradual increase in the rate of incorporation to the final rate which was similar to that of the control culture. The specific activities of the \(^{14}\text{C}-\text{uridine}\) were, however, only roughly adjusted to be the same in the two cultures and the differences in the rates of incorporation may have resulted, at least in part, from a difference in the specific activities of the \(^{14}\text{C}-\text{uridine}\) supplied to the cultures.

These results suggest that feeding high external concentrations of uridine may cause some degree of expansion of the endogenous RNA precursor pools. If the pool is expanded a higher level of unlabelled compounds will be established in the culture fed unlabelled \(^{12}\text{C}-\text{uridine}\) beforehand than in the control. When labelled \(^{14}\text{C}-\text{uridine}\) is added the specific activity of the pool would therefore rise more slowly than that of the control thus causing a delayed and reduced rate of incorporation of radioactivity into RNA. The slight delay and reduced
FIG. 8

POOL EXPANSION EXPERIMENT

A culture was incubated with $10^{-4}\text{M} \, ^{12}\text{C}$-Uridine and $^{14}\text{C}$-Uridine was added after 6 hours. In the control culture $^{12}\text{C}$- and $^{14}\text{C}$-Uridine were added together at zero time.

Radioactivity incorporated CPM $\times 10^2$ above background/ml cell culture

Time in minutes
initial rate of incorporation observed in the culture fed $10^{-4}\text{M}$ 
$^{12}\text{C}$-uridine before hand in this experiment is consistent with a small
degree of expansion of the RNA precursor pool. The difference
between the curve obtained in this culture and that obtained in the
control culture is not, however, very great and the amount of pool
expansion does not appear to be more than a small fraction of the
size of the pre-existing pool. In the McCarthy-Britten model of pool
compartmentalization such expansion would occur predominantly in the
by-pass pool since only the initial kinetics of incorporation are
affected by feeding a high concentration of unlabelled uridine to the
culture before the incubation in $^{14}\text{C}$-uridine.

A "chase" experiment

The rates of turnover of macromolecules have often been measured
with "chase" experiments. These are experiments in which a labelled
metabolite is present for a short period of incorporation and is then
replaced by the incorporation of the same metabolite unlabelled.

A chase experiment has been performed in which $2-{^{14}}\text{C}$-uridine
was fed to actively dividing six day-old cultures at a concentration
of $10^{-6}\text{M}$, specific activity 62mCi/mM. Incorporation of radioactivity
into the TCA-insoluble fraction was followed for 20 minutes at which
time a 1000 times excess of unlabelled $^{12}\text{C}$-uridine was added to the
culture and measurements of incorporation were continued.

The results are shown in Fig. 9. At this concentration incorpor-
oration of $^{14}\text{C}$-uridine into TCA-insoluble material would have proceeded
at an undiminished rate for approximately 170 minutes. 10 minutes
after the chase, however, there was a sudden reduction in the rate of
incorporation by a factor of about 4. Incorporation then continued
FIG. 9

INCORPORATION OF 2-$^{14}$C-URIDINE IN A "CHASE" EXPERIMENT.

$10^{-6}$M $^{14}$C-Uridine was added initially. $10^{-3}$M $^{12}$C-Uridine was added after 20 minutes.
with the rate falling slowly throughout the period of sampling.

The effect of the chase incubation with a high concentration of unlabelled uridine is very similar to that of depletion of the medium observed in earlier experiments. There is a sharp reduction in the rate of incorporation, after a short time lag, and it continues to fall slowly but there is no net loss of radioactivity from the total RNA for some considerable time. This again indicates two components of incorporation, one of which is very rapidly reduced by the chase and another which decreases slowly. It is clear that the specific activity of the large precursor pool is only diluted slowly by the unlabelled uridine and that net incorporation thus continues for a considerable time.

The rapid reduction to one-quarter the initial rate of incorporation and the continued incorporation for a long period after the chase does not provide suitable circumstances for the recognition and measurement of a fraction of the total RNA which is rapidly synthesised and degraded i.e. an "effective chase" cannot be performed. These results are almost identical to those obtained in similar experiments in HeLa cell cultures, Soeiro, Birnboim and Darnell (1966) and in bacteria, McCarthy and Britten (1962) and Nierlich (1968), and these workers came to the same conclusion regarding the effectiveness of chase experiments in studying turnover of RNA.
Chapter 2

The integration of exogenously applied uridine into the metabolism of the plant cell and its effects upon endogenous synthesis of nucleic acid precursors

Previous experiments have established some of the major features of the uptake of $^{14}$C-labelled uridine and its incorporation into RNA in plant cell suspension cultures. As a necessary part of the studies an investigation has been made of the pathway by which exogenously applied uridine is utilized by cell cultures, and, particularly with respect to its use as a labelled precursor of RNA, the effect it has upon the endogenous synthesis of both pyrimidine precursors of RNA.

In order to study the pathway by which uridine is utilized an analysis has been made of the major constituents of the metabolic pool that become labelled when $^{14}$C-uridine is fed to the cells. The effects of exogenously applied uridine on the endogenous synthesis of the pyrimidine precursors of RNA has been investigated by measuring the extent to which it can replace the endogenously synthesised pyrimidine bases in RNA synthesis.

The major radioactive constituents of the metabolic pool

$^{14}$C-uridine was fed to actively dividing six day-old suspension cultures of *Acer pseudoplatanus* L. at a concentration of $5 \times 10^{-4}$M, specific activity 0.5mCi/mM. The culture was incubated for 12 hours to establish steady state conditions of labelling and the cells were then harvested and carefully washed to remove any surface-adsorbed uridine. A cold acid extract of the cells was then made, purified by adsorption and elution from charcoal and fractionated by chromatography. Radioactive compounds were located by autoradiography or by use of a Panax radioactivity scanner and were identified by comparison of their
chromatographic behaviour with that of standard marker compounds.

The results are shown in Fig. 10(a) and 10(b). Both the one and two dimensional chromatograms showed only one heavily labelled spot which corresponds in its chromatographic behaviour to UMP. The two dimensional chromatogram (Fig. 10(a)) also showed a radioactive spot corresponding to CTP, a smear of radioactivity corresponding to UDP-glucose and traces of radioactivity corresponding to UDP and UTP. The radioactivity scan of the one dimension chromatogram (Fig. 10(b)) showed quite clearly that most of the radioactivity in this pool extract was present as UMP. There were very small peaks of radioactivity barely visible above the background counts corresponding to CTP and UDP-glucose and, near the starting line, peaks corresponding to UDP and UTP. This technique of one dimension chromatography is capable of separating the 5' isomers of nucleoside monophosphates from the 2'-3' isomers and comparison with standard marker compounds indicated that the large peak of radioactive UMP was predominately the 5'isomer. There was, however, no indication of any free labelled uridine in the extract in either the one or two dimension chromatographic separations.

A quantitative analysis of the levels of nucleotides throughout the growth cycle of suspension cultures of Acer pseudoplatanus L. (Brown and Short, 1969) has shown that in six day-old dividing cultures there are high levels of UDP-glucose and UMP. The other pyrimidine nucleotides, however, were present in very much lower quantities, especially the di- and triphosphates. It is therefore not surprising that a large proportion of the radioactivity of the pool is found in UMP, but if there was complete equilibration of the radioactivity between the pools of uracil-containing compounds there should also be
FIG. 10a

Radioactive compounds which appear after thin-layer chromatography of cold perchloric acid-soluble extract of cells grown in 2-\textsuperscript{14}C-uridine.

Thin layer PEI cellulose
Dimension I 0.2 M LiCl (2 min) 1.0 M LiCl (6 min) 1.6 M LiCl (to 13 cm)
Dimension II 0.5 M Sodium formate buffer pH 3.4 (30 sec) 2.0 M " " " (2 min) 4.0 M " " " (to 15 cm)

Diagram showing thin-layer chromatography results with markers for UMP, UDPG, UTP, CMP, UDP, and standards.
FIG. 10b

ONE DIMENSION CHROMATOGRAPHY OF THE SAME EXTRACT
- RADIOACTIVE COMPOUNDS LOCATED WITH A PANAX
RADIOACTIVITY SCANNER.

Thin layer PEI cellulose
Solvents 1:ON acetic acid (to 4 cm above starting line)
0:3M LiCl (" 12 cm " " " )
a high level of radioactivity in UDP-glucose, under these conditions of labelling where the pool is saturated with radioactivity. This low level of labelling in UDP-glucose relative to UMP could arise if there was a poor efficiency of extraction of UDP-glucose or a massive expansion of the size of the UMP pool. Previous experiments have indicated, however, that any pool expansion that does occur is not very great. The quantity of tissue extracted was too small to allow measurement of the absolute amounts and specific activities of each pool constituent and it is therefore not possible to determine whether there was a poor efficiency of extraction of UDP-glucose relative to UMP. A possible alternative explanation, however, is that very little synthesis of UDP-glucose occurs during the early division phase of the growth cycle although a large pool is present in the cells. Brown and Short (1969) have shown that the size of the UDP-glucose pool per cell is greatest just before the onset of cell division in these cultures and that it rapidly declines during cell division. It is therefore possible that a large proportion of the UDP-glucose required for cell wall synthesis during the division phase of growth is produced during the lag phase and that very little is being synthesised at the time when the 2-\(^{14}\)C-uridine is applied to the culture.

Since it was not possible to detect any free labelled uridine in the cell extract it appears that immediately upon entry into the cell it is very rapidly converted to UMP by a uridine kinase. Thymidine kinase has been assayed in this tissue by the ability of a soluble enzyme preparation from the cells to convert thymidine to TMP and there is also evidence that uridine can be converted to UMP by the same cell free system (M. W. Fowler personal communication). The UMP
is then converted to UDP and UTP and incorporated into RNA. The endogenous pools of UDP and UTP are known to be small and since there was very little detectable radioactivity in these compounds it is probable that they do not increase in size when uridine is fed to the cells and are rapidly turned over. The radioactive compound corresponding chromatographically to CMP indicates that uracil compounds can be converted to cytosine compounds as has been observed to occur in bacteria (Roberts, Abelson, Cowie, Bolton and Britten, 1955) by the conversion of UTP to CTP (Lieberman, 1956).

The efficiency of incorporation of radioactive uridine into RNA.

The efficiency with which exogenous $^{14}$C-uridine is incorporated into RNA was measured by comparing the specific activity of the labelled uridine supplied to the cells with the specific activities of each of the four nucleoside monophosphates in total RNA extracted from the plant cells. $^{14}$C-uridine at a concentration of $10^{-3}$M (specific activity 50μCi/mM) was fed to the sycamore suspension cultures at inoculation. This concentration of uridine is sufficient to maintain continuous incorporation for 8 days (see chapter 4). The cultures were then incubated for 6 days after which the total RNA was extracted and hydrolysed with dilute alkalis. The products of hydrolysis were separated by two dimensional chromatography and the specific activity of each nucleoside monophosphate was determined and expressed as counts per minute per mM. These specific activities were then compared with that of uridine fed to the cultures.

It can be seen from the results (Table 4) that radioactivity from $^{14}$C-uridine could only be detected in CMP and UMP. The specific activity of the UMP very closely approached that of the radioactive
TABLE 4

Efficiency of incorporation of $^{14}$C-uridine into the constituent mono-nucleotides of total RNA

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorption maxima at pH 2.0 ($\lambda_{\max}$)</th>
<th>Molar extinction ($\varepsilon_{\max}$)</th>
<th>Radio-activity cpm per ml</th>
<th>Radio-activity background (blank) subtracted cpm</th>
<th>Optical density</th>
<th>cpm per mM sample</th>
<th>Efficiency incorp. cpm/nM sample</th>
<th>Efficiency cpm/nM uridine</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLANK</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GMP</td>
<td>$\lambda_{\max}$ 258</td>
<td>$\varepsilon_{\max}$ 12.2 x 10$^3$</td>
<td>9.75</td>
<td>1.80</td>
<td>0.456</td>
<td>48.157</td>
<td>0.34%</td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>$\lambda_{\max}$ 260</td>
<td>$\varepsilon_{\max}$ 15.3</td>
<td>11.93</td>
<td>3.98</td>
<td>0.418</td>
<td>145.68</td>
<td>1.03%</td>
<td></td>
</tr>
<tr>
<td>UMP</td>
<td>$\lambda_{\max}$ 260</td>
<td>$\varepsilon_{\max}$ 10.0 x 10$^3$</td>
<td>528.00</td>
<td>520.05</td>
<td>0.388</td>
<td>13403.35</td>
<td>95.13%</td>
<td></td>
</tr>
<tr>
<td>CMP</td>
<td>$\lambda_{\max}$ 278</td>
<td>$\varepsilon_{\max}$ 12.7 x 10$^3$</td>
<td>189.75</td>
<td>181.80</td>
<td>0.229</td>
<td>10082.36</td>
<td>71.56%</td>
<td></td>
</tr>
<tr>
<td>URIDINE</td>
<td>$\lambda_{\max}$ 261</td>
<td>$\varepsilon_{\max}$ 10.1 x 10$^3$</td>
<td>1521.50</td>
<td>1513.55</td>
<td>1.085</td>
<td>14089.27</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>
uridine applied to the culture and radioactivity also appeared in CMP at high efficiency.

These results clearly demonstrate the efficient conversion of uracil-derivatives to cytosine compounds. The specific activities of both UMP and CMP from the total RNA approach that of the uridine fed to the cultures (UMP rather more so than CMP) and are therefore not diluted to a great extent by internally synthesised pyrimidine precursors of RNA or by the presence of unlabelled RNA in the extract (from the previous passage of growth of the culture in the absence of labelled uridine). It is therefore clear that the derivatives of exogenous uridine efficiently suppress endogenous synthesis of the pyrimidine precursors of RNA. It is also clear that a large proportion of the preexistent RNA in the cells synthesised during the previous passage in culture is broken down during the first few days of the present growth cycle and that the nucleotides arising from this breakdown are not efficiently reutilized for RNA synthesis.
Chapter 3

The evolution of \(^{14}\)CO\(_2\) from suspension cultures of Acer pseudoplatanus supplied \(^2\)-\(^{14}\)C-uridine

In previous experiments where \(^{14}\)C-labelled uridine had been fed to suspension cultures of Acer pseudoplatanus L, a discrepancy was observed between the total amount of radioactivity lost from the medium and the total amount which actually appeared in the cells. This could only be accounted for by loss of radioactivity as a gas and therefore the possible loss of carbon-14 as \(^{14}\)CO\(_2\) has been investigated. The kinetics of \(^{14}\)CO\(_2\) evolution from actively dividing cultures fed \(^{14}\)C-uridine have been studied together with the relationship between the external concentration of \(^{14}\)C-uridine applied to the cells and the proportion of carbon-14 released as \(^{14}\)CO\(_2\).

Evolution of \(^{14}\)CO\(_2\) by actively dividing cultures

2-\(^{14}\)C-uridine was fed to six day-old dividing cultures of Acer pseudoplatanus L, at external concentrations varying from \(10^{-6}\) to \(10^{-3}\) M and at the same specific activity of 0.5mCi/mM. The culture flasks were closed after addition of labelled uridine and the total amount of \(^{14}\)CO\(_2\) evolved by each culture was followed with time. A control culture was also set up containing the medium from a six day-old culture, filtered free of cells through nylon cloth filters and to which \(^{14}\)C-uridine was added at \(10^{-4}\) M.

The results (Figs. 11 and 12) showed that evolution of \(^{14}\)CO\(_2\) by the cultures can be detected as early as 15 minutes after addition of labelled uridine. The rate of \(^{14}\)CO\(_2\) release increased rapidly during the first hour of incubation and then settled to a linear rate which continued for varying periods of time. Previous experiments have shown that depletion of the medium, to a point where no further uptake of
FIG. 11

The Initial Kinetics of $^{14}$CO$_2$ Evolution from Cultures of Acer pseudoplatanus L. Fed $2^{14}$C-Uridine at Various External Concentrations.

The graph shows the evolution of $^{14}$CO$_2$ (in CPM x 10$^3$) over time (in hours) for different concentrations of Uridine: $10^{-3}$ M, $10^{-4}$ M, $10^{-5}$ M, and $10^{-6}$ M. The control shows no significant evolution.
$^{14}$CO$_2$ Evolution from Suspension Cultures of *Acer pseudoplatanus* L. Fed 2-$^{14}$C-Uridine Over a Longer Period of Time.

10$^{-6}$ M 2-$^{14}$C-URIDINE

10$^{-5}$ M 2-$^{14}$C-URIDINE
$10^{-4}$ M $2\cdot^{14}$C-URIDINE

$10^{-3}$ M $2\cdot^{14}$C-URIDINE
uridine could be detected, occurred after approximately 2 hours in cultures fed uridine at $10^{-6} \text{M}$, after 4 hours at $10^{-5} \text{M}$ and after 12
4 hours at $10^{-4} \text{M}$ (see Fig. 6, chapter I). It can clearly be seen that the $^{14}\text{CO}_2$ evolution, at each of the concentrations of labelled uridine, ceased after the period of time taken for effective depletion of the medium to occur. It can also be seen that the rate of $^{14}\text{CO}_2$ evolution was greatly increased by increases in the external concentration of uridine. Where $^{14}\text{C}$-uridine was fed at an initial concentration of $10^{-3} \text{M}$, $^{14}\text{CO}_2$ evolution occurred throughout the period of sampling. Previous experiments have indicated that this concentration of uridine is sufficient to maintain continuous incorporation for several days. The rate of $^{14}\text{CO}_2$ evolution, however, did not remain entirely constant after the initial increase as a further increase was observed after 9 hours incubation. The rate at later times during the period of sampling was just 1.5 times the earlier rate. Samples taken from the control culture showed that in the absence of the plant cells no radioactivity above background could be detected in the $\text{CO}_2$-trapping solution.

The rapid appearance of $^{14}\text{CO}_2$ from cultures fed $2-{^{14}\text{C}}$-uridine coupled with the fact that it only continues to be released at a high rate whilst uridine is being taken up from the medium strongly suggests that it is uridine or one of its immediate derivatives in the metabolic pool that is being degraded. The control culture results exclude the possibility that uridine might be degraded in the medium before entering the cell by extracellular enzymes or by microbial contamination. In addition, the initial kinetics suggest that the rate of $^{14}\text{CO}_2$ evolution rises and achieves the linear rate earlier at high concentration than at low concentration. This observation is inconsistent with a
degradative mechanism situated at the cell surface as such an enzymic system should give a maximum rate of $^{14}\text{C}\text{O}_2$ evolution immediately, irrespective of the concentration of uridine.

The increase in the rate of $^{14}\text{C}\text{O}_2$ evolution after approximately 9 hours in the culture fed $10^{-3}\text{M}$ $^{14}\text{C}$-uridine is an interesting observation. In a previous experiment where $^{14}\text{C}$-uridine was fed at $5 \times 10^{-4}\text{M}$ and the incorporation of radioactivity into the pool and RNA were investigated, the pool radioactivity increased for approximately 8 hours until a saturation value was reached after which there was an apparent fall in the rate of total uptake into the cells. The rate of total uptake was, however, measured as the increase in the total cell content of radioactivity with time and if an increased proportion of the labelled uridine entering the cell was degraded to $^{14}\text{C}\text{O}_2$ the actual rate of uptake may have remained unchanged. An alternative explanation is that, at this very high external concentration of uridine, there may be an adaptive rise in the activity of the degrading enzyme and that there is a time lag in this effect.

The increase in the rate of $^{14}\text{C}\text{O}_2$ evolution by the cultures with increasing external concentrations of uridine is greatest in the order of concentrations where the increase in the rate of incorporation of uridine into RNA has been shown to be greatest. It is necessary, however, to determine what proportion of $^{14}\text{C}$-uridine taken up into the cells is degraded to $^{14}\text{C}\text{O}_2$ and whether it varies with the concentration of uridine supplied.

The proportion of $2-{^{14}\text{C}}$-uridine degraded to $^{14}\text{C}\text{O}_2$

In order that a direct comparison can be made between the amount of total carbon-14 entering the cells and the amount released as $^{14}\text{C}\text{O}_2$
at varying external concentrations of uridine, a balance sheet must be compiled of the removal of radioactivity from the medium, the total uptake of radioactivity into the cells and its release as $^{14}\text{CO}_2$ in a given period of time. In order to compile such a balance sheet it is necessary to measure the amount of radioactivity removed from the medium, the amount taken up into the cells and the amount released as $^{14}\text{CO}_2$ under identical conditions of scintillation counting. All the radioactivity containing compounds must therefore be converted to the same chemical form. Samples of medium and cell material were therefore degraded with a strong oxidising mixture so that their total carbon content was converted to CO$_2$.

$2^{-14}\text{C}-\text{uridine}$ was fed to six day-old dividing cultures at $10^{-5}$, $10^{-4}$ and $10^{-3}\text{M}$ at specific activities of 0.5mCi/mM, and, at $10^{-6}\text{M}$ at a specific activity of 62mCi/mM (to allow more accurate measurements at low concentration). The cultures were then incubated for 2.0 hours at $10^{-5}\text{M}$ and $10^{-6}\text{M}$ and for 8 hours at $10^{-4}$ and $10^{-3}\text{M}$. These incubation times were chosen from the kinetic curves of $^{14}\text{CO}_2$ release as convenient points at which $^{14}\text{CO}_2$ evolution was occurring at a steady rate. $^{14}\text{CO}_2$ evolved by the cultures during this period was collected by absorption in KOH in centre wells of the culture vessels and samples of medium and cells were degraded to $^{14}\text{CO}_2$ which was also absorbed in KOH. The total amounts of radioactivity in all samples were then measured by scintillation counting.

The results of this experiment (Table 5) showed that it was possible, to a good approximation, to obtain a balance sheet between the amount of carbon-14 removed from the medium and the amounts incorporated into the cells and released as $^{14}\text{CO}_2$. It was also clear
TABLE 5

The proportion of $2^{14}$-uridine degraded to $^{14}$CO$_2$ in suspension cultures of Acer pseudoplatanus L.

<table>
<thead>
<tr>
<th>CONC. OF URIDINE</th>
<th>DURATION</th>
<th>MEDIUM BEFORE INCUBATION</th>
<th>MEDIUM AFTER INCUBATION</th>
<th>UPTAKE BY CELLS + CELL WASHINGS TOTAL CPM</th>
<th>CO$_2$ EVOLVED TOTAL CPM</th>
<th>% TOTAL UPTAKE GIVEN OFF AS $^{14}$CO$_2$</th>
<th>% RECOVERY</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-6}$M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>62mCi/mM</td>
<td>2.0</td>
<td>122,901.8</td>
<td>15073.0</td>
<td>70171.16</td>
<td>23725.0</td>
<td>25.3%</td>
<td>89.3%</td>
</tr>
<tr>
<td>$10^{-5}$M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5mCi/mM</td>
<td>2.0</td>
<td>10555.0</td>
<td>5700.0</td>
<td>1876.0</td>
<td>2314.0</td>
<td>55.2%</td>
<td>93.7%</td>
</tr>
<tr>
<td>$10^{-4}$M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5mCi/mM</td>
<td>8.0</td>
<td>102725.6</td>
<td>79500.0</td>
<td>7443.00</td>
<td>2000.20</td>
<td>72.9%</td>
<td>104.1%</td>
</tr>
<tr>
<td>$10^{-3}$M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5mCi/mM</td>
<td>8.0</td>
<td>932604.6</td>
<td>767283.0</td>
<td>28201.00</td>
<td>67569.2</td>
<td>76.1%</td>
<td>92.5%</td>
</tr>
</tbody>
</table>

CONTROLS - 2 FLASKS CONTAINING UNLABELLED CELLS - TOTAL CONTENTS DEGRADATED TO CO$_2$ AND COUNTED

<table>
<thead>
<tr>
<th>FLASK No.</th>
<th>TOTAL CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.70</td>
</tr>
<tr>
<td>2</td>
<td>37.90</td>
</tr>
</tbody>
</table>
that the proportion of the $^{14}\text{C-uridine}$ degraded to $^{14}\text{CO}_2$ was relatively small at low external concentrations of uridine but then increased as the uridine concentration increased to $10^{-4}\text{M}$. Further increases in uridine concentration, however, did not result in a very great increase in the proportion of uridine which was degraded.

The results of these experiments clearly show that $^{14}\text{C-uridine}$ fed to suspension cultures of *Acer pseudoplatanus* L., in addition to serving as a precursor of RNA, is rapidly degraded with release of the carbon-14 atom as $^{14}\text{CO}_2$. The initial kinetics of $^{14}\text{CO}_2$ evolution strongly suggest that uridine, or one of its immediate derivatives is rapidly degraded soon after entry into the cell. The immediate fall in the rate of $^{14}\text{CO}_2$ evolution when uptake of uridine from the medium ceases suggests that the compound degraded is not one of the major constituents of the pool. If this were so the rate of $^{14}\text{CO}_2$ evolution would be expected to decrease slowly after effective depletion of the medium since the amount of radioactivity in the pool is very large and only decreases very slowly after uptake has ceased.

The pathway by which breakdown occurs is not known but it must involve cleavage of the pyrimidine ring as this uridine is labelled in the 2-C position. Barnes and Naylor (1962) have demonstrated that embryos of pine seedlings or sterile pine callus tissue are capable of degrading uracil, uridine and UMP to β-alanine. β-alanine was formed rather more efficiently from uridine and uracil than from UMP. The β-alanine could then be converted to aspartic acid or pyruvate either of which could then pass into the TCA cycle and be respired with evolution of $^{14}\text{CO}_2$. Hayaishi, Nishizuka, Tatebana, Takeshita and Kuno (1961) and Nishizuka, Takeshita, Kuno and Hayaishi (1959) have demon-
strated a reversible transamination between $\beta$-alanine and pyruvate in 
Pseudomonas fluorescens.

Whatever the pathway by which it is degraded it is clear, from 
previous measurements of the specificity of $2^{-14}$C-uridine (see Table 3, 
Chapter 2) as a nucleic acid precursor, that the carbon-14 is not 
incorporated to any measurable extent in macromolecules other than 
nucleic acids. If the degradative pathway does involve production 
of amino acids it therefore appears that they are not used directly 
to supplement the cellular content of amino acids for protein synthesis.

The observation that the proportion of $2^{-14}$C-uridine degraded 
increases with increasing concentrations of uridine suggests that 
there may be competition for uridine taken up into the cell between 
the degradative pathway and nucleic acid synthesis. At low concen-
trations e. g. $10^{-6}$M only 25% of the total uridine taken up by the cell 
is degraded to $^{14}$CO$_2$ but at higher concentrations $10^{-4}$ and $10^{-3}$M this 
as risen to 75% suggesting that at low concentration the pathway for 
conversion of uridine to RNA precursors predominates and that at 
high concentrations most of the uridine enters the degradative pathway. 
A possible alternative suggestion is that there are separate mechanisms 
of entry of uridine into the cell, the entry mechanism which provides 
uridine for nucleic acid synthesis operating more efficiently at low 
external concentrations of uridine than the entry mechanism for the 
degradative pathway.
Chapter 4

The incorporation of $^{14}$C-uridine and its relationship to RNA synthesis throughout the growth cycle of suspension cultures of Acer pseudoplatanus L.

In the previous experiments a study has been made of the major features of the incorporation of uridine and its effects upon cellular metabolism in actively dividing plant cell suspension cultures. The results of these experiments have shown that a labelled nucleoside applied externally can become efficiently incorporated into RNA, and, have yielded some information of the relationship between the cellular precursor pools and RNA synthesis in actively dividing cells. The duration of the period of growth where the rate of cell division is very nearly exponential is, however, very short in the standard batch cultures and restricted to the early part of the division phase. The uptake and incorporation of labelled uridine has therefore been investigated during various stages of growth of the cultures. Long term labelling experiments have also been performed to study RNA synthesis and turnover throughout the complete growth cycle of single batch cultures.

The total content of RNA and DNA of suspension cultures of Acer pseudoplatanus L. has previously been measured by chemical assay throughout the growth cycle (Short, Brown and Street, 1969). It has been shown that the level of total RNA begins to rise immediately after inoculation and continues to rise through the lag phase. The maximum rate of accumulation of RNA per culture occurs early in the division phase, where the rate of cell division approximates most nearly to exponential growth, but the maximum rate of accumulation of RNA per cell occurs late in lag phase. RNA continues to accumulate in the culture
until late in the division phase after which time the total level in
the culture begins to fall rapidly although cell division still con-
tinues for several days. The total amount of RNA per cell reaches a
maximum value very much earlier, in the middle of the division phase,
and then falls very rapidly throughout the later stages of the division
phase.

These basic observations have provided a basis for the following
experiments using the labelled RNA precursor.

The uptake and incorporation of $2^{-14}\text{C}$-uridine at various stages in the
growth cycle

The rate of uptake and incorporation of $2^{-14}\text{C}$-uridine into RNA
has been investigated at inoculation, during the lag phase, during
early and late stages of cell division and during the stationary phase.

Replicate cultures were inoculated from the same stock culture
and were grown under identical conditions. Measurements of cell number
were taken at regular intervals. $2^{-14}\text{C}$-uridine at a concentration of
$10^{-4}\text{M}$ specific activity $0.5\text{mCi}/\text{mM}$, sufficient to establish the maximum
rate of uptake and incorporation, was fed first to a culture approx-
imately 30 minutes after inoculation and then to cultures as they
reached various ages. The total uptake of radioactivity and incor-
poration into the TCA-insoluble fraction of the cells were followed
with time for 4 hours. The initial rates of uptake and incorporation
over the first 40 minutes of incubation with labelled uridine were
calculated from the incorporation curves and expressed as counts per
minute per hour.

The results (Fig. 13(a)) show that there was appreciable uptake
and incorporation by cultures at all stages of the growth cycle.
FIG. 13
COMPARISON OF INITIAL RATES OF INCORPORATION OF 2-\textsuperscript{14}C-URIDINE FED AT DIFFERENT TIMES DURING THE GROWTH CYCLE OF SUSPENSION CULTURES OF Acer pseudoplatanus L.

(a) Incorporation / ml Culture

(b) Incorporation / 10\textsuperscript{6} cells

Initial rate of incorporation CPM x 10\textsuperscript{3} / ml cell culture / hour

Initial rate of incorporation CPM x 10\textsuperscript{3} / 10\textsuperscript{6} cells / hour

Total uptake

Cell No

TCA insoluble incorporation

Time (days)
The rate of uptake of uridine by the cultures was high at inoculation, decreased through the lag phase but then increased rapidly as the cell mass increased and finally levelled out towards the end of the division phase. The rate of uptake per $10^6$ cells (Fig. 13 (b)), however, was greatest at inoculation and rapidly declined throughout growth of the cultures. The rate of incorporation into the TCA-insoluble fraction of the cells was low at inoculation, then increased throughout the lag phase but remained constant for the remainder of the growth cycle despite the rapid rise in cell number after day 3. The rate of incorporation per $10^6$ cells (Fig. 13 (b)), after a peak at day 3, fell continuously to reach a low rate at day 10 which was subsequently maintained into stationary phase.

The results show that the capacity of the cells to take up uridine from the medium is greatest at inoculation and then declines rapidly. This clearly demonstrates that the relationship between the cells and the medium is constantly changing throughout the growth of the culture and that this affects the rate at which cells can take up small molecules from the medium. The observation that a three-fold increase in the rate of uptake of uridine per cell occurs very soon (approximately 30 minutes) after stationary phase cells are inoculated into new medium demonstrates how rapidly the permeability of such cells, which are non-dividing and in which the metabolic rate is low, to certain small molecules changes in response to a change in the composition of the culture medium.

Incorporation of labelled uridine into TCA-precipitable material occurs throughout the growth cycle at a detectable rate, even after the level of total RNA in the cultures has begun to decline (Short, Brown
and Street, 1969). It therefore appears that an appreciable amount of RNA synthesis may occur towards the end of the growth cycle although the level of total RNA is rapidly declining, which indicates that there is a detectable rate of turnover of RNA. A critical comparison of the rates of RNA synthesis at various stages of growth cannot be attempted simply by measuring the comparative rates of incorporation of radioactive precursors into RNA unless the specific activity of the immediate precursor pool can be accurately measured (Emerson and Humphreys, 1971; Nierlich, 1968). Any variations in the size or organisation of the pools or the permeability of the cells to labelled precursor would result in changes in the rate of incorporation of radioactivity into RNA without there being any actual change in the rate of RNA synthesis. It is, however, interesting to note that the highest rate of incorporation of labelled uridine into RNA per $10^6$ cells coincided with the maximum rate of accumulation of total RNA per cell recorded by Short et al (1969). This strongly suggests that the rate of RNA synthesis is greatest in the late lag phase and early division phase of the growth cycle.

The uptake and incorporation of 2-$^{14}$C-uridine throughout the growth cycle

Previous experiments had established that uridine fed at a concentration of $5 \times 10^{-4}$M could sustain a steady state of labelling throughout the period of sampling in an actively dividing culture. 2-$^{14}$C-uridine was therefore fed to cultures, at inoculation, at a concentration of $5 \times 10^{-4}$N, specific activity 0.1 mCi/mM. The total uptake and incorporation of labelled uridine were followed with time throughout the 21 day growth cycle of the culture together with
measurements of cell number. The cold TCA-insoluble fraction of the cells was degraded at regular intervals to determine the distribution of radioactivity between RNA, DNA and protein.

The results (Fig. 14) showed an initial phase of uptake and incorporation of labelled uridine lasting approximately 6 days followed by a gradual loss of radioactivity from the cell population and a very marked loss of radioactivity from the TCA-insoluble fraction of the cell. The loss of radioactivity from the TCA-insoluble fraction plotted per $10^6$ cells (Fig. 14(b)) was most dramatic as approximately 90% of the radioactivity accumulated by this fraction during the first 6 days of incubation had disappeared by day 14. The amount of radioactivity in the metabolic pool (the difference between the total and TCA-insoluble radioactivity) remained fairly constant during the phase of uptake and incorporation but expanded rapidly during the phase of rapid loss of radioactivity from the TCA-insoluble fraction of the cell. The measurements of the distribution of radioactivity between RNA, DNA and protein (Table 6) showed that up to 3 days more than 90% of the total TCA-insoluble radioactivity was present in RNA. At later times in the growth cycle an increasing proportion of this radioactivity was found in DNA but very little was present as protein or non nucleic acid material at any point during growth of the culture.

The interpretation of these results presents some difficulties. The actual increase in the total cell radioactivity and TCA-insoluble radioactivity ceased very early in the growth cycle although growth and synthesis of RNA were continuing. It can be calculated that $5 \times 10^{-4}$M uridine incorporated at 100% efficiency would provide the uracil and cytosine bases for more RNA than is known to accumulate at
FIG. 14

INCORPORATION OF $^2\text{-}{}^{14}\text{C-URIDINE}$ FED AT INOCULATION AT $5 \times 10^{-4}\text{M}$ INTO SUSPENSION CULTURES OF ACER PSEUODPLATANUS L

(a) Total and TCA insoluble incorporation per ml culture

(b) TCA insoluble incorporation per $10^6$ cells
TABLE 6
The distribution of radioactivity in the TCA-insoluble fraction of cells between RNA, DNA and protein

<table>
<thead>
<tr>
<th>Time of sampling days</th>
<th>Cold TCA insoluble radioactivity cpm/ml culture</th>
<th>Warm dil KOH resistant radioactivity cpm/ml culture</th>
<th>Hot TCA insoluble radioactivity cpm/ml culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>53.10</td>
<td>18.90</td>
<td>14.60</td>
</tr>
<tr>
<td>3</td>
<td>9940.36</td>
<td>755.80</td>
<td>504.00</td>
</tr>
<tr>
<td>8</td>
<td>16969.48</td>
<td>1910.80</td>
<td>334.95</td>
</tr>
<tr>
<td>21</td>
<td>7197.30</td>
<td>2208.22</td>
<td>441.30</td>
</tr>
</tbody>
</table>

With background (0-time sample) subtracted:

<table>
<thead>
<tr>
<th>Time of sampling days</th>
<th>Percentage RNA cold TCA-dil KOH x 100</th>
<th>Percentage DNA dil KOH-hot TCA x 100</th>
<th>Percentage protein hot TCA x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>92.55</td>
<td>2.50</td>
<td>4.95</td>
</tr>
<tr>
<td>8</td>
<td>88.80</td>
<td>9.20</td>
<td>1.90</td>
</tr>
<tr>
<td>21</td>
<td>69.35</td>
<td>24.60</td>
<td>5.90</td>
</tr>
</tbody>
</table>
any time in these cultures (Short et al., 1969). Further experiments were therefore performed to clarify this situation.

2-\textsuperscript{14}C-uridine was fed to replicate cultures (inoculated from the same stock culture) at a range of concentrations from \(10^{-5}\) to \(10^{-3}\) M specific activity \(50\mu\text{Ci}/\mu\text{M}\) at inoculation. The total uptake, incorporation and loss of radioactivity from the medium were measured throughout the growth of the culture together with the distribution of radioactivity in the TCA-insoluble fraction as in the previous experiment. In addition the release of \(\text{\textsuperscript{14}CO}_2\) and the growth parameters of cell number, dry weight and packed cell volume were measured throughout growth of the cultures.

The results are shown in Figs. 15, 16 and 17. Total uptake of labelled uridine continued until only a small proportion of the original radioactivity remained in the medium. This effective depletion of the medium occurred very rapidly in the culture fed uridine at \(10^{-5}\) M and even at \(10^{-3}\) M continuous uptake was only maintained for the first 9 days of growth of the culture. \(\text{\textsuperscript{14}CO}_2\) evolution occurred at a high rate throughout the period of uptake of uridine and was clearly responsible for the discrepancy between the amount of radioactivity lost from the medium and the actual amount which was found in the cells. The proportion of radioactivity disappearing from the medium that was given off as \(\text{\textsuperscript{14}CO}_2\) increased with increasing external concentrations of uridine as was found in previous experiments. After depletion of the medium, however, an appreciable loss of radioactivity from the total cell fraction was observed in all cultures. The initial high rate of \(\text{\textsuperscript{14}CO}_2\) evolution also fell very rapidly when the medium was depleted but an appreciable amount of \(\text{\textsuperscript{14}CO}_2\) continued to be evolved
FIG. 15

INCORPORATION OF 2-¹⁴C-URIDINE FED AT INOCULATION AT 10⁻⁵M AND EVOLUTION OF
¹⁴CO₂ DURING THE GROWTH CYCLE OF SUSPENSION CULTURES OF ACER PSEUDOPLATANUS L.

Loss of radioactivity from medium. Total uptake into the cells and ¹⁴CO₂ evolution.

Total uptake of radioactivity and incorporation into the TCA insoluble fraction of cells.
FIG. 16
INCORPORATION OF 2'-C-URIDINE FED AT INOCULATION AT 10^{-4} M AND EVOLUTION OF {^{14}CO_2} DURING THE GROWTH CYCLE OF SUSPENSION CULTURES OF ACER PSEUDOPLATANUS L.

Loss of radioactivity from medium. Total uptake into the cells and {^{14}CO_2} evolution.

Total uptake of radioactivity and incorporation into the TCA insoluble fraction of cells.
FIG. 17

INTEGRATION OF 2-14C-URIDINE FEED AT INOCULATION AT 10^3 M AND EVOLUTION OF
14CO2 DURING THE GROWTH CYCLE OF SUSPENSION CULTURES OF AGER PSEUDOHYMENIUS.

Label: Total uptake, TCA insoluble incorporation into the TCA insoluble fraction of cells.

Graph: Loss of radioactivity from medium, total uptake into the cells, and 14CO2 evolution.

Y-axis: Radioactivity CPM x 10^3/ml cell culture
X-axis: Time (days)
by the cultures. In addition there was also some evidence that at 10^{-5} and 10^{-4} M a small amount of exchange of radioactive compounds with the medium occurred between day 4 and day 8 of the growth cycle.

The total content of radioactivity in the total cell fraction and the medium were measured under conditions of scintillation counting which gave approximately the same counting efficiency of about 70\%, whilst that of the \(^{14}\)\textsubscript{CO}_2 was measured at a counting efficiency of approximately 50\%. Although it is not possible to compile an accurate balance sheet from the measurements made in this experiment a rough calculation, using the above counting efficiencies, shows that the discrepancy between the amount of labelled uridine lost from the medium and that taken up by the cells was of the order to be expected from the total amount of \(^{14}\)\textsubscript{CO}_2 evolved.

The incorporation of labelled uridine into the TCA-insoluble fraction of the cells showed some interesting features. Incorporation continued at varying rates, depending upon the concentration of uridine applied, until uptake from the medium ceased. 24 hours after effective depletion of the medium the total TCA-insoluble radioactivity of each culture was found to have decreased. This loss of total TCA-insoluble radioactivity continued throughout the remainder of the growth cycle the rate of loss in all cultures being greatest between 8 and 14 days. At high concentrations (10^{-5} M, Fig. 17) the loss of total TCA-insoluble radioactivity coincided with the period of growth cycle in which a decline in the level of total RNA in the cultures has been shown to occur (Short et al., 1969). At lower concentrations (10^{-5} and 10^{-4} M Figs. 15 and 16), however, the decline in the level of TCA-insoluble radioactivity occurred during a period of the growth cycle.
in which the level of total RNA increases at a high rate.

The radioactivity of the pool (the difference between the total uptake and TCA-insoluble incorporation curves) increased rapidly during the first day of culture but then remained constant until the medium was near to depletion. When \(^{14}\)C-uridine was supplied at \(10^{-5}\)M and \(10^{-4}\)M (Fig. 15 and 16) the amount of radioactivity in the pool became very small at the point at which the medium became depleted. Immediately after depletion of the medium the amount of radioactivity in the pool became too small to be properly estimated by the difference between the total and TCA-insoluble incorporation. After day 8, however, the radioactivity of the pool in all cultures (Figs. 15, 16 and 17) increased very rapidly to a value much greater than at any time during the phase of uptake and incorporation.

The distribution of radioactivity in the TCA-insoluble fraction (Table 7) showed again quite clearly that most of the carbon-14 labelling was found in RNA in the early part of the growth cycle and that only where uridine had been supplied at high concentration for several days did appreciable labelling of DNA and protein occur.

An important observation from these figures is that the proportion of the total TCA-insoluble radioactivity present in DNA and protein remained low whilst uptake of labelled uridine into the cells continued. After uptake had ceased, however, the proportion of radioactivity in DNA and protein increased rapidly. Close examination of the results shows that this occurred because the amount of radioactivity in DNA and protein continued to increase whilst the total amount of TCA-insoluble radioactivity was rapidly decreasing. This indicates that the decline in the level of total TCA-insoluble radioactivity
TABLE 7

The distribution of radioactivity in the TCA-insoluble fraction of

cells between RNA, DNA and protein

<table>
<thead>
<tr>
<th>Time of sampling (days)</th>
<th>Cold TCA insoluble radioactivity (cpm/ml cult.)</th>
<th>Warm dil. KOH resistant radioactivity (cpm/ml cult.)</th>
<th>Hot TCA insoluble radioactivity (cpm/ml cult.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture fed $10^{-5}$ M $2^{14}$C-uridine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>17.80</td>
<td>12.10</td>
<td>12.15</td>
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<tr>
<td>3</td>
<td>177.40</td>
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<td>6</td>
<td>129.60</td>
<td>40.15</td>
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<td>10</td>
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<td>28.40</td>
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<tr>
<td>17</td>
<td>71.00</td>
<td>41.10</td>
<td>27.60</td>
</tr>
<tr>
<td></td>
<td>With background (0-time sample) subtracted:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>159.60</td>
<td>20.00</td>
<td>10.00</td>
</tr>
<tr>
<td>6</td>
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<td>28.05</td>
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<tr>
<td>17</td>
<td>53.20</td>
<td>29.00</td>
<td>15.45</td>
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</table>

<table>
<thead>
<tr>
<th>Time of sampling (days)</th>
<th>Cold TCA insoluble radioactivity (cpm/ml cult.)</th>
<th>Warm dil. KOH resistant radioactivity (cpm/ml cult.)</th>
<th>Hot TCA insoluble radioactivity (cpm/ml cult.)</th>
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<td>Culture fed $10^{-4}$ M $2^{14}$C-uridine</td>
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</tr>
<tr>
<td>0</td>
<td>17.60</td>
<td>15.78</td>
<td>12.90</td>
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<td>645.10</td>
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<td>With background (0-time sample) subtracted:</td>
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<tr>
<td>17</td>
<td>415.20</td>
<td>175.32</td>
<td>110.80</td>
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</tbody>
</table>
### TABLE 7 contd.

The distribution of radioactivity in the TCA-insoluble fraction of cells between RNA, DNA and protein

<table>
<thead>
<tr>
<th>Time of sampling (days)</th>
<th>Cold TCA insoluble radioactivity (cpm/ml cult.)</th>
<th>Warm dil.KOH resistant radioactivity (cpm/ml cult.)</th>
<th>Hot TCA insoluble radioactivity (cpm/ml cult.)</th>
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<tbody>
<tr>
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<td>23.30</td>
<td>16.80</td>
<td>14.20</td>
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<td>2400.60</td>
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<td>117.40</td>
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<td>3824.80</td>
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<td>1088.20</td>
<td>217.30</td>
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<tr>
<td>17</td>
<td>3445.60</td>
<td>1814.10</td>
<td>1071.00</td>
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</table>

With background (0-time sample) subtracted:

<table>
<thead>
<tr>
<th>Time of sampling (days)</th>
<th>Cold TCA insoluble radioactivity (cpm/ml cult.)</th>
<th>Warm dil.KOH resistant radioactivity (cpm/ml cult.)</th>
<th>Hot TCA insoluble radioactivity (cpm/ml cult.)</th>
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<td>3801.50</td>
<td>164.80</td>
<td>276.70</td>
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<td>5520.70</td>
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<td>203.10</td>
</tr>
<tr>
<td>17</td>
<td>3422.30</td>
<td>1797.30</td>
<td>1056.80</td>
</tr>
<tr>
<td>Conc. of uridine</td>
<td>Time of sampling</td>
<td>Percentage RNA</td>
<td>Percentage DNA</td>
</tr>
<tr>
<td>-----------------</td>
<td>----------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td></td>
<td>days</td>
<td>cold TCA - dil KOH x 100</td>
<td>dil KOH - Hot TCA x 100</td>
</tr>
<tr>
<td>$10^{-5}$ M</td>
<td>3</td>
<td>87.40</td>
<td>6.27</td>
</tr>
<tr>
<td></td>
<td>6</td>
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<td>9.93</td>
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<td>25.50</td>
</tr>
<tr>
<td>$10^{-4}$ M</td>
<td>3</td>
<td>93.72</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>94.12</td>
<td>0.41</td>
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<tr>
<td></td>
<td>17</td>
<td>57.77</td>
<td>15.54</td>
</tr>
<tr>
<td>$10^{-3}$ M</td>
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<td>93.67</td>
<td>1.99</td>
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<td>86.59</td>
<td>15.72</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>47.40</td>
<td>21.64</td>
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</table>
after uptake of labelled uridine from the medium had ceased was almost entirely due to loss of radioactivity from total RNA.

The growth data show that over the range of concentrations used in this experiment (10^{-5} to 10^{-3} M Figs. 18 and 19) there does not appear to be any marked effect of uridine upon cell division, dry weight accumulation or cell expansion in these cultures.

These data illustrate some interesting features of the uptake and metabolism of exogenously applied uridine by plant cell suspension cultures, and, of the turnover of the total RNA in the cultures and its relationship to the metabolic pool. It can be seen that there are two distinct phases in the pattern of uptake and utilization of ^{14}C-labelled uridine. First, there is a phase of uptake and incorporation during which a large proportion of the uridine taken up is degraded with loss of the carbon-14 radioactivity as ^{14}CO_2. The proportion of uridine which is degraded increases with increasing concentrations supplied and explains why such large external concentrations of uridine are required to maintain continuous labelling of the cultures for several days. As soon as the medium becomes depleted, however, a second phase of uridine utilization is observed where radioactivity is lost from both the total and TCA-insoluble fractions of the cell and where ^{14}CO_2 release occurs at a much reduced rate. Radioactivity is rapidly lost from the TCA-insoluble fraction and in cultures fed 10^{-5} and 10^{-4}M ^{14}C-uridine this occurs when the level of total RNA is rapidly increasing (Short et al., 1969). This suggests that there is turnover of a large proportion of the total RNA occurring throughout the growth cycle and that a substantial fraction, if not all, of the products of RNA degradation are not readily available for RNA
FIG. 18

THE EFFECT OF URIDINE ON INCREASE IN CELL NUMBER IN SUSPENSION CULTURES OF ACER PSEUDOPLATANUS L.

CONTROL CULTURE 10^{-5} M URIDINE

0.5 1.0 1.5 2.0 2.5 3.0
Cell No./ml x 10^{-6}

0 4 8 12 16 20
Time (days)

10^{-4} M URIDINE 10^{-3} M URIDINE

0.5 1.0 1.5 2.0 2.5 3.0
Cell No./ml x 10^{-6}

0 4 8 12 16 20
Time (days)
FIG. 19
THE EFFECT OF URIDINE ON DRY WEIGHT ACCUMULATION AND PACKED CELL VOLUME OF SUSPENSION CULTURES OF ACER PSEUDOPLATANUS L.

[Graphs showing the effect of uridine on dry weight accumulation and packed cell volume of suspension cultures of Acer pseudoplatanus L.]
synthesis. If these products of RNA degradation were efficiently reutilized for RNA synthesis the radioactivity of the TCA-insoluble fraction of the culture would be expected to remain constant or fall only slightly until there was an actual loss of total RNA from the culture. The rate of decline of total TCA-insoluble radioactivity of the culture is very high especially at later times in the cycle and if correction is made for the increase in cell number (for example Fig. 14) and the increasing proportion of the TCA-insoluble radioactivity present as DNA and protein is considered, it can be seen that a large proportion of the total RNA per cell labelled during the phase of uptake of 14C-uridine is broken down before the end of the growth cycle.

In cultures where the supply of uridine is exhausted early in the growth cycle (cultures fed uridine at 10^{-4} and 10^{-5}M) most of the products of RNA degradation during the period between depletion of the medium and day 8 of incubation of the culture are either released into the medium or broken down and evolved as 14CO_2 as there is virtually no radioactivity in the acid-soluble fraction of the cell, the pool. After 8 days, however, although some of the products of RNA degradation are degraded to 14CO_2 the majority accumulate in the acid-soluble fraction of the cell forming a very large pool of radioactive compounds. This probably results from the accelerated loss of radioactivity from the TCA-insoluble fraction of the cell at 8 days, and, since the radioactivity of this pool is much greater than the maximum size of the precursor pool during the phase of uptake, and it does not appear to be a pool from which nucleotides are drawn to any extent for RNA synthesis, it is probably spatially or chemically distinct from the RNA precursor pools. The possibility exists that
in response to a high cytoplasmic level of RNA degradation products nucleotides or compounds derived from them may be compartmentalised in the vacuole.

The apparent rapid turnover of RNA is interesting since even during the early part of the division phase of the growth cycle in one cell generation time (approximately 72 hours) a large proportion of the RNA synthesised by the culture after inoculation is broken down (see culture fed $10^{-4}$M uridine, Fig. 16). The proportion of the total RNA rapidly degraded is so large that it is presumably the major components of total cellular RNA, the ribosomal and soluble RNA that are involved. Studies with bacteria (Kaempfer, Meselson and Raskas, 1968) have shown that the sub-units of ribosomes, which contain the majority of the cellular RNA, are stable for several cell generations and it is therefore of interest to determine if the two molecular species of ribosomal RNA in these cultures are rapidly synthesised and degraded.

**Fractionation of labelled RNA at various points in the growth cycle**

In order to gain some information about the labelling characteristics of the major molecular species of cellular RNA an experiment was performed in which replicate cultures were incubated in the presence of $10^{-3}$M 2-$^{14}$C-uridine specific activity $50$Ci/$\mu$M. Samples of culture were taken after 3 and 6 days during the phase of uptake and incorporation of labelled uridine and after 14 days during the phase of loss of radioactivity from RNA. The RNA of these samples was then extracted and fractionated on sucrose density gradients.

The results (Fig. 20) show the optical density and radioactivity profiles of the sucrose density gradients. The relative specific
activities of the three major species of cellular RNA, the 4S or soluble RNA and the two ribosomal components 18S and 26S, can be compared from their ratios of radioactivity to optical density. There was clearly an increase in the specific activity of all three species of RNA at 6 days compared with that at 3 days but at 14 days, after approximately 5 days in medium depleted of isotope, the specific activity of all species had fallen considerably.

These results are consistent with the observations made from the incorporation curves but do not give any indication of a preferential degradation of ribosomal RNA. Clearly the proportion of newly synthesised RNA increases between day 3 and 6. After the medium is depleted of isotope, however, the proportion of RNA synthesised whilst the isotope was present begins to fall. These results do not show whether this decrease in the specific activity of all species of RNA is a result of degradation of the labelled RNA or dilution by synthesis of new RNA. From the results of Short et al. (1969) and the incorporation curves previously described it is possible to speculate that there is both synthesis of new RNA and degradation of pre-existent RNA but there does not appear to be any preferential synthesis or degradation of any of the three major species of cellular RNA throughout the growth cycle.
Radioactive uridine has been investigated as a possible means of introducing a label into RNA in order to study the synthesis and degradation of RNA in plant cell suspension cultures during various phases of cell growth and development. In any such studies the design and interpretation of experiments will depend upon three major considerations: the characteristics of entry of externally applied precursor into the cellular pools; the possible compartmentalisation of these molecules within the pool; and the relationship between the pool and the turnover of macromolecules.

The pathway by which externally applied uridine is utilized by the cell is not known with any certainty but there are indications that it may be directly phosphorylated by a uridine kinase to form UMP. There is good evidence of the existence of uridine kinase in other plant tissues (Wanka and Wallboomers, 1967; Wasilewska et al., 1967) and there is some evidence of a uridine kinase in sycamore suspension cultures (M. W. Fowler personal communication). An analysis of the radioactive constituents of the pool during steady state conditions of incorporation of labelled uridine failed to detect any free radioactive uridine and it therefore appears that uridine is very rapidly metabolised on entry into the cell. Rapid phosphorylation of uridine may also have some function in retaining incorporated uridine within the cell since Brown and Short (1969) have shown that nucleotides do not leak out of plant cells into the culture medium and no evidence has been found in present experiments of exchange of radioactive compounds between the cells and the medium in incorporation experiments of short duration.
The radioactive UMP formed from exogenous uridine is presumably converted to UDP and then to UTP which is incorporated into RNA. Hydrolysis of total RNA extracted from cells incubated for a long period of time in labelled uridine has shown that radioactivity appears not only in the UMP residue of RNA but also in the CMP residue. This therefore demonstrates that uracil-containing compounds are converted to cytosine-containing compounds by the plant cell which is not surprising since interconversion of uracil and cytosine compounds is known to occur in bacteria (Roberts et al., 1955, McCarthy and Britten, 1963) by the conversion of UTP to CTP (Lieberman, 1956).

The rate at which radioactive precursor is incorporated into RNA at any particular time is determined by the rate of RNA synthesis and the specific activities of the immediate precursor pools. When an exogenous labelled precursor enters the cell the rate at which the specific activities of the pools increase, and then the rate at which incorporation into RNA occurs, depends upon a number of factors: the size of the endogenous pools and the rate at which the exogenous precursor equilibrates between them; the degree to which endogenous synthesis of precursors is suppressed; and the possible expansion of the endogenous pools by application of exogenous precursor. Measurement of the specific activities of the UMP and CMP residues in RNA extracted from cells which had been incubated for a long period of time with labelled uridine showed that they closely approached the specific activity of the labelled uridine supplied to the cells. This clearly indicates that exogenous uridine is to a large extent preferred to endogenously synthesised pyrimidine precursors for RNA synthesis.
The kinetics of pool formation have shown that on feeding labelled uridine to actively dividing cultures it is several hours before the acid-soluble pool becomes saturated with radioactive compounds. There is therefore either a slow equilibration of the radioactive compounds with the pre-existant pools of unlabelled compounds or very substantial pool expansion. An experiment in which the kinetics of incorporation of \(^{14}\text{C}\)-uridine were investigated in a culture preincubated with a high concentration of unlabelled \(^{12}\text{C}\)-uridine indicated that there may be some expansion of the pools but that such expansion is not very great in so far as it affects the kinetics of \(^{14}\text{C}\)-uridine incorporation. Brown and Short (1969), however, have shown that there are very large internal pools of UMP and UDP-glucose in this tissue and the long period of time taken for the total acid-soluble pool to saturate with radioactivity is therefore probably a reflection of the large size of the endogenous pools of uracil-containing compounds.

In addition to the rate at which the specific activities of the precursor pools increase the characteristics of incorporation of an exogenous radioactive precursor into RNA can also be affected by the existence of fractions of the total RNA which have different rates of synthesis and degradation, compartmentalisation of the precursor pools and the conversion of the precursor to other compounds within the cell that might also be incorporated into RNA.

A striking feature of the experimental results using \(2^{14}\text{C}\)-uridine is that there is very little delay in labelling of RNA although there is evidence of a very large nucleotide pool that labels comparatively slowly. The features of uridine incorporation in these experiments showed some marked similarities with those of the incorporation of
nucleic acid bases into the pool and RNA in bacteria (McCarthy and Britten, 1962; Buchwald and Britten, 1963). McCarthy and Britten have explained these observations by considering the possibility of compartmentalisation of the pools as represented by the schematic diagram shown in the results section (p. 39). This diagram suggests a possible mechanism for a by-pass around the large cellular pool which is consistent with the observations of incorporation of uridine at high and low concentrations. The rate of incorporation into RNA, in this scheme, is determined by the specific activity of the small immediate precursor pool P. This will depend upon the rate of entry of radioactivity into P and the extent to which the compounds in this pool mix with those derived from the large cellular pool S and from endogenous synthesis. Initially labelled uridine passes into P and its specific activity is diluted by mixing with unlabelled precursor molecules derived from pool S and from endogenous synthesis. The specific activity of S increases comparatively slowly and only after a delay do compounds passing into P from S become radioactive thus increasing the specific activity of P. The specific activity of P continues to rise until both pools are equilibrated with the radioactive compound. When uptake of uridine from the medium ceases the rate of incorporation falls abruptly and radioactive compounds pass only from the large pool into P and thence into RNA.

The results of the "chase" experiment were also consistent with this model. $^{14}$C-uridine was added at $10^{-6}$ M and incorporation was allowed to continue for 20 minutes after which $10^{-3}$ M $^{12}$C-uridine was added before radioactivity from S could make a substantial contribution to the incorporation of radioactivity into TCA-precipitable RNA. The
incorporation of radioactivity does not, however, fall to its second phase rate abruptly, there is a delay of 10 minutes, and this provides an estimate of the amount of labelled compounds in the state P. The upper limit of the quantity of labelled compounds in pool P is that amount which can sustain RNA synthesis for approximately 10 minutes.

The kinetics of 2-\(^{14}\)C-uridine incorporation into RNA in an experiment designed to test the possibility of expansion of the pools by high concentrations of exogenous uridine showed a slight initial delay of incorporation of radioactivity into TCA-precipitable RNA in cultures preincubated with a high concentration of unlabelled uridine. This suggests that there may be some expansion of the pool size of one or more of the constituents of the by-pass mechanism. The results also showed, however, that the degree of pool expansion is not very great.

One of the major features of the incorporation of labelled uridine into TCA-precipitable RNA, the increase in the rate of incorporation after approximately 35 minutes incubation could be due to a component of incorporation delayed by the conversion of uracil-compounds to cytosine-compounds. McCarthy and Britten (1962) investigated this possibility in bacteria and found that the two-phase incorporation curve could not be explained by a delayed incorporation of labelled cytosine compounds synthesised from the \(^{14}\)C-uracil supplied. The available data from the plant cell system is insufficient, however, to be able to completely exclude this possibility.

Gros et al (1961) have explained similar results obtained for the kinetics of incorporation of \(^{14}\)C-guanine in E.coli by considering the existence of two classes of RNA, one of which is rapidly turned over and the other which is comparatively stable. Salser, Janin and Levinthal
(1968) from a mathematical analysis of the kinetics of labelling of the GTP pool in *B. subtilis* and *E. coli* and Nierlich and Vielmetter (1968) from measurements of the rate of loss of radioactivity from RNA after treatment of *E. coli* with actinomycin D have argued in favour of this second hypothesis although compartmentalisation of the pools has not been excluded. In order to explain the kinetics of incorporation of uridine into RNA in the plant cell cultures the rapidly turned over RNA would have to constitute a large proportion of the total synthesis of RNA. In addition the specific activity of the pool would have to be diluted very rapidly by internal synthesis of precursor molecules to explain the abrupt fall in the rate of incorporation after uptake of uridine from the medium ceases.

In the absence of detailed information on the rates of turnover of the various species of RNA and the effects of conversion of uracil compounds to cytosine compounds on the initial kinetics of incorporation the experimental results are best explained on the basis of pool compartmentalisation. It is perhaps not surprising that the major pool of uracil compounds may be by-passed for synthesis of RNA as these compounds have other functions in the cell. UMP was found to be the major labelled constituent when 2-^14^C-uridine was fed to the cultures and since the acid-soluble fraction of these cells has been shown to contain a very large amount of UMP and UDP-glucose (Brown and Short, 1970) it is probable that the main function of these pools is to supply UDP-glucose for cell wall synthesis. In addition since the plant cell has considerable structural compartmentalisation it is possible to envisage a situation for instance where there may be a large cytoplasmic pool, concerned principally with supplying UDP-glucose for cell wall
synthesis, which is by-passed by a small pool of ribonucleotides in
the nucleus concerned with RNA synthesis.

During these studies an unexpected observation was made that a
proportion of the $^{14}$C-uridine incorporated by the cells was rapidly
degraded with release of $^{14}$CO$_2$, a process which must involve break-
age of the pyrimidine ring and further metabolism of the breakdown
products. A possible pathway by which this could occur has been suggest-
ed based on the observations of Barnes and Naylor (1962) that uracil
or uridine supplied to pine tissue was degraded to $^\beta$-alanine. The
significance of this pathway in the plant is unclear. It is possible
that it is a means by which the plant cell could obtain nitrogen or
an extra source of energy. It could, however, be a detoxication mech-
anism as such mechanisms are by no means unknown in plant tissues,
for example IAA oxidase. Very little carbon-14 from 2-$^{14}$C-uridine
enters any macromolecule in the TCA-insoluble fraction of the cell
other than RNA in these short term experiments which suggests that
the carbon skeleton of the breakdown products is not used directly
for protein synthesis. In addition, when uridine was fed to cultures
at low concentrations ($5 \times 10^{-7}$ to $10^{-6}$M) most of the radioactivity
removed from the medium passed into the pool and into RNA, and only
a relatively small fraction was released as $^{14}$CO$_2$. The proportion of
uridine degraded, however, increased rapidly with increasing concen-
tration of uridine. These observations lend support to the idea of
a detoxication mechanism.

Exogenous uridine therefore appears to be used primarily for RNA
synthesis and degradation does not appear to have any marked effect
upon the efficiency with which it is incorporated into RNA. It does,
however, create difficulties in experiments where continuous labelling
is required because attempts to supply sufficient uridine are counter-
acted by the ensuing high rate of degradation. It would be interesting
to investigate whether this effect is restricted to uracil compounds
or whether it occurs with other pyrimidines and perhaps purines.

An investigation of the incorporation of $^{14}$C-uridine during the
various phases in the growth cycle of suspension cultures of Acer
pseudoplatanus L. has shown that nucleosides are taken up from the
medium and incorporated into RNA at varying rates during all phases
of growth. Long term labelling experiments in which the $^{14}$C-uridine
was added to the cultures, at various concentrations. at inoculation,
have revealed some interesting facts concerning the synthesis and
degradation of total RNA throughout the growth of the cultures. The
most striking feature is the substantial net loss of radioactivity
from total RNA after $^{14}$C-uridine in the medium is exhausted. A large
proportion of the RNA synthesised after inoculation of the culture
(the $^{14}$C-labelled RNA) is degraded after one cell generation time has
elapsed from the point at which uptake of $^{14}$C-uridine from the medium
ceased. This can occur in the early part of the growth cycle when
the accumulation of total RNA is proceeding at maximum rate and is
not associated with a decline in total RNA. The products of RNA deg-
radation, therefore, are clearly not efficiently reutilized for RNA
synthesis. If the depletion of the medium occurs during the lag phase
or the early division phase the products of the ensuing RNA degradation
are either converted to a compound which can pass out into the medium
or are further degraded and released as $^{14}$CO$_2$. At later stages in the
division phase, however, the degradation products pass into the TCA-
soluble fraction of the cell the size of which increases several times.
These observations underline the possible compartmentalisation of the
pools as it appears that a large proportion, if not all, of the products of RNA degradation are organized in a spatial or chemical state which renders them unavailable for RNA synthesis. The suggestion has been made, since the size of the acid soluble pool at later times in the division phase of growth is very large, that some of these radioactive compounds may exist in the vacuole.

These observations raise some important questions concerning the stability of the major species of cellular RNA. The degree of degradation is so great that it must involve breakdown of the three major species of cellular RNA, the soluble RNA and the two species of ribosomal RNA. Sucrose density gradient fractionation of RNA extracted from cultures of several ages, before and after uptake of labelled uridine from the medium had ceased indicated that there is no preferential degradation of ribosomal or soluble RNA. It therefore appears that a considerable proportion of all major species of cellular RNA are turned over during one cell generation. It is quite clear that in rapidly growing cells of higher organisms (Rake and Graham, 1964) and in exponentially growing bacteria (Davern and Meselson, 1960; Kaempfer et al., 1968) ribosomal RNA is not rapidly turned over and is stable for several cell generations. In rat liver cells, however, labelled ribosomal RNA is found to decay exponentially with a half-life of from two to five days, considerably less than the half-life of the average liver parenchymal cell (Loeb, Howell and Tomkins, 1965; Hadjolov, 1966).

In the unicellular alga *Chlamydomonas reinhardtii* most of the ribosomal RNA is turned over during sexual differentiation (Siersma and Chang, 1971) and this latter example is particularly interesting as the products of RNA breakdown expanded the TCA-soluble pool and were not reutilized
for RNA synthesis. It would therefore be interesting to investigate the half life of ribosomes in the batch sycamore suspension cultures using double labelling or density labelling techniques to separate the newly synthesised ribosomes from the preexistent ribosomes. It would also be necessary to compare such results from the batch cultures with the results of similar experiments carried out on cell cultures maintained in a number of steady states of growth in the apparatus described by Wilson, King and Street (1971). It is possible that the apparent rapid rate of turnover of ribosomal RNA may be a consequence of the batch culture system in so far as an exponential rate of growth is never sustained for any length of time and the growth rate of the culture begins to decrease very soon after the onset of cell division.

The results of the experiments performed in this section have shown that labelled uridine supplied exogenously to suspension cultures of Acer pseudoplatanus L. is rapidly taken up and efficiently utilized as a specific precursor of RNA. The disadvantage of using this RNA precursor is the fact that where it is supplied at high concentrations a large proportion of the uridine taken up into the cells is rapidly degraded and in the case of 2-\(^{14}\)C-uridine the radioactivity is released as \(^{14}\)CO\(_2\). This may, however, be a detoxication mechanism which enables the plant cell to tolerate high concentrations of exogenous uridine since there were no detectable effects of uridine upon growth over a wide range of concentrations.
SECTION II

RIBOSOMAL RNA SYNTHESIS IN
RAPIDLY DIVIDING SUSPENSION
CULTURES OF ACER PSEUDOPLATANUS L.
Introduction

The synthesis and assembly of ribosomes has been extensively investigated in animal cells, particularly in mammalian tissue cultures, and the subject has been reviewed by Perry (1967) and Darnell (1968).

Ribosomes throughout nature contain two large molecular species of RNA which have sedimentation coefficients of 23S and 16S in bacteria, and 28S and 18S in mammalian cells. In the majority of other organisms the sedimentation coefficients of these RNA species fall between the values for the bacterial and the mammalian RNA species. In addition, most ribosomes contain a relatively small molecular species of RNA with a sedimentation coefficient of approximately 5S.

In mammalian cells a large molecule of sedimentation coefficient 45S is synthesised in the nucleolus (Perry, 1962; Scherrer, Latham and Darnell, 1963). The molecular weight of this molecule has been estimated at 4.4 x 10^6 daltons (Weinberg, Loening, Willems and Penman, 1967; McConkey and Hopkins, 1969) and it has been shown to be methylated either during or very soon after its synthesis (Greenberg and Penman, 1966). It is then processed, through a sequence of intermediate stages which occur in the nucleolus to form the 28S and 18S ribosomal RNA species found in the mature ribosome (Penman, Smith and Holtzman, 1966; Warner, Soeiro, Birnboim, Girard and Darnell, 1966; Weinberg et al., 1967).

The molecular weight of the precursor is approximately twice the sum of the molecular weights of the 28S and 18S ribosomal RNA components (1.7 x 10^6 and 0.7 x 10^6 daltons) and the excess RNA which is not methylated appears to be discarded during the processing sequence (Weinberg et al., 1967; Zimmerman and Holler, 1967; Jeantur, Amaldi
and Attardi, 1968). It appears that there are two sequences rich in 2'-O-methyl nucleotides, one at the 5'-terminus of the 45S precursor and one near the centre of the molecule, and two sequences of RNA which contain few 2'-O-methyl nucleotides, one at the 3'-hydroxyl terminus and the other between the two methylated segments (Quagliorotti, Hidvegi, Wikman and Busch, 1970; Choi and Busch, 1970). The nucleolar 45S precursor is apparently cleaved specifically to 41S nucleolar RNA, losing non-methylated regions in the process. The 41S molecule is then cleaved to give a 32S precursor to the 28S ribosomal RNA and a 20S precursor to the 18S RNA. Two minor molecular species are often seen in the processing sequence, a 36S molecule which arises after 41S cleavage and a 23 or 24S molecule which appears before the 20S precursor to 18S RNA. Weinberg and Penman (1970) concluded that these species were probably only produced when the order of cleavage steps was altered but Egawa, Choi and Busch (1971) have purified the 23S component and have obtained evidence from hybridization experiments that it is not a degradation product of 28S RNA and that it appears to be a precursor of 18S RNA.

The 32S precursor has a prolonged stay in the nucleolus whilst the smaller RNA component, the 18S, matures rapidly from the 23S precursor and is transported into the cytoplasm (Girard, Penman and Darnell, 1964). Association of ribosomal proteins with ribosomal RNA appears to begin immediately after, or even coincident with, the synthesis of ribosomal precursor RNA and continues until the mature 60 and 40S ribosomal subunits, containing 28S and 18S RNA, appear in the cytoplasm. The 5S RNA is apparently not part of the 45S precursor (Brown, 1968) and appears to be added at a later stage
from a separate pool through which new molecules must pass before entering into ribosome manufacture (Knight and Darnell, 1967).

The function and fate of the large amount of excess RNA in the 45S precursor is obscure. The degraded fragments are extremely rich in G + C (guanine + cytosine) content (Willems, Wagner, Laing and Penman, 1968) and are probably completely unmethylated. They cannot be detected in vivo (Weinberg and Penman, 1970) and are probably degraded very rapidly.

Ribosome biosynthesis has also been studied, in much less detail, in a variety of other organisms. The indications at present are that the very high molecular weight 45S precursor, which contains 40% excess RNA, is peculiar to the mammals. Gall (1966) demonstrated a 40S ribosomal precursor in the newt Triturus which was processed to a 30S precursor and thence to 28S and 18S ribosomal RNA's. Landesman and Gross (1969) described a 40S precursor in Xenopus and Das, Micou-Eastwood, Ramamurthy and Alfert (1970) have described a similar pattern of synthesis in eggs of Urechis caupar. Loening, Jones and Birnstiel (1969) have estimated the molecular weights of the Xenopus precursors as 2.4 - 2.6 x 10^6 daltons for the 40S precursor and 1.78 x 10^6 daltons for the 30S precursor and have concluded that only a relatively small amount of non-ribosomal excess is lost during processing. A very similar situation is found in the insect Chironomous in which a 38S precursor gives rise to a 30S precursor and then to 28S and 18S ribosomal RNA (Ringborg, Daneholt, Edstrom, Egyhazi and Lambert, 1969).

In higher plants, until recently, no such high molecular weight ribosomal precursor could be found, a presumed precursor in pea root-tips (Loening, 1967b) was found to be unstable and frequently lost
during extraction. Rogers, Fraser and Loening (1970) have subsequently described a rapidly labelled high molecular weight RNA precursor in the pea root-tip and in artichoke-tuber tissue. It has an estimated molecular weight of $2.3 \times 10^6$ daltons and in the pea appears to consist of two components migrating very close together on acrylamide gels whilst in the artichoke the peak appears heterogeneous indicating that it may consist of several components. These precursors are processed, a component of $1.4 \times 10^6$ daltons appears and then the $1.3 \times 10^6$ and $0.7 \times 10^6$ mature ribosomal RNA. There was also evidence of a $0.9 \times 10^6$ daltons component which may be a precursor to the $0.7 \times 10^6$ ribosomal RNA. A system has been reported in carrot tissue (Leaver and Key, 1970) where in addition to the $2.3 \times 10^6$ daltons precursor there is evidence of a larger $2.8 \times 10^6$ daltons component. This has an apparent base composition similar to that of ribosomal RNA although its kinetics of labelling do not show clearly whether or not it arises before the $2.3 \times 10^6$ daltons component. Seitz and Richter (1970) have described a 32S precursor in cell suspension cultures of parsley and very recently a precursor with a molecular weight of $3.5 \times 10^6$ daltons, which gives rise to a $2.2 \times 10^6$ daltons precursor and to $1.35 \times 10^6$ and $0.85 \times 10^6$ daltons ribosomal RNA, has been described in *Euglena gracilis* (Brown and Haselkorn, 1971).

The synthesis and processing of ribosomal RNA in plant tissues clearly requires further investigation. The observations of Leaver and Key (1970) suggest that there may either be different systems in different species of higher plants or that there may be variation in the stability and the ease with which the largest precursors are extracted. Plant cell suspension cultures provide a system in which
the flow of labelled precursors through the ribosomal RNA precursors to complete ribosomes can be followed. Suspension cultures of *Acer pseudoplatanus* L. have previously been shown to have a high rate of total RNA synthesis in the early part of the division phase of the growth cycle (Short, Brown and Street, 1969) and some of the major features of the incorporation of labelled RNA precursor into the cellular pools and RNA have been determined. Such cultures, which are close to an exponential rate of growth in which the cell type is relatively homogeneous compared to other higher plant tissues, have therefore been used to study higher plant synthesis of ribosomal RNA.
Methods and Materials

Radiochemicals

$^{14}$C-uridine and $^{14}$C (methyl)-methionine were obtained from the Radiochemical Centre Ltd. Amersham at specific activities of 62mCi/mM and 50mCi/mM respectively.

Growth and labelling of the cell cultures

Suspension cultures of Acer pseudoplatanus L. were grown as previously described in the standard synthetic medium, at 25°C, and at an inoculation density of between 4 and $6 \times 10^5$ cells/ml. Isotopes in aqueous solution were added directly to actively dividing cultures with a Hamilton syringe under sterile conditions.

Cell fractionation and extraction of undegraded RNA

The preparation of undegraded RNA from plant cell suspension cultures is difficult since the high molecular weight species are readily lost through ribonuclease action. In order to minimize contamination by ribonuclease all buffers were freshly prepared and millipore-filtered before use. All glassware was acid-cleaned and great care was taken to avoid touching any part of the extraction apparatus with bare fingers. It is, however, predominantly the endogenous plant nucleases which are responsible for this instability of high molecular weight plant RNA, and an extraction procedure has therefore been developed, based on the procedure of Parish and Kirby (1966) and similar to that described by Loening, Jones and Birnstiel (1969), which minimizes ribonuclease action.

Cells were harvested very rapidly by passing the culture suspension through nylon filters under vacuum. The collected tissue was then immediately frozen in liquid nitrogen and stored at -70°C for extraction.
The frozen tissue was suspended in approximately 5 tissue volumes of homogenization medium at 0°C containing 0.3M sucrose, 0.05M KCl, 5mM magnesium acetate, 0.05M Tris buffer, adjusted to pH 7.8 at 0°C with HCl, and 200μl of DEP (diethyl oxidiformate - Kodak Ltd.; Solymosy, Fedorcsak, Gulyas, Parkas and Ehrenberg, 1968). The tissue was then homogenized in a chilled motor-driven Teflon-in-glass homogenizer for 20 seconds at 0°C and was immediately centrifuged at 1000g for 5 minutes at 2°C to give a crude nuclear-enriched pellet and a cytoplasmic supernatant.

Detergents for RNA extraction were immediately added to the homogenate of the whole tissue or to the cell fractions, 1% w/v TNS (tri isopropyl naphthalene sulphonate, Kodak Ltd.) and 6% w/v 4-AS (4-amino salicylate), as concentrated stock solutions in the extraction buffer, and the solution was shaken for 2 minutes at 0°C. One vol. of freshly redistilled phenol/10% v/v m-cresol previously equilibrated with the extraction buffer and containing 0.1% w/v 8-hydroxyquinoline was added at room temperature and the mixture was shaken for 3 min and then cooled in an ice bath. The aqueous phase was separated from the phenol by centrifugation at 3000g for 5 min at 2°C and was carefully removed together with the interphase protein. The residue in the phenol layer was then re-extracted by adding 0.5 vol. of fresh buffer plus detergents and shaking in a water bath at 60°C for 3 min. The extraction tube was then cooled rapidly to 2°C by immersion in methanol and dry ice, centrifuged at 3000g for 5 min and the aqueous phase was removed and added to the original extract.

The phenol/m-cresol extraction was repeated twice, using 0.5 vol. phenol/m-cresol, keeping the interphase protein with the aqueous
supernatant until the last extraction to ensure maximum extraction of RNA.

Nucleic acids were then precipitated from the aqueous phase by the addition of 0.05 vol. of 3M sodium acetate plus 2 vol. of absolute ethanol at -18°C and storing for several hours at -18°C. The first alcohol precipitate was collected by centrifuging at 10,000g for 20 min at 0°C and was reprecipitated twice from solution in 0.15M sodium acetate buffer pH 6.0, by the addition of 2.5 vol. of absolute ethanol at -18°C. The final precipitate was washed once in 70% ethanol containing 0.15M sodium acetate and the tubes were drained to remove most of the ethanol. It was then dissolved in a small volume of gradient or electrophoresis buffer and the O.D.260 was determined in a Unicam S.P.800 spectrophotometer against a blank containing the respective buffer.

In some experiments DNA was removed by dissolving the first alcohol precipitate in 0.05M KCl, 5mM magnesium acetate, 0.05M Tris buffer, pH 7.8 with HCl, at 0°C and adding deoxyribonuclease (Sigma electrophoretically purified, 1.0mg/ml stock solution in the same buffer) to a final concentration of 50μg/ml. The solution was then incubated for 30 mins at 0°C dispersing any DNA-gel with a glass rod. The incubation was then stopped by the addition of 0.05 vol. sodium acetate 3M and 2.5 volumes of absolute ethanol at -18°C.

**Fractionation of RNA on sucrose density gradients**

A stock solution of 40% w/v sucrose was made up in the gradient buffer 0.01M Tris, pH 7.6 with HCl, 0.2M KCl and 1mM EDTA. The solution was then boiled for 10 minutes with 6μl/ml DEP (Solymosy et al., 1968) to remove any ribonuclease. The sucrose solution was
then diluted to 5% w/v and 20% w/v with the same buffer and 5 - 20% continuous linear gradients were produced, by mixing the two solutions in a two-chambered vessel, in 17.0 ml. Beckman cellulose nitrate ultracentrifuge tubes, previously washed for several days with 1mM EDTA. The gradients were then placed carefully in a refrigerator and cooled to 2°C.

The RNA from the final ethanol precipitation of the extraction procedure was dissolved in a small volume of the gradient buffer at 0°C. A volume of buffer containing 50 - 80μg of RNA (calculated from the O.D. at 260 assuming a concentration of 1mg/ml to have an O.D. of 20 in a 1cm light path) were carefully loaded onto the surface of the sucrose density gradients using a drawn-out pasteur pipette. The gradients were then placed in the pre-cooled buckets of an S.W. 27 rotor and were centrifuged for a time varying between 10 and 15 hours at 27,000 rpm in a Beckman L 265-B ultracentrifuge.

The gradients were then fractionated in a home-built device attached to a Unicam SP 800 spectrophotometer (Fig. 21). The gradients were very slowly and carefully displaced by injecting 50% w/v sucrose into the bottom of the centrifuge tubes from a constant drive perfusor pump (B. Braun Ltd., Melsungen, Germany) through a 1.0 cm light path silica flow cell positioned in the spectrophotometer. This instrument was set at a constant wavelength of 260nm and the optical density peaks were printed out on a recorder with the scale expansion factor set at 5x giving a full scale deflection of 0.4 O.D. unit.

**Determination of radioactivity**

5 drop fractions were collected from the outlet tube of the flow
FIG. 21

DENSITY GRADIENT FRACTIONATOR

Diagram of arrangement of apparatus for density gradient analysis.

Drop forming nozzle for fraction collecting

Constant speed perfuser pump

Unicam SP 800 spectrophotometer

Quartz flow cell 10 cm light path

Edge of bench

Gradient tube clamping device

Gradient tube

1.2 mm bore teflon tubing

Stainless steel outlet tube

PTFE tube mounting

Rubber O-ring

Cellulose nitrate gradient tube

Silicone rubber grommet

Syringe needle

Spring steel pressure plate

PTFE clamp

PTFE moulding

Wooden base
cell, by hand, in 1/2" x 1/2" glass vials and were stored frozen at -18°C. These fractions were allowed to thaw and liquid scintillator was then added directly to the vials. The toluene/Triton X-100 scintillator of Paterson and Greene (1966) was used since aqueous samples containing varying concentrations of sucrose can be counted with very little variation in counting efficiency provided that the aqueous sample constitutes at least 12% and less than 20% of the total volume of the scintillation mixture. The composition of the scintillator was:

\[
5.5 \text{ gm/l PPO} \\
2:1 \text{ v/v toluene/Triton X-100}
\]

Carbon-14 labelled samples were counted with a counting efficiency of approximately 35% in a Beckman LS-100 liquid scintillation system and the maximum variation between samples was generally less than 5% as indicated by external standard channels ratio measurements. The results are therefore expressed directly as counts per minute. Background radioactivity was estimated by counting identical fractions from a gradient containing unlabelled RNA and was subtracted from each fraction of the gradients of labelled RNA.

**Estimation of sedimentation coefficients**

The sedimentation coefficients which are quoted for the RNA species discussed in this section have not been measured accurately. The sedimentation coefficients of the ribosomal RNA components have been assumed to be 18 and 25S (where S refers to a Svedberg unit) which are the values published for higher plant ribosomal RNA by Stutz and Noll, 1967. The sedimentation coefficients of high molecular weight species of RNA have been estimated very approximately by comparison of their mobilities on linear sucrose gradients with those of the 18 and 25S
components of plant ribosomal RNA. A linear relationship between mobility and sedimentation coefficient of various RNA species on linear sucrose density gradients was assumed.

**Fractionation of RNA by gel electrophoresis**

Polyacrylamide gels were prepared according to Loening (1966). Acrylamide was purified by recrystallisation from chloroform and bis-acrylamide was recrystallised from acetone. Suitable volumes of the acrylamides, dissolved in electrophoresis buffer, were mixed and were degassed in vacuo at room temperature for about 20 seconds.

N,N,N',N'-tetramethylethylene diamine (0.033ml) and 10% w/v ammonium persulphate (0.33ml) were added per gm of acrylamide and the solution was pipetted into 4\(\frac{1}{2}\)" x 1\(\frac{1}{4}\)" perspex tubes (manufactured in the departmental workshop by reaming out perspex tubes to a constant internal diameter and polishing). Water was then layered carefully over the gels with a draw-out pasteur pipette.

As soon as the gels had polymerised, dialysis tubing (previously boiled for 20 minutes in lmM EDTA) was fitted over the lower end of the electrophoresis tubes and held in position with a rubber O-ring. The tubes were then placed in a Shandon disc-electrophoresis tank. The electrophoresis buffer consisted of 36mM Tris, 30mM sodium dihydrogen orthophosphate, lmM EDTA (Loening, 1969) to which was added 0.2% w/v sodium dodecyl sulphate (SDS). Pre-electrophoresis was carried out for 1.0 hour at room temperature to run SDS into the gels and remove the polymerisation catalysts.

RNA from the final ethanol precipitation of the extraction procedure was dissolved in a small volume of electrophoresis buffer containing 6% w/v sucrose. Approximately 50μg in 50μl of buffer were
layered very carefully on top of the gels with a drawn-out pasteur pipette. The buffer compartments of the electrophoresis tank were then filled with electrophoresis buffer containing SDS and electrophoresis was continued at room temperature at 6mA/gel for approximately 3.5 hours. The gels were then removed from the perspex tubes and were washed in distilled water for 0.5 to 2.0 hours.

Scanning of the gels for optical density was carried out using a Gilford 240 spectrophotometer fitted with a linear transport model 2415 accessory. The gels were scanned at 265nm in a parallel-sided 10 x 0.6cm quartz cuvette with a light path of 0.6cm and a slit width of 0.2cm.

**Determination of radioactivity**

After optical density scanning the gels were frozen at exactly their scanned length in an open-ended trough made of heavy grade aluminium foil. The gels were placed in the trough with their upper scanned end against a rubber stopper and the other end free. The troughs were then placed on powdered dry ice and the gels were allowed to freeze slowly. The frozen gels were sliced into 1mm segments with a Mickle gel slicer (H. Mickle Ltd., Gomshall, Surrey). Each gel was frozen onto the platform of the instrument with dry ice and was allowed to thaw slightly before cutting. The slices were then stuck onto glass fibre filter discs 1.0cm in diameter (Whatman GF/C) and were dried in an oven at 55°C for several hours. The filter discs were folded against the inside surface of 1⅛" x ⅜" disposable glass vials which were then inserted into the counting vials. Samples were counted in a Beckman LS-100 scintillation spectrometer in the following liquid scintillator:-
All samples were counted under identical conditions and the results are expressed directly as counts per minute. Background radioactivity was estimated by counting gel slices from a gel containing unlabelled RNA and was subtracted from the counts recorded for each gel slice from the gels containing labelled RNA.

**Determination of molecular weight**

A linear relationship between the electrophoretic mobility of RNA in the Tris-sodium phosphate buffer and the logarithm of its molecular weight was assumed (Loening, 1969). *E.coli* RNA, kindly donated by Dr. G. Turnock, and plant RNA were mixed in roughly equal amounts and were applied to the gels. Electrophoresis was carried out for 3.5 hours at 6mA/gel. The gels were then removed from the electrophoresis tubes, washed and scanned for optical density at 265nm. The electrophoretic mobility of each species of RNA was determined by measuring the distance migrated from the origin as shown by the optical density profile. The two components of *E.coli* ribosomal RNA were assumed to have molecular weights of $1.07 \times 10^6$ and $0.56 \times 10^5$ daltons (Loening, 1968 - a compromise of published values Stanley and Bock, 1965; Hamilton, 1967). The molecular weights of the two components of the plant ribosomal RNA were then estimated by comparison of their electrophoretic mobilities with those of the *E.coli* RNA standards.
Chapter 1

Analysis of rapidly labelled RNA from suspension cultures of Acer pseudoplatanus L. by sucrose density gradient zonal sedimentation

The pattern of synthesis of plant cell RNA has been investigated by a kinetic analysis of the labelling of the various fractions of RNA in plant cell suspension cultures. Six day-old actively dividing cultures of Acer pseudoplatanus L. which are in exponential growth and in which the rate of total RNA synthesis has been shown to be high have been used for the investigation.

Sucrose density gradient fractionation of total RNA

Fig. 22 shows the optical density profile of a density gradient fractionation of the total RNA extracted from the cells by the modified Parish and Kirby method. The three major peaks, consisting of the two ribosomal RNA components and the soluble RNA, have been found to be present in similar ratios in extracts from cultures in different phases of the growth cycle (see Fig. 20, Chapter 4, Section I). Short, Brown and Street (1969) have also shown, by fractionation of total RNA on MAK (methylated serum albumin-Kieselguhr) columns, that the ratios of the amounts of the major species of RNA do not change through the growth cycle of sycamore suspension cultures. The sedimentation coefficients have not been measured accurately but are estimated at approximately 25S and 18S for the two ribosomal RNA components and 4S for the soluble RNA component. The 25S component was present in almost exactly twice the amount of the 18S ribosomal component as is expected since ribosomes consist of two easily dissociable subunits, a $\frac{2}{3}$ and a $\frac{1}{3}$ piece by weight, the larger subunit containing 25S RNA and the smaller subunit containing 18S RNA.
FIG. 22

DENSITY GRADIENT FRACTIONATION OF A PREPARATION OF TOTAL CELLULAR RNA.

Volme gradient (ml)

Soluble RNA

Ribosomal RNA

End of Gradient
Analysis of rapidly-labelled nuclear RNA

A large amount of cytological, cyto genetic and biochemical evidence, mainly from work on animal tissues, has indicated that the majority of cellular RNA synthesis occurs in the nucleus. An analysis has therefore been made of the nuclear RNA labelled by a short incubation of the cell culture with $^{14}$C-uridine.

A six day-old dividing culture was fed $2\cdot^{14}$C-uridine at $5 \times 10^{-6}$M and at a specific activity of 62mCi/mM, and was incubated for 40 minutes. The cells were then harvested and fractionated and RNA was extracted from a crude nuclear-enriched pellet. DNA was not digested. The RNA was then fractionated on a 5-20% w/v linear sucrose density gradient by centrifuging at 27,000 rpm for 10.0 hours. The gradient was scanned for optical density and analysed for radioactivity.

The density gradient fractionation of this rapidly labelled RNA is shown in Fig.23. The optical density profile contained the three major peaks of total cell RNA since the cell fractionation procedure produced only a crude nuclear fraction containing a small amount of cytoplasmic RNA, carried over as markers. There was no optical density peak which could be attributed to DNA and it was assumed any DNA which remained in the RNA preparation after the Parish-Kirby extraction procedure was not extensively sheared into smaller molecules and therefore sedimented very rapidly at the bottom of the gradient tube. The radioactivity profile, however, contained four discrete components which did not correspond with the peaks of optical density. There were two large peaks of radioactivity (2 and 3), one rather broad which sediments at between 37 and 40S and one component sedimenting just before the large ribosomal component at about 28-30S. In
Cells were labelled with $2-^{14}$C-Uridine for 40 minutes and RNA was extracted from a crude nuclear fraction.
addition, there were two smaller peaks visible above the heterogeneous background, one with a very high sedimentation velocity of approximately 45S and one which sedimented slightly faster than the 18S ribosomal RNA with a sedimentation coefficient of approximately 20S.

It is therefore possible to distinguish several distinct molecular species of RNA that are rapidly labelled at a high specific activity with $^{14}$C-uridine and which apparently occur only in the nucleus. These RNA preparations were fractionated on sucrose density gradients in high ionic strength buffer containing 1mM EDTA which provides suitable conditions for the recognition of single covalently-linked molecules and which excludes the possibility that any of these large molecules may be complexes of smaller molecules.

These rapidly labelled RNA molecules closely resemble the high molecular weight ribosomal precursors reported in nuclear preparations of the RNA of other higher plant tissues (Rogers, Fraser and Loening, 1970; Leaver and Key, 1970). It is therefore necessary to determine whether these molecular species isolated from sycamore cells have kinetics of labelling consistent with their possible function as precursors of ribosomal RNA.

**Time course of labelling of ribosomal RNA**

In order to investigate the time course of labelling of ribosomal RNA a continuous labelling experiment was performed to follow the flow of radioactivity into the various species of RNA. 2-$^{14}$C-uridine was fed to six day-old cultures at $5 \times 10^{-6}$M (specific activity 62mCi/mM), a concentration which has been shown to sustain continuous incorporation of radioactivity into RNA at an undiminished rate for at least 2 hours. Cultures were harvested after 10, 20, 40 and 60
minutes and were fractionated to give a crude nuclear fraction and a
cytoplasmic fraction. RNA was extracted from the fractions and analysed by sucrose density gradient centrifugation.

The time course of labelling of the fractions of RNA during continuous labelling is shown in Fig. 24. After 10 minutes most of the radioactivity in the nuclear fraction was "polydisperse or heterogeneous" indicating that molecules of a wide range of molecular sizes were labelled. There was perhaps some suggestion of a small peak of radioactivity arising at about 45S and another at about 38S and there was some labelling of the soluble RNA. In the cytoplasmic fraction there was appreciable labelling of the soluble RNA and a much lower level of heterogeneous radioactivity. After 20 minutes a definite peak of radioactivity had appeared at the 38S position in the nuclear fraction but the 45S peak was still not very clear. After 40 minutes the four peaks described previously had appeared distinctly although the cytoplasmic fraction still only contained radioactivity in heterogeneous and soluble RNA. After 60 minutes the 38S component was still clearly visible and its content of radioactivity was still increasing. The 50S and 20S components had been obscured by the appearance of radioactivity in the mature 16 and 25S ribosomal RNA and an appreciable amount of labelled ribosomal RNA had appeared in the cytoplasmic fraction. The 45S peak was no longer visible and was either not extracted or obscured by the increased level of heterogeneous labelling.

These results have demonstrated a flow of radioactive labelling into three distinct classes of RNA: (i) a very rapid incorporation of radioactivity into the soluble RNA in the nucleus and in the cytoplasm;
FIG 24

TIME COURSE OF LABELLING OF RNA

Cells were incubated with $2^{14}$C-Uridine. RNA was extracted from crudely separated nuclear and cytoplasmic fractions after 10, 20, 40 and 60 minutes and fractionated on sucrose density gradients.
(ii) a rapid flow of radioactivity into a polydisperse or heterogeneous fraction of RNA in the nucleus followed by a steady increase in the heterogeneous labelling in the cytoplasm; and (iii) a rapid incorporation of radioactivity into several distinct species of RNA of high molecular weight in the nucleus followed by a delayed appearance of radioactivity in ribosomal RNA in the cytoplasm.

The four distinct high molecular weight species of RNA, restricted to the crude nuclear fraction, therefore have kinetics of labelling consistent with their possible function as precursors to ribosomal RNA. The 38S molecule clearly appears before the 30S and 20S components, a very similar result to that obtained by $^{32}\text{P}$-labelling of nuclear RNA in carrot (Leaver and Key, 1970) and in pea and artichoke (Rogers, Fraser and Loening, 1970) in which the base compositions of these species have been shown to be similar to ribosomal RNA. These workers have suggested that the molecule of $2.4 \times 10^6$ daltons molecular weight (38S) gives rise to both the $1.4 \times 10^6$ daltons (30S) and $0.9 \times 10^6$ daltons (20S) components which are the immediate precursors of the $1.3 \times 10^6$ (25S) and $0.7 \times 10^6$ daltons (18S) ribosomal RNA components. These results agree with this hypothesis but there is also some evidence of a larger molecular species with a sedimentation coefficient similar to that of the 45S mammalian ribosomal RNA precursor. The kinetics of labelling of this molecule, however, cannot be distinguished sufficiently clearly, in continuous labelling experiments with externally applied labelled nucleoside, to be able to determine whether or not it is a precursor of the other high molecular weight RNA molecules. It is possible that the earliest appearance of this 45S molecule is obscured by the rapidly produced heterogeneous radio-
activity.

The sequence of processing of these species of RNA molecules requires further investigation, especially the status of the inferred 45S molecule and there are three possible approaches to this problem. First, there is the possibility of performing a "chase" experiment in which the RNA is first labelled and then incorporation of radioactivity is stopped abruptly by addition of unlabelled precursor to the medium. The disappearance of labelling from the individual components could then be followed with time to determine the sequence of processing of these high molecular weight RNA molecules. Earlier experiments with the plant cells have shown, however, that the introduction of radioactive precursors into the cellular pool cannot be followed by an effective chase. This is true also of animal cells (Soeiro, Birnboim and Darnell, 1966; Warner, Soeiro, Birnboim, Girard and Darnell, 1966) and bacteria (Nierlich, 1967) the incorporation of radioactive precursor continuing for a long period after excess unlabelled precursor has been added to the medium. Second, there is the possibility of following the kinetics of RNA labelling in the subnuclear fractions (the nucleolus and nucleoplasm) as a means of separating the heterogeneous and ribosomal RNA precursor labelling, as performed in animal cells (Penman, Smith and Holtzman, 1966). This is technically very difficult with plant cells and it is not at present possible to perform this kind of fractionation without degradation of the high molecular weight RNA. Third, it is possible that ribosomal RNA could be specifically labelled by supplying the cultures with methyl-labelled methionine. In animal cells it has been shown that ribosomal RNA has methyl groups attached to the nucleic acid
bases and to the 2'-OH of the ribose of RNA (Brown and Attardi, 1965). The direct precursor of the RNA methylation reaction was known to be S-adenosyl methionine and therefore methyl-labelled methionine can be used to label ribosomal RNA with reduction of the heterogeneous labelling, since this fraction is not labelled to any great extent, if at all, by methyl-labelled methionine (Greenberg and Penman, 1966).

It was therefore decided to investigate the possibility of labelling plant ribosomal RNA at the precursor level with methyl-labelled methionine and reducing the heterogeneous radioactivity so that the presumed ribosomal RNA precursors may be distinguished at an earlier stage of their synthesis and a possible sequential relationship established.

**Labelling of plant RNA with $^{14}$C (methyl)methionine**

An incorporation curve of $^{14}$C (methyl)methionine into the TCA-insoluble fraction of the cell Fig.25 showed that it was rapidly taken up by suspension cultures of *Acer pseudoplatanus* L. and incorporated into macromolecules. A six day-old culture was therefore fed $^{14}$C (methyl)methionine at a concentration of $5 \times 10^{-6} \text{M}$ and a specific activity of $50 \mu\text{Ci/mM}$ and was incubated for 40 minutes. The cells were then harvested and fractionated, and RNA was extracted from the crude nuclear fraction and fractionated by sucrose density gradient centrifugation at 27,000 rpm for 10.5 hours.

The results of this experiment are shown in Fig.26(a). The radioactivity profile indicated that there was some methylation of the 38S RNA component but the gradient above this point became increasingly contaminated with radioactivity. The nature of this contamination was investigated by taking samples from the gradient and treating them
FIG. 25
INCORPORATION CURVE OF $^{14}$C-(METHYL)-METHIONINE

[Graph showing incorporation curve with time (hours) on the x-axis and radioactivity CPM x 10^-3 on the y-axis. The curve starts with a steep increase and levels off after 3 hours.]
FIG. 26

FRACTIONATION OF RNA LABELED WITH $^{14}\text{C}$(METHYL) METHIONINE BY SUCROSE DENSITY GRADIENT CENTRIFUGATION

Cells were labelled for 40 minutes with $^{14}\text{C}$(methyl) methionine and RNA was extracted from a crude nuclear fraction.

(a) Gradient fractions counted directly

(b) Gradient fractions treated with hot 5% w/v TCA
with an equal volume of hot 10#/v TCA, containing 100μg/ml carrier BSA, at 90°C for 20 minutes. The samples were then allowed to cool and precipitates were collected on glass fibre filters and washed five times with cold 5% TCA. The filters were then dried and counted as previously described in Section I. The radioactivity profile of the gradient after hot acid treatment is shown in Fig.26(b). Clearly the majority of the radioactivity of the gradient was hot acid-soluble which indicated that it was not protein. There was therefore either a very strange pattern of labelling of nucleic acid or contamination by another class of macromolecules labelled by 14C (methyl)-methionine.

These observations, together with the obvious advantages of obtaining greater resolution in the separation of high molecular weight species of RNA, have led to the use of polyacrylamide gel electrophoresis for fractionation of RNA. The labelled macromolecules which appear to be contaminating the 14C (methyl)-methionine labelled preparation, although of sufficient molecular size to move a substantial distance on a density gradient, may not possess a very large number of charged groups and may therefore migrate only a very short distance in an electric field, compared to nucleic acids. Polyacrylamide gel electrophoresis may therefore enable the pattern of methylation of RNA to be followed in these plant suspension cultures.
Fractionation of rapidly labelled high molecular weight RNA from plant cell suspension cultures by polyacrylamide gel electrophoresis

A technique for the fractionation of high molecular weight species of RNA by polyacrylamide gel electrophoresis was described by Loening (1967) and has since been used to study synthesis and processing of ribosomal RNA in a variety of plant and animal tissues. The technique offers the possibility of more extensive and precise separations than are possible by density gradient centrifugation. In addition it is also possible to make an estimation of the molecular weight of RNA species since the relationship between electrophoretic mobility and the logarithm of the molecular weight has been shown to be linear with a range of viral (Bishop, Claybrook and Spiegelman, 1967) and ribosomal RNA's (Loening, 1969). It must be remembered, however, that this is not an absolute method and will be affected by any marked changes in the secondary structure of RNA.

Determination of molecular weight

The molecular weights of the two ribosomal RNA components from the sycamore cells were determined by comparing their mobilities with those of ribosomal RNA of known molecular weight in the same gel. Escherichia coli ribosomal RNA was used as the standard since its molecular weight has been determined by several methods at \(1.07 \times 10^6\) daltons for the large molecular weight species and \(0.56 \times 10^6\) daltons for the small molecular species (Loening, 1968; Stanley and Bock, 1965; Stutz and Noll, 1967).

The separation of the plant and bacterial ribosomal RNA by gel electrophoresis is shown in Fig.27. An identical gel containing only
FIG. 27
DETERMINATION OF MOLECULAR WEIGHTS OF PLANT RIBOSOMAL RNAs BY COMPARISON OF ELECTROPHORETIC MOBILITY WITH BACTERIAL RIBOSOMAL RNA.

SYCAMORE RNA + E. COLI RNA

SYCAMORE RNA

Upper scale - cm migrated
Lower scale - Mol wt daltons x 10^6
sycamore cell RNA was run at the same time to identify the plant species clearly. The distance migrated by each peak of optical density was measured and the molecular weights of the plant ribosomal RNA components were calculated at $1.3 \times 10^6$ daltons for the large component and $0.69 \times 10^6$ daltons for the smaller component.

In order to obtain an accurate determination of apparent molecular weight a single known marker is insufficient; at least two markers are required since the relative mobilities of RNA from different sources varies with different gel concentrations (Loening, 1969). E. coli RNA, however, was the only readily available marker RNA for this determination and the results obtained were almost identical with published values for higher plant RNA (Rogers, Fraser and Loening, 1970; Leaver and Key, 1970).

**Analysis of rapidly labelled nuclear RNA**

The pattern of rapidly labelled nuclear RNA has been analysed by polyacrylamide gel electrophoresis for comparison with the pattern obtained by density gradient centrifugation. A six day-old culture was incubated with $5 \times 10^{-6}$ M 2-$^{14}$C-uridine (specific activity 62mCi/mM) for 40 minutes. The cells were then harvested and fractionated, and nucleic acids were extracted from a crude nuclear fraction. The extract was split into two halves, one of which was layered directly onto the gels whilst the other was first treated with deoxyribonuclease to digest DNA.

The radioactivity profile of rapidly-labelled nuclear RNA, together with optical density markers, is shown in Fig.28. It can clearly be seen that the results obtained were very similar to those obtained by density gradient centrifugation. The mobility of an RNA
FIG. 28

FRACTIONATION OF RAPIDLY LABELLED RNA BY
GEL ELECTROPHORESIS

Cells were labelled with 2-$^{14}$C-uridine and RNA was extracted from a crude nuclear preparation.

DNA Not Digested

Deoxyribonuclease Treated

Upper scale - cm migrated
Lower scale - Mol wt. daltons x 10$^{-6}$
component on a gel is inversely related to its sedimentation velocity on a density gradient and the largest molecular species therefore travel the least distance on a gel. The four peaks of radioactivity were slightly more clearly defined than on a density gradient and their molecular weights have been tentatively estimated, by extrapolation from the ribosomal RNA components, as $3.8 \times 10^6$ daltons for the 45S component, $1.4 \times 10^6$ daltons for the 28-30S component and $0.9 \times 10^6$ daltons for the 20S component. The estimated molecular weight for the largest radioactive component was rather less than might have been expected from its sedimentation velocity on sucrose density gradients but since both the estimation of molecular weight and sedimentation coefficient were made by extrapolation from the ribosomal RNA markers neither measurement may be accurate. The tendency of gels to be more dilute at their upper end could lead to an underestimation of the molecular weights of the largest molecular species and since the sedimentation coefficients of the sycamore cell ribosomal RNA have not been measured accurately an actual value of less than 25S for the large ribosomal component could lead to an overestimation of the sedimentation coefficient of the 45S precursor. This largest molecular species will therefore be referred to as the 45S or $3.8 \times 10^6$ daltons component for convenience only and this does not necessarily imply an accurate description of its sedimentation properties and molecular weight.

The 45S or $3.8 \times 10^6$ daltons component migrates very close, in some gels, to an optical density peak believed to be DNA. Treatment of the total nucleic acid extract with deoxyribonuclease removed the optical density peak whilst the radioactivity peak remained intact.
This very large molecular species has therefore been demonstrated in preparations of RNA from nuclear-enriched extracts of cells continuously labelled for 40 minutes with $^{14}\text{C}$-uridine, and fractionated by density gradient centrifugation and by polyacrylamide gel electrophoresis.

**Labelling of nuclear RNA from plants with $^{14}\text{C}$ (methyl)-methionine**

Fractionation of an RNA extract from cells labelled with $^{14}\text{C}$ (methyl)-methionine by density gradient centrifugation has suggested that there may be methylation of the presumed ribosomal RNA precursor molecules. The experiment has therefore been repeated and the RNA fractionated by gel electrophoresis. A six day-old culture was incubated with $5 \times 10^{-6}$N (specific activity $50\text{mCi/mL}$) $^{14}\text{C}$ (methyl)methionine for 40 minutes. The cells were then harvested and fractionated, and nucleic acids were extracted from a crude nuclear-enriched preparation.

The optical density and radioactivity profiles of the gel electropherogram are shown in Fig. 29. Three of the molecular species labelled with $^{14}\text{C}$-uridine could be clearly distinguished and there was a noticeable reduction in the level of the heterogeneous labelling. There was, however, very substantial methylation of DNA at the upper end of the gel which obscured the region in which the $3.8 \times 10^{6}$ daltons component was situated (as seen in the $^{14}\text{C}$-uridine-labelled preparations).

It is therefore possible to demonstrate methylation of the $2.5 \times 10^{6}$ (38S), $1.4 \times 10^{6}$ (28-30S) and the $0.9 \times 10^{6}$ (20S) daltons RNA using $^{14}\text{C}$ (methyl)-methionine as the methyl donor. The heterogeneous labelling, observed where $^{14}\text{C}$-uridine was used to label the cells, was greatly reduced which shows that the heterogeneous RNA is not
FIG. 29

FRACTIONATION OF RNA LABELLED WITH $^{14}$C-(METHYL)-METHIONINE BY GEL ELECTROPHORESIS

Cells were labelled with $^{14}$C-(methyl)-methionine for 40 minutes and RNA was extracted from a crude nuclear fraction.

Upper Scale - cm migrated
Lower Scale - Mol. wt. daltons x $10^6$
methylated to any great extent. This suggests that methylation is a specific process which occurs either during or immediately after the synthesis of the high molecular weight ribosomal RNA precursors and that it does not occur in the ribonucleotide pools or by non-specific binding of methyl groups to RNA.

This experiment has not, however, established whether methylation occurs only during or immediately after synthesis of the largest precursor molecule or whether methyl groups are attached directly to the $2.5 \times 10^6$ (38S), $1.4 \times 10^6$ (26-30S) and $0.9 \times 10^6$ (20S) daltons RNA components. In animal cells methylation occurs during synthesis of the 45S precursor molecule; the methylated bases and ribosides are then conserved during processing but no further methylation takes place (Greenberg and Penman, 1966). Clearly further experiments must be performed with the sycamore cell cultures in which methyl-labelled methionine is used to determine the initial site or sites of methylation of plant RNA. Treatment of the nucleic acid preparations with deoxyribonuclease may establish whether or not the $3.8 \times 10^6$ (45S) daltons component (demonstrated by $^{14}$C-uridine labelling of plant cells) is methylated and intensive investigation of the kinetics of methyl methionine labelling of these RNA species may enable a clearer picture of the sequence of processing of plant ribosomal precursor RNA to be obtained.
Discussion

In this section of work techniques have been developed for the extraction and fractionation of undegraded RNA from suspension cultures of *Acer pseudoplatanus* L. Short periods of incubation of these cultures with $^{14}$C-uridine have resulted in the labelling of several discrete components of high molecular weight RNA, clearly visible above a background of heterogeneous labelling, in a crudely prepared nuclear fraction of the cell. These components have very similar patterns of separation on polyacrylamide gels and on density gradients, and are unaffected by treatment of the preparations with deoxyribonuclease. The peaks of radioactivity do not correspond to the optical density profile of the majority species of RNA and these rapidly labelled species of RNA therefore only constitute a very small proportion of the total RNA.

Kinetic studies of the labelling of the molecules clearly have shown that a component of molecular weight $2.5 \times 10^6$ daltons, and sedimentation coefficient of approximately 38S, is clearly labelled before two further components with molecular weights of $1.4 \times 10^6$ and $0.9 \times 10^6$ daltons and sedimentation coefficients of approximately 30S and 20S. In addition, there is also evidence of a very large molecular species, molecular weight approximately $3.8 \times 10^6$ daltons and sedimentation coefficient approaching 45S, which appears as a radioactive peak at the same time as the $2.5 \times 10^6$ daltons component. After some delay radioactivity then begins to accumulate in the $1.3 \times 10^6$ (25S) and $0.67 \times 10^6$ daltons (18S) components of ribosomal RNA in the cytoplasm. The kinetics of labelling of these molecules are therefore consistent with their possible function as precursors to
ribosomal RNA and are very similar to those obtained by $^{32}$P-labelling in the pea root-tip and artichoke-tuber tissues (Rogers, Fraser and Loening, 1970) and in carrot root-disc tissue (Leaver and Key, 1970). Precursor-product relationships are however difficult, if not impossible, to establish on kinetic grounds alone (Rake and Graham, 1964). This is especially true of RNA molecules since the introduction of radioactive precursors into the cellular pool cannot be followed by an effective "chase" and incorporation of radioactivity into RNA continues for some time after excess unlabelled precursor has been added to the medium.

In order to discover the relationship of the newly labelled species to the majority species of RNA other approaches are necessary. In mammalian tissues this has been accomplished, first, by obtaining chemical evidence of the relationship between rapidly-labelled RNA and the majority species of RNA, and second, by determination of the time taken for maximal label to accumulate in the newly labelled species before the first appearance of labelling in various chemically related molecules. Chemical evidence has been obtained by labelling cells with $^{32}$PO$_4^{3-}$, isolating the various RNA species by zonal sedimentation and determining the base compositions of molecules with different sedimentation coefficients. The 45S and 32S rapidly labelled peaks of nuclear RNA were found to have a high (> 60%) guanine + cytosine (G + C) content similar to that of ribosomal RNA, whereas the heterogeneous RNA had a low G + C content similar to that of DNA (Scherrer, Latham and Darnell, 1963; Soeiro, Birnboim and Darnell, 1966; Warner et al., 1966). These results suggest a possible role for the 45S RNA in ribosome biosynthesis and further decisive evidence
has come from experiments in which actinomycin D has been used to block RNA synthesis by the inhibition of transcription of DNA. Cells were labelled long enough for the 45S precursor to become heavily radioactive but not long enough for radioactivity to appear in the 28S and 18S ribosomal RNA and then actinomycin D was added and the fate of the 45S radioactivity was followed in the absence of further RNA synthesis. It was found that the 45S label quickly disappeared in actinomycin-treated cells and coincident with its disappearance radioactivity appeared in the 32S and 18S RNA (Scherrer, Latham and Darnell, 1963; Girard, Penman and Darnell, 1966; Penman, 1966). After longer periods in actinomycin the 32S radioactivity shifted predominantly to the 28S RNA (Scherrer, Latham and Darnell, 1963).

Rogers, Fraser and Loening (1970) and Leaver and Key (1970) have shown that the base composition of the 2.4 x 10^6 and the 1.4 x 10^6 daltons components of rapidly labelled plant RNA (measured by the distribution of ³²P-labelling amongst the constituent nucleoside monophosphates) are similar to those of the 1.3 x 10^6 and 0.7 x 10^6 daltons ribosomal RNA, but no meaningful base composition for the 0.9 x 10^6 daltons component has yet been obtained. It is therefore reasonable to assume, from their kinetics of labelling and from additional information from more extensive studies of other systems, that the rapidly-labelled high molecular weight RNA species found in sycamore suspension culture tissue are precursors to ribosomal RNA. The sequence of processing of these precursors in plants, however, is far from clear and the possible function of the 0.9 x 10^6 daltons (20S) component as a precursor of the 0.7 x 10^6 daltons (18S) ribosomal RNA is uncertain. The labelling of the 2.5 x 10^6, 1.4 x 10^6...
and \(0.9 \times 10^6\) daltons components with \(^{14}C\) (methyl)methionine as a methyl donor in the sycamore tissue is further evidence of their function as ribosomal RNA precursors, holds out the possibility of performing kinetic experiments without the complication of the heterogeneous RNA obscuring the earliest appearance of the ribosomal RNA precursors.

Sycamore tissue also contains a very large molecule of molecular weight of approximately \(3.8 \times 10^6\) daltons and a sedimentation coefficient approaching 45S. In pea root-tip and carrot root-disc tissues a large component is also found but in these tissues it is only slightly larger than the \(2.4-2.5 \times 10^6\) daltons component. This large component could not be clearly distinguished kinetically from the \(2.4-2.5 \times 10^6\) daltons component although Leaver and Key (1970) have shown that the base composition is similar to ribosomal RNA. Its position in the processing sequence was therefore not clear.

In these cases, where two apparent precursors are found, it would appear to be the one of lower molecular weight which would be split exactly into the two immediate precursors to the \(1.3 \times 10^6\) and \(0.7 \times 10^6\) daltons ribosomal RNA. The high molecular weight precursor would then include a variable amount of excess RNA above the maximum weight of \(2.3 \times 10^6\) daltons \((1.4 \times 10^6 + 0.9 \times 10^6\) daltons). This would account for the differences between species.

If this interpretation is correct then the \(2.3 \times 10^6\) daltons precursor can be compared with the 41S \((3.1 \times 10^6 - 3.4 \times 10^6\) daltons) component in HeLa cells which is cleaved to 32S \((2.1 \times 10^6 - 2.4 \times 10^6\) daltons) and 20S \((0.9 \times 10^6 - 1.0 \times 10^6\) daltons) without loss of excess RNA (Weinberg and Penman, 1970). All the eukaryotes would then have in common a ribosomal precursor of a minimum molecular weight...
sufficient to give the immediate precursors to ribosomal RNA. This minimum is $2.3 \times 10^6$ daltons in plants, $2.5 \times 10^6$ daltons in *Xenopus* (Loening *et al.*, 1969) and about $3.3 \times 10^6$ daltons in HeLa cells (Weinberg and Penman, 1970). Different species would then have a first precursor which contains a variable excess of RNA above the minimum, from $0 - 0.5 \times 10^6$ daltons in pea and carrot, about $0.1 \times 10^6$ daltons in *Xenopus* to about $1 \times 10^6$ daltons in mammals. In birds the excess appears to be slightly smaller than that in mammals (Perry, Cheng, Freed, Greenberg, Kelley and Tartof, 1970). Loening, Jones and Birnstiel (1969) and Perry *et al* (1970) have suggested that evolution of the mammalian precursor is a result of a shift in the initiation or termination site to previously non-transcribed lengths of ribosomal DNA. The discovery of a possible precursor to ribosomal RNA in sycamore suspension culture tissue with a sedimentation coefficient approaching 45S and a molecular weight in the region of $3.8 \times 10^6$ daltons is therefore very interesting since if this is indeed the initial precursor then its size very closely approaches that of the mammalian 45S precursor and its content of excess RNA would then be at least $1.0 \times 10^6$ daltons.

Clearly further investigation of this molecular species is necessary. First, a more detailed study of its kinetics of labelling, if possible with methyl-labelled methionine, must be carried out. Attempts must then be made to develop a technique for performing an "actinomycin D chase", such as performed in animal cells, in order to follow the fate of this molecule in the absence of further RNA synthesis. Chemical evidence is also necessary and in addition to determination of the base composition an analysis of the sequences of
oligonucleotides from an enzymic digest of this molecular species, such as that carried out in HeLa cells (Amaldi and Attardi, 1968; Jeantur, Amaldi and Attardi, 1968) would show decisively whether or not this component consists of one molecular species containing methylated ribosomal RNA sequences and unmethylated non-ribosomal sequences.

This investigation has therefore provided some information of the synthesis of ribosomal RNA in exponentially growing cultures of plant cells and has raised some interesting questions about the apparent differences and evolution of ribosomal RNA synthesis in different classes of eukaryotes.
SECTION III

STUDIES OF THE INCORPORATION OF LABELLED

THYMIDINE, URIDINE AND LEUCINE IN

SYNCHRONOUSLY DIVIDING SUSPENSION

CULTURES OF ACER PSEUDOPLATANUS
The growth and development of higher plants is the outcome of cell division, cell growth and differentiation. Division of embryonic cells is necessary for the establishment of a population of cells which can undergo differentiation and control of the sequence of events of the division cycle of plant cells is therefore one of the most important processes underlying growth and development.

Until very recently studies of the various events of the cell cycle in plant cells have been restricted to cytological investigations of individual cells in actively dividing tissues such as root-tips. Chemical analyses and investigations of macromolecular biosynthesis must be restricted to histochemical and autoradiographic techniques in such studies thereby placing severe limitations on the amount and type of information that can be obtained. Detailed morphological studies, chemical analyses and measurement of the metabolic processes that accompany and control the events of the cell cycle require a population of cells which are growing and dividing synchronously.

Such synchronous populations of bacteria, yeasts, algae, ciliates and amoebae have been used to describe the series of morphological and biochemical events that occur during the cell cycle, and to identify the factors which control the orderly sequence of biochemical processes that accompany cell division. In higher organisms, however, such studies have been restricted to cultured mammalian and amphibian cells.

The establishment of large scale synchronous populations of higher plant cells, which could be maintained through a succession of cell cycles, would enable the fundamental processes involved in their
cell growth and differentiation to be investigated and compared with those of other eukaryotic and procaryotic cells.

Synchronous divisions in cultured explants of the Jerusalem artichoke have been described by Yeoman, Evans and Naik (1966) and Yeoman and Evans (1967). Studies of the changes in DNA, RNA and protein during the first synchronous division cycle have been carried out and are summarised in a review by Yeoman (1970). Although this work has yielded some valuable information this system is limited by the fact that the synchrony rapidly declines after the first division and since the synchronously dividing cells exist within large tissue explants it is difficult to study, in isolation, the effect of external stimuli on the control of cell division.

Some early work on the growth of plant cell suspension cultures of *Convolvulus* (Torrey, Reinert and Merkel, 1962) and of *Rubus fruticosus* (Street and Henshaw, 1963) indicated that there were short-lived peaks of mitotic indices at various points during growth. These were shown to occur in cell aggregates of particular sizes by fractionation of the suspensions on filters indicating that within separate fractions of the culture there was some degree of cell division synchrony. Much better evidence of cell division synchrony was obtained in the finely dispersed suspension cultures of *Acer pseudoplatanus* L. (Street, 1967). Synchronous divisions were obtained in up to 70% of the cells when the cultures were initiated from stock cultures which had been in the stationary phase of growth for three days or longer. The high inoculum density in these cultures, however, limited the number of possible synchronous divisions to three as the culture then approached its maximum cell density. Under these con-
ditions the high degree of synchrony obtained in the first division was rapidly lost in subsequent cell generations.

Two later developments have enabled a high degree of cell division synchrony to be maintained through several cell generations in these suspension cultures. First, it was shown (Stuart and Street, 1969) that cultures could be successfully initiated from cell densities as low as $1.5\times10^3$ cells per ml. At these low densities, although the lag phase may be lengthened, a high proportion of the cells participate in the growth of the cultures and as many as seven cycles of cell division can be completed before the culture reaches its final density. Second, an apparatus has been developed for the culture of large volumes of cell suspension (Wilson, King and Street, 1971) which enables sufficient culture to be grown to provide cellular material for the large number of samples required for biochemical analysis during a cell cycle. Growth curves have been presented (Wilson et al., 1971) which show that up to six divisions can be obtained in which a high degree of synchrony is obtained. The synchronising mechanism is considered to be one of starvation and re-growth (Williamson and Scopes, 1960), but it is not known whether it is a nutrient, a growth regulator or a combination of these factors that becomes depleted during stationary phase and which is responsible for induction of synchronous cell division when the culture is inoculated into new medium.

The work which will be described here consists of some preliminary experiments designed to establish the overall patterns of DNA, RNA and protein synthesis during the cell cycle of such cultured sycamore cells. Howrd and Pelc (1953), from their work on mammalian cells,
suggested that the cell cycle could be represented in terms of progression around a clock face and that the various events of the cell cycle could be divided into a mitotic phase, a phase of DNA synthesis (the S phase) and two phases, one before DNA synthesis (the pre-synthetic phase or $G_1$) and one after DNA synthesis before mitosis (the post-synthetic phase or $G_2$). The terms $G_1$, S and $G_2$ have since been frequently used to describe the subdivision of the interphase between mitosis and cytokinesis in eukaryote cells. The purpose of this preliminary investigation has therefore been to attempt to define the higher plant cell cycle in terms of mitosis, cytokinesis and DNA synthesis, and to obtain some indication of the pattern of synthesis of RNA and protein. The experiments have been carried out in collaboration with two colleagues P. J. King and Dr. N.W. Fowler and some of the data described has been provided by these workers. The author was concerned particularly with the measurement of the incorporation of radioactive thymidine, uridine and leucine.
Materials and Methods

Radiochemicals

2\textsuperscript{-14}C-uridine (62mCi/mM), \textsuperscript{14}C-leucine universally labelled (344 mCi/mM), 6-\textsuperscript{3}H-thymidine (22Ci/mM) and 2\textsuperscript{-14}C-thymidine (50mCi/mM) were obtained from the Radiochemical Centre Ltd. Amersham.

Synchronous cell culture

Synchronous cell division in suspension cultures of Acer pseudo-platanus L. was induced by initiating the cultures at a low cell density of approximately 2.6 - 3.0 \times 10^3 cells per ml from stock cultures which had been in stationary phase for at least 7 days. The culture medium was as previously described in Table I but also contained kinetin at a concentration of 0.25mg/l. The culture vessel had a capacity of 41 and was fitted with a magnetic stirrer, an aerating device and an electronically controlled automatic sampling device (Wilson, King and Street, 1971).

Measurement of the rates of incorporation of specific isotopes into macromolecules

The rates of incorporation of specific radioactive precursors of DNA, RNA and protein were measured at 6 hour intervals through a cell cycle.

10ml aliquots of culture were removed from the 41 culture vessel and were incubated for 1.0 hour with \textsuperscript{10}^{-4}M 2\textsuperscript{-14}C-uridine (specific activity 5.0 or 0.5mCi/mM), 2\textsuperscript{-14}C-thymidine (specific activity 0.5 mCi/mM) or 6-\textsuperscript{3}H-thymidine (specific activity 5.0mCi/mM), and U\textsuperscript{14}C-leucine (specific activity 0.5mCi/mM) in separate \textsuperscript{25}ml erlenmeyer flasks on a rotary shaker (120 rpm) at \textsuperscript{25}\textdegree C. Four replicate 2.0ml samples of culture were taken from each incubation flask with an
automatic pipette and were added to an equal volume of 10% w/v TCA, containing a 100 times excess concentration of unlabelled uridine, thymidine or leucine, at 0°C. These samples were then allowed to stand at 0°C for at least 2 hours and the TCA-insoluble precipitates were collected on glass fibre filters, washed, dried and assayed for radioactivity as previously described in Section I.

The cold TCA-precipitates of cells incubated with labelled thymidine were extracted with 0.3N KOH, containing 100μg/ml calf-thymus DNA (Sigma Ltd.) as carrier, for 18 hours at 37°C. DNA was then precipitated from this extract and was collected on glass fibre filters, washed, dried and assayed for radioactivity as previously described in detail in Section I.
Chapter I

The rates of incorporation of specific precursors of RNA, DNA and protein through the cell cycle of synchronous suspension cultures of *Acer pseudoplatanus* L.

The data which will be presented has been obtained from experiments carried out on two separately initiated cultures. The initiation of the cultures and measurements of cell number, total DNA, RNA and protein were carried out by P. J. King and measurements of enzyme activities were carried out by Dr. M. W. Fowler.

Growth of the cultures

The two cultures in which a cell cycle has been studies are denoted as culture 73 and culture 77. Culture 73 was initiated at a cell density of $2.0 \times 10^4$ cells/ml and culture 77 was initiated at $3.0 \times 10^4$ cells/ml.

The growth curves of these two cultures are shown in Fig. 30 plotted as the log cell number/ml culture against incubation time in days. The percentage increase in cell number at each division and the mean doubling time are indicated on both growth curves. It can clearly be seen that there were well-defined steps in the cell number curve, the percentage increases in cell number ranging from 63-123% with most values between 70 and 95% and occurring over a short period of time. This is indicative of a high degree of cell division synchrony and it was clearly possible to obtain at least five or six complete cell cycles in which there was no marked tendency toward a decrease in the percentage increase of cell number at each cytokinesis. The mean generation time was, however, rather variable and in culture 77 tended to become shorter as the growth of the culture proceeded.
FIG. 30
SYNCHRONOUS CELL DIVISION IN SUSPENSION CULTURES OF
ACER PSEUDOPLATANUS L.

CULTURE 73

CULTURE 77

Cell No./ml x 10^-4 (Log scale)

Days of incubation

84% 82 hr
82% 60 hr
93% 72 hr
123% 61 hr
93% 76 hr
FIG. 31

THE CELL CYCLE INVESTIGATED IN CULTURES 73 AND 77

The positions and duration of mitosis and cytokinesis are shown together with a regression analysis of the cell number data.
The cell cycles enclosed in rectangles are the cell cycles in which some biochemical and physiological processes have been measured. As the growth of the cultures proceeds the degree of synchrony eventually declines and in choosing the cell cycle to be investigated a compromise must be made between obtaining a high degree of synchrony, which is most reliably obtained early in the growth of the culture, and having sufficient cell material for biochemical and physiological studies. Fig. 31 shows a regression analysis of the cell cycles chosen for analysis in cultures 73 and 77. The mean of the cell number line was always within the 95% confidence limits for the regression line indicating that there was no significant increase in cell number across the plateau between cytokinesis. (Data from P. J. King).

The incorporation of specific precursors of DNA, RNA and protein in relationship to the accumulation of total DNA, RNA and protein

The rates of incorporation of specific radioactive precursors of DNA, RNA and protein have been measured at 6 hour intervals throughout the cell cycles indicated in Fig. 31 to attempt to establish, in conjunction with measurements of total DNA, RNA and protein, the areas of the cell cycle where the majority of synthesis of these macromolecules may occur. The isotopes were fed at a concentration of $10^{-4}$M in order to ensure that a high rate of incorporation of each radioactive compound into the cells was maintained throughout the period of incubation. Two replicate incubation flasks were set up for each isotope and five samples were taken from each flask. The cells were incubated for 1.0 hours and then the total incorporation of radioactivity into the TCA-insoluble fraction of the cells was measured. The TCA-insoluble precipitates from the thymidine labelled aliquots of
culture were further treated with a Schmidt-Thannhauser procedure to isolate DNA. 2-\(^{14}\)C-thymidine was used in culture 73 and 6-\(^{3}\)H-thymidine was used in culture 77.

The rates of incorporation of radioactivity are plotted per ml of culture and per \(10^6\) cells at 6 hour intervals throughout the cell cycle. The position and duration of mitosis (determined by measurement of mitotic indices - P. J. King) and cytokinesis are shown on each diagram.

**Incorporation of labelled thymidine**

The rates of incorporation of 2-\(^{14}\)C-thymidine in culture 73 and 6-\(^{3}\)H-thymidine in culture 77 per ml and per \(10^6\) cells at 6 hour intervals through the cell cycle are shown in Fig. 32 together with measurements of total DNA per \(10^6\) cells.

The rate of accumulation of total DNA per \(10^6\) cells was high in both cultures for 30-35 hours preceding cytokinesis (Fig. 32). In culture 73 there was an initial period in the cell cycle when the level of DNA per cell remained constant but in the cycle of shorter duration in culture 77 the level of DNA began to rise immediately after cytokinesis. The incorporation rates of radioactive thymidine were high during periods of the cell cycle when the rate of accumulation of DNA was high, between 34 and 58 hours in culture 73 and throughout the cell cycle in culture 77 but with two peaks of incorporation, one just after cytokinesis and one during mitosis, which correlated with the highest rates of DNA accumulation. A marked decrease in the rate of incorporation occurred just before and during cytokinesis in both cultures and coincided with a period when the DNA content per cell remained constant. A further feature common to
FIG. 32

CHANGES IN TOTAL DNA PER CELL DURING THE CELL CYCLE OF SUSPENSION CULTURES OF ACER PSEUDOPLATANUS L.

CULTURE 73

Time after cytokinesis (hrs.)

Total DNA μg/10^6 cells

CULTURE 77

Time after cytokinesis (hrs.)

Total DNA μg/10^6 cells
CHANGES IN THE RATES OF INCORPORATION OF LABELLED THYMIDINE DURING THE CELL CYCLE OF SUSPENSION CULTURES OF ACER PSEUDOPLATANUS L.

CULTURE 73  RATES OF $^{14}$C-THYMIDINE INCORPORATION

CULTURE 77  RATES OF $^{3}$H-THYMIDINE INCORPORATION
both cultures was a high rate of thymidine incorporation immediately after cytokinesis.

It can be considered that there was an S phase in both cultures of 30-36 hours duration and in culture 73, in which the cell doubling time was 67 hours, there was some evidence of a pre-synthetic or $G_1$ phase during which the content of DNA per cell remained constant and thymidine incorporation was low. In culture 77 the cell doubling time was reduced to 48 hours possibly as a result of the reduction or total loss of the $G_1$ phase as there was no indication of a period in the cell cycle during which the DNA content per cell remained constant or during which thymidine incorporation was low.

The rate of incorporation of labelled thymidine into DNA depends not only upon the rate of DNA synthesis but also upon the specific activity of the immediate precursor pool, the dTTP pool. Changes in the rate of thymidine incorporation can therefore result from changes in the size of the dTTP pool during cell growth, from changes in the rate of uptake of thymidine from the medium or from changes in the rate of utilization of exogenous thymidine. It is therefore possible to obtain marked variations in the rate of thymidine incorporation without there being any change in the rate of DNA synthesis. The enzymes responsible for the utilization of exogenous thymidine are thymidine kinase and thymidylate kinase. One of these enzymes thymidine kinase was assayed through the cell cycles investigated in both culture 73 and 77 by Dr. M. W. Fowler (see Fig. 33). There was a very striking correlation between thymidine kinase activity and the rates of thymidine incorporation in culture 73 and a similar pattern in culture 77 (Fig. 33) which throws some doubt on whether the rates
FIG. 33

CHANGES IN THE ACTIVITY OF THYMIDINE KINASE DURING THE CELL CYCLE OF SUSPENSION CULTURES OF ACER PSEUDOPLATANUS.

THYMIDINE KINASE ACTIVITY

CULTURE 73

THYMIDINE KINASE ACTIVITY

CULTURE 77

THYMIDINE KINASE ACTIVITY
of thymidine incorporation were a measure of the rates of DNA synthesis or of the rate of entry of thymidine into the dTTP pool.

The object of these experiments was to attempt to define the pattern of DNA synthesis in the cell cycle with simple techniques in the hope that there might be periods of the cell cycle when there was virtually no incorporation of thymidine into DNA and periods where there was a very high rate of incorporation. Clearly this is not the case although there are large fluctuations in the rate of thymidine incorporation, especially in culture 73. It is therefore only possible to infer from the rates of thymidine incorporation, in so far as they correlate with rates of accumulation of total DNA, when the majority of DNA synthesis occurs in the cell cycle.

**Incorporation of labelled uridine and leucine**

The rates of incorporation of $^{14}$C-uridine and $^{14}$C-leucine per ml of culture and per $10^6$ cells, measured at 6 hour intervals through the cell cycle in culture 73 and culture 77, are shown in Fig. 34 together with measurements of total RNA and protein per $10^6$ cells (uridine was fed at a specific activity of 5mCi/mM in culture 73 and 0.5mCi/mM in culture 77).

The total protein and RNA content per cell accumulated in an almost exactly parallel manner through the cell cycles in both culture 73 and culture 77. Both the protein and RNA contents of the cells increased throughout the interphase to reach a peak immediately following mitosis and it can be seen that their rates of accumulation generally correspond with the rates of incorporation of $^{14}$C-uridine and $^{14}$C-leucine. A feature of both cultures was a high rate of incorporation of $^{14}$C-uridine after mitosis and after cytokinesis with:
FIG. 34

Changes in total protein and RNA per cell during the cell cycle of suspension cultures of Acer pseudoplatanus L.
CHANGES IN THE RATES OF INCORPORATION OF LABELLED URIDINE AND LEUCINE DURING THE CELL CYCLE OF SUSPENSION CULTURES OF ACER PSEUDOPLATANUS L.

CULTURE 73

RATES OF $^{14}$C-URIDINE INCORPORATION

CULTURE 77

RATES OF $^{14}$C-URIDINE INCORPORATION

CULTURE 73

RATES OF $^{14}$C-LEUCINE INCORPORATION

CULTURE 77

RATES OF $^{14}$C-LEUCINE INCORPORATION

- CPM/m$^3$ cells/hr

- Time after cytokinesis (hrs)
minimum values recorded during mitosis and cytokinesis. $^{14}$C-leucine incorporation also showed a peak immediately following cytokinesis and a rate was then established which did not decline until the cells had entered mitosis. In common with the thymidine incorporation data there was a low level of incorporation of both $^{14}$C-uridine and $^{14}$C-leucine during cytokinesis.

These results indicate that RNA and protein synthesis occurred throughout the interphase period of the cell cycle in these cultures with a reduction in the rate of synthesis during mitosis. The criticism levelled against using measurements of the rate of thymidine incorporation as a means of measuring the rate of DNA synthesis also applies to the use of uridine and leucine incorporation as a measure of RNA and protein synthesis. However, in so far as the incorporation rates correlate well with the rate of accumulation of total RNA and protein the rate of incorporation may well genuinely reflect the rates of synthesis of RNA and protein. In addition these results are very similar to those obtained in other systems. RNA synthesis decreases during early prophase in mammalian tissue cultures and synthesis ceases completely about mid-prophase and is not resumed again until telophase (Feinendegen, Bond, Sleeve and Painter, 1960; Prescott and Bender, 1962; Taylor, 1960). During the absence of RNA synthesis in hamster cells engaged in mitosis the rate of protein synthesis declines rapidly, reaching a minimum in telophase and rising quickly thereafter (Prescott and Bender, 1962). Mittermayer, Braun and Rusch (1964) showed that in synchronized populations of the slime mould Physarum there were two periods of high uridine incorporation indicating a biphasic synthesis of RNA, one peak just before
and one peak just after mitosis. This is very similar to the results obtained in both culture 73 and 77.
A system of cell culture has been described in which suspensions of higher plant cells can be successfully maintained in synchronous cell division for several full cell cycles. This is probably the first higher plant system in which a population of free cells and small aggregates can be maintained in synchronous division. It also differs from many of the other available synchronous systems in higher organisms in that it can properly be described as a "synchronous" system rather than a system which has been "synchronised". The initial synchronising method is considered to be one of starvation and re-growth but once synchrony is established it is maintained for several full cell cycles without the need for any further manipulations.

This development obviously provides an excellent opportunity to study in detail the biochemical events that occur during the higher plant cell cycle and the factors which control cell division. The work of P. J. King has shown that there is a very high degree of cell division synchrony and well-defined peaks of mitotic index. The purpose of this preliminary investigation has been to attempt to define, using simple techniques, the major areas of the cell cycle in terms of DNA synthesis and to establish the periods when RNA and protein synthesis occur.

Howard and Pelc (1953) first used the terms which are now generally used to describe the eukaryotic cell cycle, the pre-synthetic phase or $G_1$, the synthetic phase or $S$, the post-synthetic phase or $G_2$ and the mitotic phase. In most eukaryote systems already studied the $S$ phase is restricted to a definite period of the interphase between
mitosis whilst in rapidly dividing bacteria it occurs throughout
interphase. There are often, however, very large differences in the
relative lengths of duration of the G₁, S and G₂ phases between
different eukaryote systems, and sometimes within the same system,
and there is often almost complete suppression of the G₁ or G₂ phase.
In the Jerusalem artichoke, the only other synchronous population of
higher plants studied in detail, a well defined S phase and G₁ phase
have been observed but the G₂ phase appears to be absent. (Yeoman,
personal communication). This is very similar to the pattern of DNA
synthesis obtained in culture 73 of the sycamore suspension culture
system. Both the rate of accumulation of total DNA and the rate of
thymidine incorporation indicated an S phase and a G₁ phase but in a
later culture, culture 77 in which the cell doubling time was much
shorter, total DNA accumulated at a high rate throughout the cell
cycle and the rate of thymidine incorporation was transient. This
aspect of the cell cycle clearly requires further investigation to
determine whether the S phase actually occupies a large proportion
of interphase or whether the degree of synchrony of DNA replication
is as high as the degree of cell division synchrony. It may be possible
to improve the technique of thymidine labelling by using a shorter
pulse of thymidine fed at lower concentration, and ideally, if it
were possible to measure the specific activity of the dTTP pool
during a thymidine pulse, it would be possible to calculate the actual
amount of thymidine incorporated into DNA in unit time as a measure
of DNA synthesis. The isolation and measurement of the size of the
dTTP pool by spectrophotometry would require large amounts of tissue
but it may be possible to adopt the technique described by Iozzarin
and Winslow (1970) to measure the pool size by its content of \( P^{32} \)-labelling.

The results of uridine and leucine incorporation experiments showed some cyclic changes in the rates of incorporation of both labelled compounds which in general correlated with measurements of the rate of accumulation of total RNA and protein during the cell cycle. These results indicated that both RNA and protein synthesis occurred throughout the majority of the cell cycle but were both suppressed during mitosis. These observations agree with those of synchronous populations of cultured mammalian cells. Clearly it should now be possible to follow in detail the synthesis and degradation of the various species of RNA, and of individual enzymes and structural proteins, during the higher plant cell cycle. Such studies may lead to the discovery of the mechanisms which control the sequence of events and the rate of cell division.
A preliminary study of nucleic acid metabolism in suspension cultures of *Acer pseudoplatanus* L. has been carried out by K. C. Short (1969). Short measured the changes in the levels of total RNA and DNA throughout the growth cycle of batch cultures in chemically defined synthetic media and in complex media, and also showed that there were no detectable changes in the ratios of the amounts of the major components of total RNA, or in the base composition of total RNA, throughout the growth cycle.

Further studies of the synthesis and degradation of RNA in the cultured plant cells required the development of a method of introducing a radioactive tracer into RNA and techniques of extracting and fractionating radioactively labelled RNA in an undegraded form. Some preliminary experiments performed by Short (1969) have indicated that $P^{32}$-labelled ribose is not readily utilized by actively dividing sycamore suspension cultures and a specific precursor of RNA, labelled uridine, has therefore been investigated as a possible means of introducing a radioactive label into RNA.

As a necessary part of the measurement of the time course of incorporation of $^{14}$C-uridine into RNA a study of the role of the metabolic pool has been carried out. The system in cultured sycamore cells has some interesting features, for example, a large fraction of the labelled uridine is incorporated into RNA without measurable delay whilst the remainder equilibrates with a large pool of phosphorylated compounds. In addition, it has been shown that these cells have a mechanism by which exogenous uridine is rapidly degraded, and, in the case of $2-^{14}$C-uridine, the radioactivity is released from the
culture as $^{14}_{\text{C}}\text{CO}_2$. These observations are of interest not only with regard to the way in which exogenous uridine is handled by the plant cell but also have significant implications for studies of metabolic pools and nucleoside metabolism in general.

The results of long term labelling experiments in which the incorporation of $^{14}_{\text{C}}$-uridine (fed to cultures at varying concentrations at inoculation) throughout the growth cycle was investigated have indicated that a large proportion of the total cellular RNA is turned over during one cell generation time in the batch cultures. There was also strong evidence of separate pools for RNA precursor molecules and RNA degradation products. This implies that there may be a nucleotide cycle, at least with regard to the synthesis of the majority of total cellular RNA, in which nucleotides are synthesised from amino acid precursors and incorporated into RNA but on degradation of the RNA do not pass back into the precursor pools without further metabolism.

The information gained from the labelling experiments has been used, in conjunction with the development of techniques of extraction and fractionation of undegraded RNA, to design experiments to study the pattern of synthesis of RNA in rapidly dividing cell cultures. These studies have revealed the presence of distinct high molecular weight species of RNA which are rapidly labelled in the nucleus and which have been tentatively identified as precursors of ribosomal RNA. One of these suspected ribosomal RNA precursors is a molecule with a molecular weight of approximately $3.8 \times 10^6$ which is much larger than any such molecule yet reported in higher plant systems. The possibility exists that the process of maturation of ribosomal RNA in this tissue
is similar to that in mammalian tissues where a large proportion of the initial precursor is degraded during the processing sequence.

At the present time it appears to be a general feature of ribosome biosynthesis in eukaryotes and prokaryotes that ribosomal RNA is derived from precursor molecules of higher molecular weight by a series of specific cleavages. There are now some indications that eukaryote messenger RNA may arise in a similar way. Messenger RNA molecules undoubtedly exist in the cytoplasm of eukaryotic cells, but precisely how they get there is something of a mystery. Extensive work with mammalian tissues has shown that a large proportion of the heterogeneous high molecular weight RNA, which is rapidly labelled by short pulses of specific radioactive precursors in the nucleus, and which it was once tempting to assume is messenger RNA, is degraded without ever leaving the nucleus (Attardi, Parnas, Huang and Attardi, 1966; Soeiro, Vaughan, Warner and Darnell, 1968). Soeiro and Darnell (1970), from studies of the competitive hybridisation of nuclear heterogeneous RNA and messenger RNA from cytoplasmic polyribosomes of HeLa cells with DNA, have suggested that a small proportion of the high molecular weight heterogeneous RNA may be cleaved in some specific way and pass to the cytoplasm as a functional messenger. This view is strengthened by the work of Nissing and Sekeris (1970) who have reported that low concentrations of protein isolated from intranuclear ribonucleoprotein particles in rat liver rapidly cleave 50-60S DNA-like RNA into 30S pieces. Further incubation results in the formation of 4S to 16S RNA's. Detailed analysis of these RNA's and the endonuclease which produces them may well throw light on the maturation of messengers.
Studies of the origin and fate of the rapidly labelled high molecular weight nuclear RNA are therefore important not only for the elucidation of the mechanism and control of ribosome biosynthesis but may also lead to the recognition of species of RNA as messenger RNA and throw some light on the control of their synthesis, processing and transport to the cytoplasm.

The recent development, in this laboratory, of improved techniques of controlling the growth of plant cells (Wilson et al., 1971) has provided a very suitable system for further study of ribosomal RNA maturation and the origin and fate of the high molecular weight heterogeneous RNA in higher plants. Suspension cultures of *Acer pseudoplatanus* L. can now be maintained in a number of steady states of growth and it is possible by manipulation of the cultural conditions to shift a population of cells from one steady state to another in which the rate of cell division and the type of cell produced are markedly changed. This system provides an opportunity to study the effects of changes in the control of cell division and development on the synthesis and sequence of processing of ribosomal RNA, and its association with ribosomal proteins. Such an approach may make it possible to determine the level at which ribosome biosynthesis is controlled and the effects of specific growth regulators, nutrients and other stimuli on the protein synthesising capacity of higher plant cells.

In addition to the steady state cultures an improved batch culture system has been developed in which cells can be maintained in synchronous division for a number of full cell cycles. Some preliminary experiments have been performed to attempt to establish the
pattern of synthesis of total RNA, DNA and protein, through the cell cycle. The changes in rates of incorporation of specific labelled precursors of RNA, DNA and protein have been measured throughout the cell cycle and have been shown, in general, to correlate with changes in the rates of accumulation of total RNA, DNA and protein. There were indications of peaks of DNA synthesis just before mitosis and just after cytokinesis and peaks of RNA synthesis before and after mitosis and immediately after cytokinesis. These investigations, however, clearly require further work with improved techniques.

The potential of this culture system is such that it may soon be possible to study in detail the internal factors which control the progress of the higher plant cell cycle and their interaction with external factors which affect rates of cell division and determine the kind of cell that is produced.
Section I

Summary

Incorporation and metabolism of labelled uridine in suspension cultures of Acer pseudoplatanus L.

1) $^{14}\text{C}$-uridine was found to be rapidly taken up from the culture medium, even where present in only trace amounts, and a proportion was incorporated specifically into RNA without detectable delay whilst the remainder equilibrated with a large pool of phosphorylated compounds within the cell.

2) High concentrations of uridine in the culture medium did not appear to have any great effect upon the size of the endogenous RNA precursor pools. Any pool expansion that did occur was small compared with the pre-existent size of the endogenous pools.

3) It has been shown that the RNA precursor pools cannot be "effectively chased" since incorporation of radioactivity into RNA continued for some time after addition of a large excess concentration of unlabelled uridine to the medium.

4) Analysis of the radioactive compounds in the pool showed that $5'$ UMP was the major labelled constituent and that there were small amounts of labelled UDP-glucose, CMP, UDP and UTP. There was no detectable $^{14}\text{C}$-uridine which suggests that it was rapidly phosphorylated on entry into the cell.

5) There was shown to be a high efficiency of incorporation of labelled uridine into both the UMP and CMP residues of total RNA indicating that there was efficient conversion of uracil-compounds to cytosine-compounds within the cells and that exogenous uridine to a large extent suppresses endogenous synthesis of both pyrimidine
6) Cultured sycamore cells have the capacity to rapidly degrade 2-\(^{14}\)C-uridine with release of the radioactivity as \(^{14}\)CO\(_2\). The proportion of uridine taken up from the medium that was degraded to \(^{14}\)CO\(_2\) increased as the concentration of exogenous uridine increased, from 25% at \(10^{-6}\)M to 75% at \(10^{-3}\)M. It has been suggested that this may be a detoxication mechanism.

7) \(^{14}\)C-uridine was taken up at varying rates by the cultures and incorporated into RNA at all stages of the growth cycle. Incorporation of radioactivity into RNA continued during periods of growth when the total cell content of RNA was known to be rapidly decreasing indicating a substantial rate of turnover of total RNA.

8) Long term labelling experiments have indicated that a high proportion of the total cell RNA was turned over during the generation time of exponential phase cells and that a large proportion of the degradation products are not re-utilized for RNA synthesis. Fractionation of the total cell RNA has shown that there was no preferential degradation of any of the major species of total RNA.

9) The possible effects of uridine upon growth of the cultures were investigated by feeding a range of concentrations from \(10^{-5}\)M to \(10^{-3}\)M to cultures at inoculation. It was found, however, that uridine had no detectable effects upon cell division, dry weight accumulation or cell expansion throughout the growth cycle of the cultures.

Section II

Ribosomal RNA synthesis in rapidly dividing cultures of Acer pseudoplatanus L.

1) Analysis of the flow of radioactivity into nuclear and cytoplasmic
RNA, fractionated by sucrose density gradient zonal sedimentation, has revealed the presence of several discrete peaks of rapidly labelled high molecular weight nuclear RNA which have kinetics of labelling consistent with their possible function as precursors of cytoplasmic ribosomal RNA.

2) These high molecular weight species of RNA had estimated sedimentation coefficients of approximately 45S, 38-40S, 28-30S and 20S. The 38S molecule appeared before the 28 and 20S molecules which in turn appeared before the 25S and 18S components of ribosomal RNA, but the position of the 45S molecule in the sequence of processing, as revealed by its kinetics of labelling, was unclear.

3) The 38S molecule was shown to be labelled when cells were supplied with $^{14}$C(methyl)-methionine.

4) These rapidly labelled high molecular weight ribosomal RNA precursors have also been detected in preparations of nuclear RNA fractionated by gel electrophoresis and their molecular weights have been estimated at $3.8 \times 10^6$ daltons for the 45S molecule, $2.3 \times 10^6$ daltons for the 38S molecule, $1.4 \times 10^6$ daltons for the 28S molecule and $0.9 \times 10^6$ daltons for the 20S molecule.

5) Labelling of the cells with $^{14}$C(methyl)-methionine resulted in labelling of the $2.4 \times 10^6$ daltons (38S), the $1.4 \times 10^6$ daltons (28S) and the $0.9 \times 10^6$ daltons (20S) components with a marked reduction in the labelling of heterogeneous RNA.

Section III

Studies of the incorporation of labelled thymidine, uridine and leucine in synchronously dividing cultures of Acer pseudoplatanus L.

1) Rates of incorporation of labelled thymidine, measured at 6 hour
intervals through a cell cycle in two separate cultures correlated closely with rates of accumulation of total DNA and thymidine kinase activity. Peaks of incorporation were obtained immediately before mitosis and after cytokinesis. In one culture with a cell doubling time of 67 hours an S phase of 30-36 hours duration immediately before mitosis was indicated but in a later experiment using a culture with a cell doubling time of 48 hours DNA synthesis occurred throughout interphase.

2) Uridine incorporation occurred throughout interphase and two peaks occurred in both cultures investigated, one immediately following mitosis and one following cytokinesis. Low levels of uridine incorporation were obtained during mitosis and cytokinesis. These results correlated with rates of accumulation of total RNA.

3) Incorporation of labelled leucine showed a peak immediately following cytokinesis and a rate was then established which did not decline until the cells had entered mitosis. These results correlated with rates of accumulation of total protein, and, in common with both thymidine and uridine incorporation a low rate of incorporation was recorded during cytokinesis.
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ABSTRACT

As a necessary part of the measurement of the time course of incorporation of a specific precursor of RNA, $^{14}$C-uridine, into the various species of cellular RNA, a study of the role of the metabolic pool has been carried out. $2^{-14}$C-uridine was rapidly incorporated by suspension cultures of Acer pseudoplatanus L. over a wide range of concentrations. A substantial proportion was incorporated specifically into RNA without measurable delay whilst the remainder equilibrated with a large pool of phosphorylated compounds within the cell. In addition, as the external concentration of $2^{-14}$C-uridine was increased, an increasing proportion of the uridine entering the cells was rapidly degraded with release of $^{14}$CO$_2$ from the culture.

The pattern of labelling of RNA throughout the growth cycle has indicated that a large proportion of the total cellular content of RNA is turned over during one cell generation time in batch cultures and that there are separate pools for RNA precursor molecules and degradation products.

Investigation of the time course of incorporation of $2^{-14}$C-uridine into the various species of total RNA, fractionated by sucrose density gradient sedimentation and by gel electrophoresis, in actively dividing cell cultures has revealed the presence of several distinct species of rapidly labelled, high molecular weight nuclear RNA which have been tentatively identified as precursors to ribosomal RNA. One of these RNA components has an estimated molecular weight of $3.8 \times 10^6$ daltons which is much greater than any yet reported in higher plant tissues.

A culture system in which cells can be maintained in synchronous
cell division for several full cell cycles has been described. Measurement of the changes in the rates of incorporation of labelled thymidine, uridine and leucine through the cell cycle have shown that they correlate, in general, with changes in the rates of accumulation of total DNA, RNA and protein. An S phase of from 30-35 hours was indicated and there was evidence of peaks of RNA synthesis after mitosis and after cytokinesis.